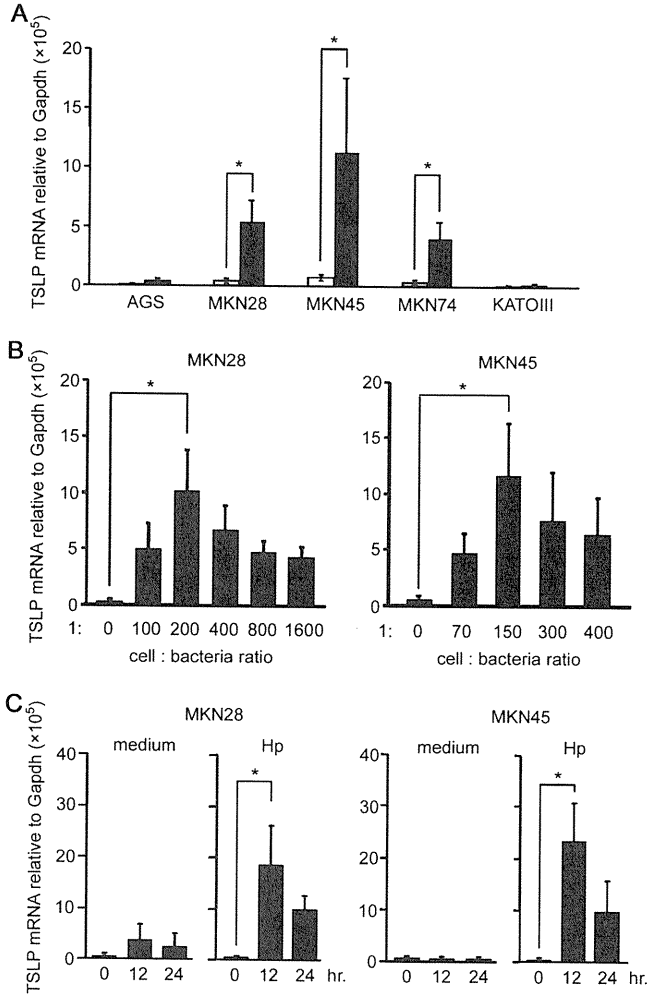


FIG. 1. TSLP expression in gastric epithelial cells after *H. pylori* colonization. (A) Various human gastric epithelial cell lines were cultured for 24 h with (closed bars) or without (open bars) *H. pylori* at 1 cell per 150 bacteria. (B) MKN28 and MKN45 cells were cultured for 24 h with *H. pylori* at the indicated cell/bacterium ratios. (C) MKN28 and MKN45 cells were cultured for the indicated times with *H. pylori* at 1 cell per 150 bacteria. Expression levels of mRNA encoding TSLP were measured using real-time quantitative RT-PCR. The data represent the means of three independent experiments. The error bars represent standard deviations (SD). *, $P < 0.05$.

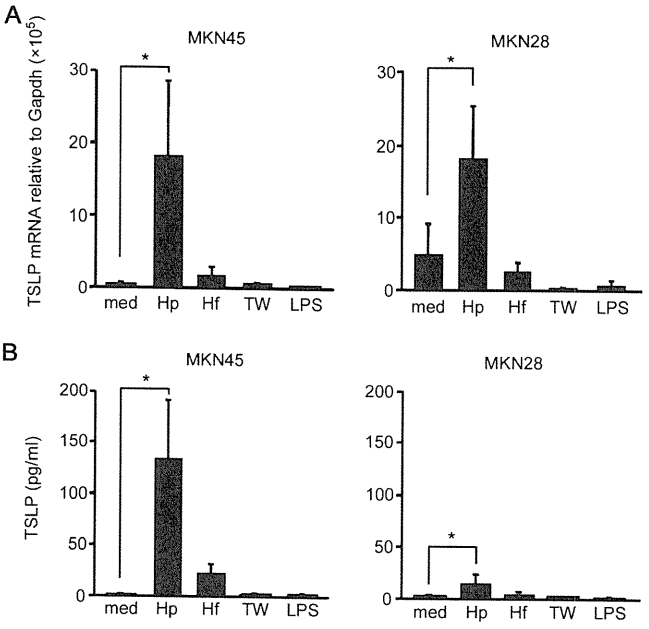


FIG. 2. TSLP expression in gastric epithelial cells, depending on direct contact with *H. pylori*. MKN45 and MKN28 cells were cultured for 24 h in the presence of 1 μg/ml LPS, *H. felis* (Hf), or *H. pylori* at 1 cell per 150 bacteria with (TW) or without (Hp) using Transwell, or medium alone (med). The expression levels of TSLP mRNA were measured using real-time RT-PCR (A) or those of TSLP protein in the culture supernatant were measured by protein ELISA (B). The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

sessed by protein ELISA. In contrast to the exposure to *H. pylori*, gastric epithelial cells did not upregulate TSLP expression after *H. felis* colonization (Fig. 2A and B). It seemed likely that one mechanism by which *H. pylori*, but not *H. felis*, could mediate induction of TSLP expression was by the secretion of specific proinflammatory factors. However, when gastric epithelial cells were separated from *H. pylori* by culture in a Transwell, induction of TSLP expression was not observed (Fig. 2A and B). Indeed, one of the proinflammatory factors, the Toll-like receptor 4 ligand LPS (from *Escherichia coli*), also failed to induce TSLP expression in gastric epithelial cells (Fig. 2A and B). Taken together, these data suggest that direct contact of *H. pylori* with human gastric epithelial cells is essential for induction of TSLP expression in these cells.

**Direct contact of *H. pylori* triggers human gastric epithelial cells to produce MIP-3α.** Chemokine production by epithelial cells plays a critical role in the migration of immune cells in inflamed mucosal lesions, and *H. pylori* infection induces upregulation of MIP-3α gene expression in gastric epithelial cells in humans and mice (26, 49). Next, we examined whether *H. pylori* colonization can induce production of chemokines attracting myeloid lineage cells, such as MCP-1 (also called CCL-2), MIP-1α (CCL3), MIP-1β (CCL4), and MIP-3α (CCL20), together with TSLP, in gastric epithelial cells. MKN45 cells were cultured for 24 h with *Helicobacter* bacteria, and chemokine production in the culture supernatant was assessed by protein ELISA. In contrast to MCP-1, MIP-1α, or MIP-1β, production of MIP-3α was strongly upregulated after colonization by *H. pylori*, but not *H. felis*, in gastric epithelial cells (Fig. 3). In addition, when gastric epithelial cells were separated from *H. pylori* by culture in a Transwell, no induction of MIP-3α production was observed (Fig. 3). Moreover, LPS (from *Escherichia coli*) failed to induce MIP-3α production in gastric epithelial cells (Fig. 3). Taken together, these data suggest that direct contact of *H. pylori* with human gastric epithelial cells is also essential for induction of MIP-3α production in these cells.

**TSLP-containing supernatants from *H. pylori*-colonized gastric epithelial cells enhanced surface CD80 expression in DCs.** Human $CD11c^+$ blood immature DCs respond to the stimulation of TSLP, and TSLP stimulation enhances CD80 expression in DCs (23, 44). To examine whether TSLP-containing supernatants from the *H. pylori*-infected gastric epithelial cells

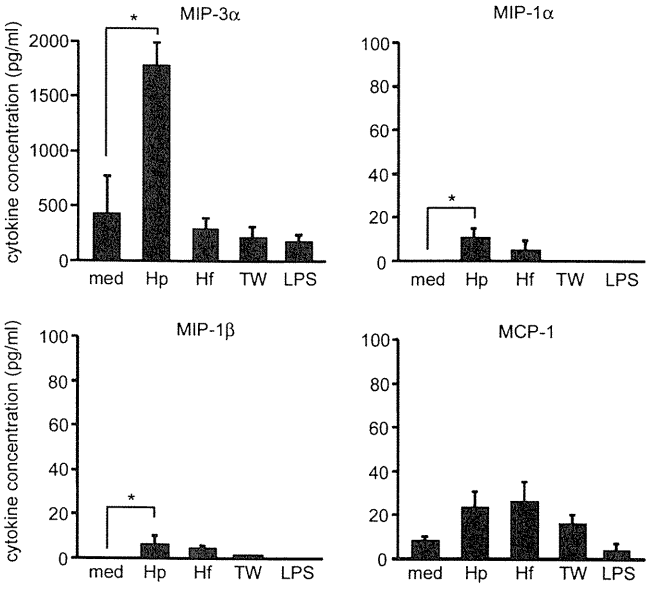


FIG. 3. Chemokine production by gastric epithelial cells, depending on direct contact with *H. pylori*. The cells were cultured as described in the legend to Fig. 2. MIP-3α, MIP-1α, MIP-1β, and MCP-1 were measured in the culture supernatant by protein ELISA. The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

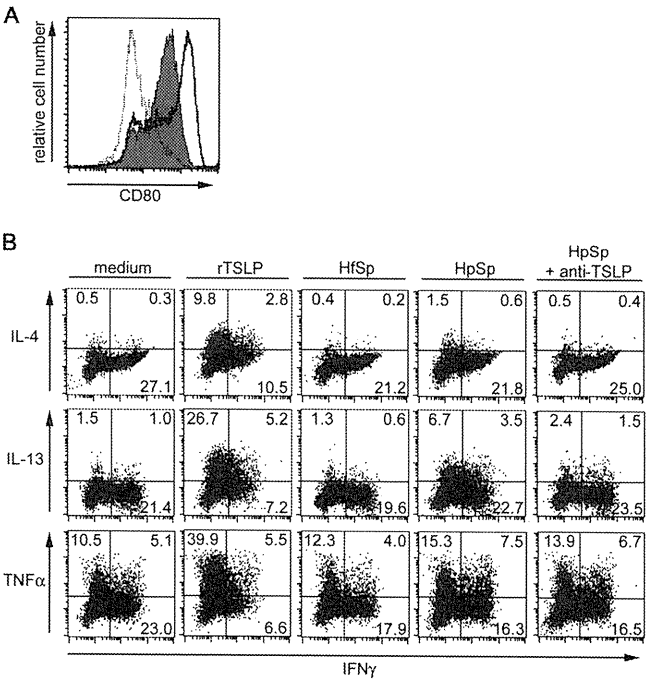


FIG. 4. CD80 expression on myeloid DCs and the cytokine-producing capacity of $CD4^+$ T cells are expanded by activated DCs. (A) Purified $CD11c^+$ myeloid DCs were cultured with recombinant TSLP (solid line, open histogram) or supernatants from MKN45 cells with (filled histogram) or without (dotted line, open histogram) exposure to *H. pylori* at 1 cell per 150 bacteria. After 24 h of incubation, the surface expression of CD80 on DCs was determined by flow cytometry. The data represent one of three experiments. (B) Purified DCs were cultured with rTSLP or supernatants from MKN45 cells exposed to *H. felis* (HfSp), *H. pylori* (HpSp) at 1 cell per 150 bacteria, or medium alone (medium). For neutralization of TSLP, supernatants were incubated with anti-human TSLP (20 μg/ml) (HpSp + anti-TSLP). After 24 h of incubation, DCs were cocultured with allogeneic naïve $CD4^+$ T cells at a 1:5 ratio of DCs to T cells. After 7 days of coculture, T cells were restimulated for 6 h with PMA plus ionomycin, and production of indicated T-cell-derived cytokines was determined by intracellular cytokine staining. Shown are dot blot profiles of the indicated cytokine-producing cells. The numbers indicate the percentages of cells in each quadrant. The data represent one of three independent experiments.

enhanced cell surface expression of CD80 in DCs, we isolated blood myeloid DCs and incubated them with supernatants from the *H. pylori*-infected gastric epithelial cells. After 24 h of incubation, we analyzed the surface expression of CD80 on DCs by flow cytometry. Recombinant TSLP protein induced upregulation of surface CD80 expression in DCs (Fig. 4A). Notably, after DCs were incubated with supernatants from *H. pylori*-infected epithelial cells, the conditioned cells also enhanced surface CD80 expression (Fig. 4A).

**DCs conditioned by TSLP-containing supernatants prime naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines.** TSLP-activated DCs induce differentiation of inflammatory Th2 cells, and DCs activated with TSLP and CD40 ligand induce the differentiation of effectors producing both Th1 and Th2 cytokines (23, 42, 44). Next, we conditioned DCs with TSLP-containing supernatants from the *H. pylori*-colonized gastric epithelial cells and cocultured these DCs with allogeneic naïve $CD4^+$ T cells at a 1:5 ratio of DCs to T cells. After 7 days of coculture, we evaluated the cytokine production capacity using intracellular cytokine staining of primed T cells restimulated with PMA plus ionomycin. This staining demonstrated that DCs conditioned with supernatants from gastric epithelial cells without *Helicobacter* colonization (medium) primed $CD4^+$ T cells to produce IFN-γ and TNF-α, but not IL-4 or IL-13 (Fig. 4B). In contrast, DCs activated with recombinant TSLP protein (rTSLP) primed $CD4^+$ T cells to produce IL-4, IL-13, and TNF-α as expected. Although DCs incubated with supernatants from *H. felis*-colonized gastric epithelial cells (HfSp) did not change the Th1 cytokine production profile of cocultured $CD4^+$ T cells, DCs incubated with supernatants from *H. pylori*-colonized gastric epithelial cells (HpSp) did change it to a mixed Th1 and Th2 profile in which cocultured T cells produced IL-4 and IL-13, together with IFN-γ and TNF-α. After incubation of the TSLP-containing supernatants with neutralizing antibodies to human TSLP, however, the conditioned DCs (HpSp plus anti-TSLP) did not prime T cells to produce high levels of IL-4 and IL-13. These data suggest that DCs conditioned with supernatants from the *H. pylori*-colonized gastric epithelial cells promote naïve $CD4^+$ T cells to differentiate into effectors producing both Th1 and Th2 cytokines and that TSLP in the supernatants is responsible for the conditioning of DCs to prime $CD4^+$ T cells to produce Th2 cytokines.

***H. pylori*-colonized gastric epithelial cells sequentially upregulate TSLP and B-cell-activating factor BAFF.** BAFF (also called BLyS) is a powerful regulator of B-cell biology (2, 17, 33, 39). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). Next, we tested whether *H. pylori* colonization on gastric epithelial cells can upregulate BAFF gene expression in gastric epithelial cells. Such cells were cultured with *H. pylori*, and the

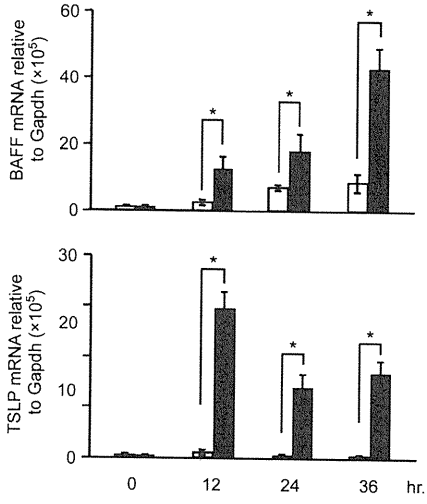


FIG. 5. TSLP and BAFF expression in gastric epithelial cells after *H. pylori* colonization. MKN28 cells were cultured for the indicated times with (closed bars) or without (open bars) *H. pylori* at 1 cell per 150 bacteria. The expression levels of mRNAs encoding TSLP and BAFF were measured using real-time quantitative RT-PCR. The data represent the means of three independent experiments. The error bars represent SD. *, $P < 0.05$.

expression levels of mRNA encoding BAFF were measured using real-time quantitative RT-PCR. In contrast to gastric epithelial cells not exposed to *H. pylori*, gastric epithelial cells that were exposed upregulated BAFF expression (Fig. 5). Although induction of TSLP expression peaked at 12 h after *H. pylori* colonization, induction of BAFF expression was detected after 12 h of *H. pylori* colonization and increased even after 36 h. These findings suggest that *H. pylori* colonization induces upregulation of TSLP and subsequently of BAFF expression in gastric epithelial cells.

**Expression of TSLP is induced in mucosal lesions from *H. pylori*-infected gastritis patients.** Finally, we evaluated the expression of TSLP in mucosal lesions from patients with *H. pylori*-infected follicular gastritis in which B-cell activation, including Th2 responses, was apparently involved. Frozen sections of mucosal lesions from follicular gastritis were stained with anti-TSLP antibodies. As shown in Fig. 6, immunoglobulin isotype control antibodies did not produce any positive staining, and there was no detectable immunostaining for TSLP in normal gastric mucosa. However, we found anti-TSLP staining of epithelial cells in the inflamed gastric mucosa from patients with *H. pylori*-infected follicular gastritis. TSLP expression was associated with the presence of $CD11c^+$ DCs within the inflamed gastric mucosa and was also associated with the presence of DC–lysosome-associated membrane protein (DC-LAMP, which is a DC activation marker)-positive cells. These data suggest that expression of TSLP is enhanced in mucosal lesions from *H. pylori*-induced follicular gastritis patients, but not in normal gastric mucosa, and also that TSLP expressed by gastric epithelial cells may play an important role in DC-mediated T-cell activation of a process related to Th2 inflammation in *H. pylori*-induced chronic gastritis.

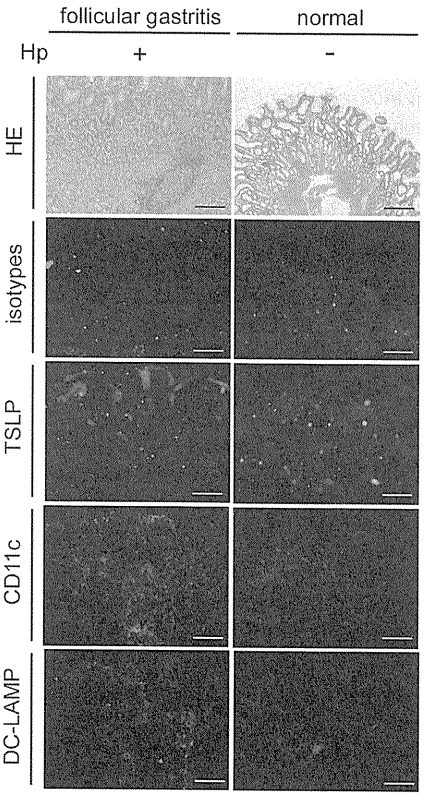


FIG. 6. Immunohistological staining of gastric mucosa. *H. pylori* (Hp)-infected inflamed mucosa containing a lymphoid follicle from a patient with follicular gastritis (left column) and non-*H. pylori*-infected normal mucosa (right column) were stained with hematoxylin and eosin (HE), immunoglobulin isotype control antibodies (isotypes), anti-human TSLP (TSLP), anti-CD11c (CD11c), or anti-DC-LAMP (DC-LAMP). All scale bars, 100 μm.

## DISCUSSION

In the present study, we demonstrated that *H. pylori* triggered human gastric epithelial cells to produce TSLP, together with the DC-attracting chemokine MIP-3α and B-cell activating factor BAFF. DCs conditioned by *H. pylori*-infected epithelial cells expressed high levels of costimulatory molecules and primed naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines.

*H. pylori*-induced atrophic gastritis is characterized by marked infiltration of $CD4^+$ T cells that produce IFN-γ (12, 14). Development of *H. pylori*-induced atrophic gastritis is severely impaired in mice lacking $CD4^+$ T cells or IFN-γ production (7, 32). In addition, development of Th1 cell-mediated atrophic gastritis is also severely impaired in *Helicobacter*-infected Peyer's patch-null mice, which normally develop well-organized lymphoid organs, except for Peyer's patches. In these mice, the marked colonization of bacteria in the gastric mucosa can be detected (18, 25).

Although the gastric mucosa originally does not have a lymphoid apparatus, *H. pylori* infection triggers the development of MALT-like structures consisting of lymphoid aggregates and organized B-cell follicles in patients with *H. pylori*-induced gastritis (10, 11, 34). High-level expression of B-cell-activating chemokine 1 (BCA-1) (also called CXCL13) and its receptor,

CXC chemokine receptor (CXCR) 5, is observed in lymphoid aggregates and in the mantle zone of secondary lymphoid follicles in *Helicobacter*-induced follicular gastritis, suggesting that the chemokine-chemokine receptor interaction triggers the recruitment of lymphocytes (24). However, the mechanisms underlying the triggering of B-cell activation, including Th2 responses apparently involved in *H. pylori*-induced chronic gastritis, are not fully understood.

Chronic inflammatory processes in both autoimmunity and infection are characterized by the infiltration of a variety of immune cells, such as T cells, macrophages, and DCs, but also B cells and plasma cells. These cellular elements often organize the *de novo* formation of B-cell follicles and T-cell areas (1). In mouse models of *H. pylori*-induced follicular gastritis, the inflamed gastric mucosa contains B-cell follicles with germinal centers, T-cell areas, and high endothelial venules (28, 31). On the other hand, the constitutive expression of BAFF in secondary lymphoid tissues is essential for sustaining the long-term survival of mature B cells *in vivo* (17, 33). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). In addition, a recent human study suggested an association between circulating levels of BAFF in ectopic germinal centers of the salivary gland in primary Sjögren's syndrome (16, 37). Taken together, these results suggest that TSLP, together with BAFF, produced by *H. pylori*-infected gastric mucosa leads to inflammatory Th2 responses and maintenance of B-cell activation.

TSLP is involved in a variety of immune responses in the mucosal immune system in humans and mice. Epithelial cells in the tonsils and intestines release TSLP (13, 30, 38, 43, 48, 50). Human TSLP-secreting tonsillar epithelial cells produce BAFF to induce class switching by stimulating B cells (48). TSLP-conditioned DCs induce homeostatic noninflammatory Th2 responses and produce a proliferation-inducing ligand (APRIL) (13, 30, 43, 50). This results in enhancement of IgA2 class switching by intestinal epithelial cells under physiological conditions. In addition, TSLP-mediated signaling plays critical roles in host-protective Th2 cytokine-dependent immunity to the intestinal nematode pathogen *Trichuris* (40, 50). TSLP is involved in the regulation of Th1-type inflammation in a mouse model of colitis (40). We showed that *H. pylori*, one of the major pathogens in the human gastrointestinal tract, triggered gastric epithelial cells to produce TSLP, implying that TSLP may be crucial in a variety of immune responses in the gastrointestinal tract, including the stomach.

In mice, TSLP appears to suppress Th1 responses by acting on DCs in the mucosal immune system. In infection of mice by *Trichuris*, neutralization of TSLP or deletion of the TSLP receptor (TSLPR) in normally resistant mice resulted in defective expression of Th2 cytokines and persistent intestinal infection (40). In the intestinal inflammation in these mice, expression of inflammatory cytokines, such as IFN-γ, IL-17, and IL-12/23p40, was abundant. Neutralization of IFN-γ rescued the Th2 response and restored antiworm immunity in TSLP-deficient mice. In humans, although TSLP-activated DCs promote $CD4^+$ T cells to differentiate into inflammatory Th2 cells, TSLP primes DCs to produce large amounts of IL-12 following CD40 ligand stimulation. DCs activated with TSLP and CD40 ligand induce the differentiation of naïve $CD4^+$ T cells into effectors producing both Th1 and Th2 cytokines (23, 42, 44). These data suggest that, in the human system, induction of Th1 responses mediated by IL-12 is not suppressed under TSLP-induced Th2 inflammation and that IL-12-mediated negative regulation of Th2 responses is not effective in TSLP-induced Th2 inflammation. In this study, DCs conditioned by *Helicobacter*-infected epithelial cells primed naïve CD4 T cells to differentiate into effectors producing both Th1 and Th2 cytokines. Taken together, although Th1 cytokine-producing $CD4^+$ T cells markedly infiltrate inflamed mucosa in *Helicobacter*-induced chronic gastritis, these Th1 cytokines may not suppress Th2 cytokine production by TSLP-conditioned DCs.

Several recent studies have shown that microorganism-derived stimulation and/or proinflammatory cytokines upregulate expression of both TSLP and MIP-3α in human epithelial cells, and these upregulations are directly controlled by NF-κB (15, 21, 22, 36). In addition, although it is unclear whether BAFF expression is under the direct control of NF-κB, a recent study demonstrated that Toll-like receptor 3 ligand poly(I · C) induced upregulation of TSLP and subsequently of BAFF production in human tonsillar epithelial cells (48). In this study, we demonstrated that *H. pylori* triggered human gastric epithelial cells to produce TSLP, together with MIP-3α and BAFF. Because *H. pylori* products induce NF-κB activation in gastric epithelial cells (8, 27, 41, 47), *H. pylori*-induced NF-κB activation in gastric epithelial cells may directly and/or indirectly upregulate the expression of TSLP, MIP-3α, and BAFF in these cells.

In conclusion, we have demonstrated that *H. pylori* triggered gastric epithelial cells to produce TSLP, MIP-3α, and BAFF and that DCs conditioned by *Helicobacter*-infected epithelial cells triggered differentiation of T cells with a mixed Th1 and Th2 profile. These results, and the finding that TSLP was expressed by the epithelial cells of human follicular gastritis, suggest that *H. pylori* can directly trigger epithelial cells to produce TSLP and that TSLP-mediated DC activation may be involved in Th2 responses triggering B-cell activation in *H. pylori*-induced gastritis.

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## REFERENCES

1. **Aloisi, F., and R. Pujol-Borrell.** 2006. Lymphoid neogenesis in chronic inflammatory diseases. Nat. Rev. Immunol. **6**:205–217.
2. **Brink, R.** 2006. Regulation of B cell self-tolerance by BAFF. Semin. Immunol. **18**:276–283.
3. **Chiba, T., H. Seno, H. Marusawa, Y. Wakatsuki, and K. Okazaki.** 2006. Host factors are important in determining clinical outcomes of *Helicobacter pylori* infection. J. Gastroenterol. **41**:1–9.
4. **Dick, E., A. Lee, G. Watson, and J. O'Rourke.** 1989. Use of the mouse for the isolation and investigation of stomach-associated, spiral-helical shaped bacteria from man and other animals. J. Med. Microbiol. **29**:55–62.
5. **Dixon, M. F., R. M. Genta, J. H. Yardley, and P. Correa.** 1996. Classification

and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston, 1994. Am. J. Surg. Pathol. **10:**1161–1181.

6. **Dunn, B. E., M. Altmann, and G. P. Campbell.** 1991. Adherence of *Helicobacter pylori* to gastric carcinoma cells: analysis by flow cytometry. Rev. Infect. Dis. **13**(Suppl. 8):S657–S664.
7. **Eaton, K. A., M. Mefford, and T. Thevenot.** 2001. The role of T cell subsets and cytokines in the pathogenesis of Helicobacter pylori gastritis in mice. J. Immunol. **166:**7456–7461.
8. **Farinha, P., and R. D. Gascoyne.** 2005. *Helicobacter pylori* and MALT lymphoma. Gastroenterology **128:**1579–1605.
9. **Fox, J. G., M. Blanco, J. C. Murphy, N. S. Taylor, A. Lee, Z. Kabok, and J. Pappo.** 1993. Local and systemic immune responses in murine *Helicobacter felis* active chronic gastritis. Infect. Immun. **61:**2309–2315.
10. **Genta, R. M., and H. W. Hamner.** 1994. The significance of lymphoid follicles in the interpretation of gastric biopsy specimens. Arch. Pathol. Lab. Med. **118:**740–743.
11. **Genta, R. M., H. W. Hamner, and D. Y. Graham.** 1993. Gastric lymphoid follicles in *Helicobacter pylori* infection: frequency, distribution, and response to triple therapy. Hum. Pathol. **24:**577–583.
12. **Harris, P. R., L. E. Smythies, P. D. Smith, and A. Dubois.** 2000. Inflammatory cytokine mRNA expression during early and persistent *Helicobacter pylori* infection in nonhuman primates. J. Infect. Dis. **181:**783–786.
13. **He, B., W. Xu, P. A. Santini, A. D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D. M. Knowles, M. Rescigno, and A. Cerutti.** 2007. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity **26:**812–826.
14. **Itoh, T., Y. Wakatsuki, M. Yoshida, T. Usui, Y. Matsunaga, S. Kaneko, T. Chiba, and T. Kita.** 1999. The vast majority of gastric T cells are polarized to produce T helper 1 type cytokines upon antigenic stimulation despite the absence of *Helicobacter pylori* infection. J. Gastroenterol. **34:**560–570.
15. **Izadpanah, A., M. B. Dwinell, L. Eckmann, N. M. Varki, and M. F. Kagnoff.** 2001. Regulated MIP-3alpha/CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. Am. J. Physiol. Gastrointest. Liver Physiol. **280:**G710–G719.
16. **Jonsson, M. V., P. Szodoray, S. Jellestad, R. Jonsson, and K. Skarstein.** 2005. Association between circulating levels of the novel TNF family members APRIL and BAFF and lymphoid organization in primary Sjögren's syndrome. J. Clin. Immunol. **25:**189–201.
17. **Kalled, S. L.** 2006. Impact of the BAFF/BR3 axis on B cell survival, germinal center maintenance and antibody production. Semin. Immunol. **18:**290–296.
18. **Kiriya, K., N. Watanabe, A. Nishio, K. Okazaki, M. Kido, K. Saga, J. Tanaka, T. Akamatsu, S. Ohashi, M. Asada, T. Fukui, and T. Chiba.** 2007. Essential role of Peyer's patches in the development of *Helicobacter*-induced gastritis. Int. Immunol. **19:**435–446.
19. **Lee, A., J. G. Fox, G. Otto, and J. Murphy.** 1990. A small animal model of human *Helicobacter pylori* active chronic gastritis. Gastroenterology **99:**1315–1323.
20. **Lee, A., S. Krakowka, J. G. Fox, G. Otto, K. A. Eaton, and J. C. Murphy.** 1992. Role of *Helicobacter felis* in chronic canine gastritis. Vet. Pathol. **29:**487–494.
21. **Lee, H. C., and S. F. Ziegler.** 2007. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFκB. Proc. Natl. Acad. Sci. USA **104:**914–919.
22. **Lee, H. C., M. B. Headley, M. Iseki, K. Ikuta, and S. F. Ziegler.** Inhibition of NF-κB-mediated TSLP expression by retinoid X receptor. J. Immunol. **181:** 5189–5193.
23. **Liu, Y. J., V. V. Soumelis, N. Watanabe, T. Ito, Y. H. Wang, R. de Waal Malefyt, M. Omori, B. Zhou, and S. F. Ziegler.** 2007. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu. Rev. Immunol. **25:**193–219.
24. **Mazzucchelli, L., A. Blaser, A. Kappeler, P. Schärli, J. A. Laissue, M. Baggiolini, and M. Uguccioni.** 1999. BCA-1 is highly expressed in *Helicobacter pylori*-induced mucosa-associated lymphoid tissue and gastric lymphoma. J. Clin. Invest. **104:**R49–R54.
25. **Nagai, S., H. Mimuro, T. Yamada, T. Baba, K. Moro, T. Nochi, H. Kiyono, T. Suzuki, C. Sasakawa, and S. Koyasu.** 2007. Role of Peyer's patches in the induction of *Helicobacter pylori*-induced gastritis. Proc. Natl. Acad. Sci. USA **104:**8971–8976.
26. **Nishi, T., K. Okazaki, K. Kawasaki, T. Fukui, H. Tamaki, M. Matsuura, M. Asada, T. Watanabe, K. Uchida, N. Watanabe, H. Nakase, M. Ohana, H. Hiai, and T. Chiba.** 2003. Involvement of myeloid dendritic cells in the development of gastric secondary lymphoid follicles in Helicobacter pylori-infected neonatally thymectomized BALB/c mice. Infect. Immunol. **71:**2153–2162.
27. **O'Keeffe, J., and A. P. Moran.** 2008. Conventional, regulatory, and unconventional T cells in the immunologic response to *Helicobacter pylori*. Helicobacter **13:**1–19.
28. **Oshima, C., K. Okazaki, Y. Matsushima, M. Sawada, T. Chiba, K. Takahashi, H. Hiai, T. Katakai, S. Kasakura, and T. Masuda.** 2000. Induction of follicular gastritis following postthymectomy autoimmune gastritis in *Helicobacter pylori*-infected BALB/c mice. Infect. Immun. **68:**100–106.
29. **Otto, G., S. H. Hazell, J. G. Fox, C. R. Howlett, J. C. Murphy, J. O'Rourke, and A. Lee.** 1994. Animal and public health implications of gastric colonization of cats by *Helicobacter*-like organisms. J. Clin. Microbiol. **32:**1043–1049.
30. **Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G. M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno.** 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat. Immunol. **6:**507–514.
31. **Shomer, N. H., J. G. Fox, A. E. Juedes, and N. H. Ruddle.** 2003. *Helicobacter*-induced chronic active lymphoid aggregates have characteristics of tertiary lymphoid tissue. Infect. Immun. **71:**3572–3577.
32. **Smythies, L. E., K. B. Waites, J. R. Lindsey, P. R. Harris, P. Ghiara, and P. D. Smith.** 2000. *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-γ, gene-deficient mice. J. Immunol. **165:**1022–1029.
33. **Stadanlick, J. E., and M. P. Cancro.** 2008. BAFF and the plasticity of peripheral B cell tolerance. Curr. Opin. Immunol. **20:**158–161.
34. **Stolte, M., and S. Eidt.** 1989. Lymphoid follicles in antral mucosa: immune response to *Campylobacter pylori*? J. Clin. Pathol. **42:**1269–1271.
35. **Suerbaum, S., and P. Michetti.** 2002. *Helicobacter pylori* infection. N. Engl. J. Med. **347:**1175–1186.
36. **Sugita, S., T. Kohno, K. Yamamoto, Y. Imaizumi, H. Nakajima, T. Ishimaru, and T. Matsuyama.** 2002. Induction of macrophage-inflammatory protein-3alpha gene expression by TNF-dependent NF-kappaB activation. J. Immunol. **168:**5621–5628.
37. **Szodoray, P., P. Alex, M. V. Jonsson, N. Knowlton, I. Dozmorov, B. Nakken, N. Delaleu, R. Jonsson, and M. Centola.** 2005. Distinct profiles of Sjögren's syndrome patients with ectopic salivary gland germinal centers revealed by serum cytokines and BAFF. Clin. Immunol. **117:**168–176.
38. **Tanaka, J., K. Saga, M. Kido, H. Nishiura, T. Akamatsu, T. Chiba, and N. Watanabe.** 2009. Proinflammatory Th2 cytokines induce production of thymic stromal lymphopoietin in human colonic epithelial cells. Dig. Dis. Sci. doi:10.1007/s10620-009-0979-x.
39. **Tangye, S. G., V. L. Bryant, A. K. Cuss, and K. L. Good.** 2006. BAFF, APRIL and human B cell disorders. Semin. Immunol. **18:**305–317.
40. **Taylor, B. C., C. Zaph, A. E. Troy, Y. Du, K. J. Guild, M. R. Comeau, and D. Artis.** 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. J. Exp. Med. **206:**655–667.
41. **Viala, J., C. Chaput, I. G. Boneca, A. Cardona, S. E. Girardin, A. P. Moran, R. Athman, S. Mémet, M. R. Huerre, A. J. Coyle, P. S. DiStefano, P. J. Sansonetti, A. Labigne, J. Bertin, D. J. Philpott, and R. L. Ferrero.** 2004. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. **5:**1166–1174.
42. **Watanabe, N., S. Hanabuchi, M. A. Marloie-Provost, S. Antonenko, Y. J. Liu, and V. Soumelis.** 2005. Human TSLP promotes CD40-ligand-induced IL-12 production by myeloid dendritic cells but maintains their Th2 priming potential. Blood **105:**4749–4751.
43. **Watanabe, N., S. Hanabuchi, V. Soumelis, W. Yuan, S. Ho, R. de Waal Malefyt, and Y. J. Liu.** 2004. Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4+ T cell homeostatic expansion. Nat. Immunol. **5:**426–434.
44. **Watanabe, N., V. Soumelis, and Y. J. Liu.** 2005. Human thymic stromal lymphopoietin triggers dendritic cell-mediated allergic inflammation and CD4+ T cell homeostatic expansion. Adv. Exp. Med. Biol. **560:**69–75.
45. **Watanabe, N., Y. H. Wang, H. K. Lee, T. Ito, Y. H. Wang, W. Cao, and Y. J. Liu.** 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature **436:**1181–1185.
46. **Watanabe, T., M. Tada, H. Nagai, S. Sasaki, and M. Nakao.** 1998. *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. Gastroenterology **115:**642–648.
47. **Wilson, K. T., and J. E. Crabtree.** 2007. Immunology of *Helicobacter pylori*: insights into the failure of the immune response and perspectives on vaccine studies. **133:**288–308.
48. **Xu, W., B. He, A. Chiu, A. Chadburn, M. Shan, M. Buldys, A. Ding, D. M. Knowles, P. A. Santini, and A. Cerutti.** 2007. Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat. Immunol. **8:**294–303.
49. **Yoshida, A., H. Isomoto, J. Hisatsune, M. Nakayama, Y. Nakashima, K. Matsushima, Y. Mizuta, T. Hayashi, Y. Yamaoka, T. Azuma, J. Moss, T. Hirayama, and S. Kohno.** 2009. Enhanced expression of CCL20 in human Helicobacter pylori-associated gastritis. Clin. Immunol. **130:**290–297.
50. **Zaph, C., A. E. Troy, B. C. Taylor, L. D. Berman-Booty, K. J. Guild, Y. Du, E. A. Yost, A. D. Gruber, M. J. May, F. R. Greten, L. Eckmann, M. Karin, and D. Artis.** 2007. Epithelial-cell-intrinsic IKK-β expression regulates intestinal immune homeostasis. Nature **446:**552–556.

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# G-protein β3 subunit 825CC genotype is associated with postprandial distress syndrome with impaired gastric emptying and with the feeling of hunger in Japanese

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**Abstract**

***Background*** *G-protein dysfunction related alteration of intracellular signal transduction might be linked to various abnormalities of functional gastrointestinal (GI) disorders. Serotonin (5-hydroxytryptamine; 5-HT) as well as G-protein is also key signaling molecule sensorimotor functions in the GI tract. Thus, this study aims to evaluate the correlation between gastric emptying and GNβ3 and 5-HTs polymorphisms in functional dyspepsia (FD) as defined by Rome III classification.* ***Methods*** *Seventy-four patients presenting with typical symptoms of FD (epigastric pain syndrome: EPS, n = 24; postprandial distress syndrome: PDS, n = 51) and sixty-four healthy volunteers were enrolled. Gastric motility was evaluated with the $T_{max}$ value using the $^{13}$C-acetate breath test. We used Rome III criteria to evaluate upper abdominal symptoms and SRQ-D scores to determine depression status. GNβ3-C825T, 5-$HT_{1A}$-C1019G, 5-$HT_{2A}$-G1438A, 5-$HT_{3A}$-C42T, and 5-$HT_{4A}$-G353 + 6A polymorphisms were analyzed in DNA from blood samples of enrolled subjects. Genotyping was performed by polymerase chain reaction.* ***Key Results*** *There was a significant relationship (P = 0.045) between GNβ3 825CC genotype and PDS patients without gastro-esophageal reflux symptoms with impaired gastric emptying. In Japanese, GNβ3 825CC genotype in FD patients was significantly associated (P = 0.0485) with the feeling of hunger compared with GNβ3 825CT and TT genotypes.* ***Conclusions & Inferences*** *Our results suggest that the GNβ3 825CC genotype is significantly associated with PDS patients without gastro-esophageal reflux with impairments of gastric emptying and also with the feeling of hunger in patients with FD. Further studies are needed to clarify whether the GNβ3 825CC genotype is linked to disturbances of gastric emptying via altered signal transduction responses.*

***Keywords*** *functional dyspepsia, gastric motility, GNβ3, polymorphism, postprandial distress syndrome.*

## INTRODUCTION

Recently, Functional dyspepsia (FD) has been subclassified into two new disease categories under the Rome III classification, epigastric pain syndrome (EPS) and postprandial distress syndrome (PDS).[1] Although Rome III criteria exclude gastro-esophageal reflux symptoms from the clinical symptoms of FD patients, some degree of overlap between the symptoms of non-erosive reflux disease (NERD) and FD is inevitable. Impairment of gastric motility such as gastric emptying is strongly associated with the pathophysiology of FD, one of the most common gastrointestinal (GI) disorders.[2] Disturbances of physiological gastric emptying occur with a variety of symptoms ranging from premature saturation, fullness, nausea, vomiting, epigastric pain, and acid reflux in patients with delayed emptying in FD patients. We have previously reported that $T_{max}$ value as a marker of gastric emptying in PDS patients was significantly greater compared with that of healthy volunteers.[3] We have reported that prokinetics such as mosapride citrate improve clinical symptoms through affecting $T_{max}$ value in proton pump inhibitor (PPI)-resistant NERD patients with

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impaired gastric emptying.[4] These results suggest that $T_{max}$ value is one of the useful marker for considering the management of FD and NERD patients.

G-protein is composed of different $\alpha$, $\beta$, and $\gamma$ subunit isoforms, the $\beta\gamma$ subunit forming a functional monomer. On receptor activation, both $\alpha$ and $\beta\gamma$ subunits dissociate from the receptor and in turn modulate a large variety of intracellular effector system. Accordingly, G-protein dysfunction potentially could block intracellular signal transduction. The GN$\beta$3 gene encodes the G$\beta$3 subunit of heterotrimeric G proteins, which are key components of intracellular signal transduction that are widely present in cells of the body.[5] Thus, G-protein dysfunction related alteration of intracellular signal transduction might be linked to various abnormalities of functional GI disorders including disturbed gut sensory or motor function,[6–8] dysfunction of the autonomic nervous system,[9] and underlying psychiatric disturbances.[10] A common C825T polymorphism has been described in the gene GN$\beta$3 that encodes the $\beta$3 subunit of heterotrimeric G-proteins. Homozygous 825C allele carriers (CC genotype) form only minute amounts of the $\beta$3 splice variant and, thus, are characterized by diminished signal transduction responses.[11] In recent studies, clinical symptoms such as unexplained abdominal symptoms and meal-unrelated dyspepsia in FD have also been associated with the various polymorphism including GN$\beta$3 polymorphism.[12,13] Moreover, familial clustering of FD has been reported, suggesting that a genetic factor may also play a significant role in developing FD.[14]

In addition, serotonin (5-hydroxytryptamine; 5-HT) as well as G-protein is key signaling molecule sensorimotor functions in the GI tract. 5-$HT_{1A}$ receptor agonists with anxiolytic properties delays gastric emptying[15] and relaxes the proximal stomach in human.[16] 5-$HT_{2A}$ receptor has been reported to be involved in the modulation of enteric neuronal activity.[17] 5-$HT_3$ receptor agonist, MKC-733 has also delayed gastric emptying in association with relaxation of the proximal stomach. 5-$HT_4$ receptor as well as 5-$HT_3$ play an important role in GI sensory and motor functions.[18] Lelyveld *et al.* have studied whether there was a significant relationship among three genotypes including 5-$HT_3$ and clinical symptoms in FD patients based on Rome II classification in Austria.[19]

In this study, we aimed to clarify whether GN$\beta$3 genotype as well as genotypes of 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{3A}$, and 5-$HT_{4A}$ could be associated with clinical symptoms and gastric emptying via impairment of receptor dysfunction, reduction of serotonin levels, and the response for serotonin in FD patients based on Rome III classification.

## MATERIAL AND METHODS

### Patients

Seventy-four consecutive patients presenting with typical symptoms of FD (EPS, $n = 24$; PDS, $n = 51$) and sixty-four healthy volunteers were enrolled after upper GI endoscopy and abdominal ultrasonography. Patients were diagnosed according to Rome III criteria.[20] Healthy volunteers were also recruited from the volunteers among Japanese medical staffs of Nippon Medical School, who have no clinical history of gastroduodenal disease including clinical symptom of FD symptoms. Exclusion criteria included severe heart disease, renal or pulmonary failure, liver cirrhosis, severe systemic illness, and history of malignant disease. Patients with previous gastroduodenal surgery, duodenal ulcer scar, diabetes mellitus, and recent use of NSAIDs, PPIs or anticoagulants at endoscopy were also excluded. *Helicobacter pylori* infection was determined by both the $^{13}$C-urea breath test and by histological identification. Written informed consent was obtained from all subjects prior to undergoing upper GI endoscopy and abdominal ultra-sonography for evaluation of their dyspeptic symptoms. The study protocol was approved by the Ethics Review Committee of Nippon Medical School Hospital.

### Clinical symptoms

Clinical symptoms of FD were evaluated according to Rome III criteria[20] and must have included at least one of the following: bothersome postprandial fullness, early satiation, epigastric pain, or epigastric burning. Diagnostic criteria for PDS included bothersome postprandial fullness occurring after ordinary-sized meals and/or early satiation that prevented finishing a regular meal, with either symptom occurring at least several times a week. Diagnostic criteria for EPS included all of the following: pain or burning that is intermittent, localized to the epigastrium, and of at least moderate severity at least once per week. Diagnostic criteria for PDS and EPS were fulfilled with symptoms occurring for the last 3 months and the onset of symptoms occurring at least 6 months prior to diagnosis. Abdominal symptoms including the feeling of hunger were assessed by using the modified questionnaire that has been applied in previous studies.[1,21,22] We assessed abdominal symptoms including the feeling of hunger using the modified Glasgow dyspepsia severity score,[23] which consist of frequency (never; score 0, on only 1 or 2 days; score 1, on approximately 1 day per week; score 3, on approximately 50% of days; score; score 4, on most days; score 5), duration of symptoms (maximal score 5) and intensity of symptoms (maximal score 3). Status of depression was evaluated by SRQ-D (Self-Rating Questionnaire For Depression) score.[24]

### Measurement of gastric motility

Sodium acetate (water soluble) for emptying of liquids was used as tracer (Cambridge Isotope Laboratories; MA, USA). Probes were analyzed by non-dispersive infrared spectroscopy (IRIS, Wagner Analyzentechnik; Bremen, Germany). The subject's own production of 300 mmol $CO_2$ per $m^2$ body surface and per hour were set as default. We used an Integrated Software Solutions program to calculate the half gastric emptying time ($T_{1/2}$) and the lag phase ($T_{max}$; min) as the point of maximum gastric emptying according to Hellmig *et al.*[25] $T_{max}$ value greater than 60 min, representing the mean $T_{max}$ in healthy volunteers plus SD, was defined to represent relative disturbances in gastric emptying according to

the diagnostic criteria of the Japan Society of Smooth Muscle Research and our study.[3,26]

## Study protocol for gastric emptying of liquids

The liquid test meal consisted of 100 mg of $^{13}$C-acetate dissolved in 200 mL of liquid meal (Racol, 1 mL kcal$^{-1}$; Otsuka Pharmacia Company, Tokyo, Japan). Breath samples were collected 0, 10 s, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min, 60 min, 75 min, and 90 min after ingestion of the test meal at 10:00 a.m. Patients were instructed not to drink, eat, or smoke during the test.

## Genotyping

We have developed or optimized the following assays for genetic variation. Genotypes were confirmed or selectively assessed for GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{3A}$, 5-HT$_{4A}$ genotypes by direct sequencing using an ABI 7500 Fast. Gene polymorphisms were determined by methods in the literature. Real-time polymerase chain reaction using TaqMan chemistries (Applied Biosystems, Foster City, CA, USA) was used to determine alleles present in each sample. Real-time polymerase chain reactions were performed in an Applied Biosystems 7500 Fast machine (Applied Biosystems). TaqMan primer-probe assays for GNβ3 SNPs C825T (rs:5443; C-2184734-10), 5-HT$_{1A}$ SNPs C1019G (rs:6296; C-11904666-10), 5-HT$_{2A}$ SNPs G1438A (rs:6311; C-7488465-10), and 5-HT$_{3A}$ SNPs C42T (rs:1062613; C-2184734-10), 5-HT$_{4A}$ SNPs G353 + 6A (rs:2278392; C-15965377-10) were purchased from Applied Biosystems. In briefly, each reaction volume was 10 μl and consisted of 5 μl of a TaqMan Genotyping Master Mix (Applied Biosystems), 0.25 μl of a 40 × primer probe assay mix (Applied Biosystems), $H_2O$ 3.75 μl and 1 μl (10 ng) genomic DNA. Amplification conditions consisted of 95 °C, 10 min; 40 cycles of: 95 °C, 15 s; 60 °C, 60 s; followed by 50 °C, 2 min. And then analyzed using automated software (SDS 2.1; Applied Biosystems) to determine the genotype of each sample.

## Measurement of plasma ghrelin levels in FD patients

We measured plasma ghrelin levels to evaluate their association with polymorphism of GNβ3 825CT. Blood samples were obtained after an overnight fast of > 12 h, immediately transferred to chilled polypropylene tubes containing $Na_2$EDTA and aprotinin, then centrifuged at 4 °C. One tenth of the volume of 1N HCl was immediately added to the separated plasma. The acylated and des-acylated forms of ghrelin were measured using commercially available ELISA kits according to the manufacturer's instructions (Active Ghrelin ELISA Kit and Desacly-Ghrelin ELISA Kit, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). The intra- and inter-assay coefficients of variation (CV) were 6.5% and 9.8% for acylated ghrelin, and 3.7% and 8.1% for des-acylated ghrelin.

## Statistical analysis

For statistical evaluation of group data, Students' *t*-test for paired data and analysis of variance (ANOVA) for multiple comparisons were followed by Scheffe's F test. Mann–Whitney *U* test was used for analysis of categorical data. To determine factors that associated with the disturbance of gastric emptying, multiple logistic regression analysis was used at 95% confidence intervals and associated *P* values. A *P* value < 0.05 was statistically significant.

# RESULTS

## Characteristics of FD patients and healthy volunteers

The age, sex, and BMI in FD and healthy volunteers were not statistically different (Table 1). SRQ-D score in FD patients was also significantly higher ($P < 0.001$) compared with that of healthy volunteers. Both of $T_{1/2}$ and $T_{max}$ values in FD patients were significantly ($P < 0.001$, $P < 0.001$) higher compared with those of healthy volunteers. The proportion of disturbed gastric emptying in FD patients (43.2%) was significantly ($P < 0.01$) higher compared with that of healthy volunteers (4.7%) (Table 1).

## GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes in FD patients

GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes distribution in FD were 14CC (18.9%), 44CT (59.5%), 16TT (21.6%); 44GG (59.5%), 28GC (37.8%), 2CC (2.7%); 17CC (23.0%), 35CT (47.3%), 22TT (29.7%); 58CC (78.4%), 16CT (21.6%); 7AA (9.4%), 29GA (39.2%), 38GG (51.4%), respectively. Meanwhile, in the healthy controls, GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes distribution were 17CC (26.6%), 28CT (43.7%), 19TT (29.7%); 33GG (51.6%), 28GC (43.7%), 3CC (4.7%); 18CC (28.1%), 30CT (46.9%), 16TT (25%); 49CC (76.5%), 14CT (21.9%), 1TT (1.6%); 7AA (10.9%), 22GA (34.4%), 35GG (54.7%), respectively. Each genotype distribution was not significantly different in FD patients and healthy volunteers (Table 2).

## Multiple logistic analysis for $T_{max}$ value in FD patients

As various clinical symptoms in FD patients are partly involved in the disturbance of gastric motility, we tried

**Table 1** Characteristics of the patients

| | FD | Healthy volunteer |
|---|---|---|
| Subjects (*n*) | 74 | 64 |
| Age | 59.2 ± 14.2 | 37.2 ± 9.13 |
| Sex (M/F) | 36/38 | 57/7 |
| BMI | 22.2 ± 2.57 | 22.9 ± 2.63 |
| SRQ-D | 9.94 ± 0.71 | 6.14 ± 0.49 |
| $T_{1/2}$ | 94.5 ± 3.54 | 72.8 ± 1.62 |
| $T_{max}$ | 59.2 ± 1.74 | 46.7 ± 0.95 |
| Disturbed gastric emptying (%) | 43.2 | 4.7 |

FD, functional dyspepsia.

**Table 2** GN$\beta$3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$ and 5-HT$_{4A}$ genotypes in FD patients

| Variables *n* (%) | Genotype CC | CT | TT | OR CC *vs* others | *P* value |
|---|---|---|---|---|---|
| GN$\beta$3-G825C polymorphism and FD | | | | | |
| Healthy volunteers (*n* = 64) | 17 (26.6) | 28 (43.7) | 19 (29.7) | Reference | |
| FD (*n* = 74) | 14 (18.9) | 44 (59.5) | 16 (21.6) | 0.645 | 0.283 |
| Variables *n* (%) | Genotype GG | GC | CC | OR GG *vs* others | *P* value |
| 5-HT$_{1A}$-C1019G polymorphism and FD | | | | | |
| Healthy volunteers (*n* = 64) | 33 (51.6) | 28 (43.7) | 3 (4.7) | Reference | |
| FD (*n* = 74) | 44 (59.5) | 28 (37.8) | 2 (2.7) | 1.38 | 0.316 |
| Variables *n* (%) | Genotype CC | CT | TT | OR CC *vs* others | *P* value |
| 5-HT$_{2A}$-G1438A polymorphism and FD | | | | | |
| Healthy volunteers (*n* = 64) | 18 (28.1) | 30 (46.9) | 16 (25.0) | Reference | |
| FD (*n* = 74) | 17 (23.0) | 35 (47.3) | 22 (29.7) | 0.762 | 0.488 |
| Variables *n* (%) | Genotype CC | CT | TT | OR CC *vs* others | *P* value |
| 5-HT$_{3A}$-G42T polymorphism and FD | | | | | |
| Healthy volunteers (*n* = 64) | 49 (76.5) | 14 (21.9) | 1 (1.6) | Reference | |
| FD (*n* = 74) | 58 (78.4) | 16 (21.6) | 0 (0) | 1.11 | 0.798 |
| Variables *n* (%) | Genotype AA | GA | GG | OR AA *vs* others | *P* value |
| 5-HT$_{4A}$-G353 + 6A polymorphism and FD | | | | | |
| Healthy volunteers (*n* = 64) | 7 (10.9) | 22 (34.4) | 35 (54.7) | Reference | |
| FD (*n* = 74) | 7 (9.4) | 29 (39.2) | 38 (51.4) | 1.35 | 0.825 |

to clarify whether these parameters including age, BMI, sex, SRQ-D score, FD symptoms, *H. pylori* infection, and five genotypes are linked to $T_{max}$ value as a marker of gastric emptying. Multiple logistic regression analysis revealed that there was no significant relationship between these parameters and $T_{max}$ value in FD patients (Table 3).

## GN$\beta$3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes in PDS patients with or without impaired gastric emptying

We then compared five genotypes (GN$\beta$3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$) in FD patients with or without disturbance of gastric emptying. We divided FD patients into two groups which are disturbed with gastric emptying ($T_{max}$ value > 60 min) and normal gastric emptying ($T_{max}$ value < 60 min). We could not find a significant correlation ($P = 0.620$; $P = 0.760$; $P = 0.365$; $P = 0.570$; $P = 0.691$) between $T_{max}$ value and genotype of GN$\beta$3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ in seventy-four FD patients, respectively. The proportion of GN$\beta$3 825C/C in FD patients with disturbed gastric emptying was relatively greater ($P = 0.06$) compared with that of healthy volunteers (data not shown).

To investigate whether there is any significant difference in five genotypes and $T_{max}$ value as a marker of gastric emptying in PDS patients, we compared genotypes with PDS patients with or without disturbed gastric emptying. We found that there was no significant relationship ($P = 0.501$; $P = 0.131$; $P = 0.924$; $P = 0.490$; $P = 0.390$) between five genotypes and PDS patients with impaired gastric emptying, respectively (Table 4).

**Table 3** Multiple logistic analysis for impaired $T_{max}$ value in FD patients ($n$ = 74)

| Factor | Odds ratio (95% CI) | $P$ value |
|---|---|---|
| Age | 1.034 (0.998–1.071) | 0.061 |
| BMI | 1.032 (0.857–1.242) | 0.742 |
| SEX | 1.100 (0.438–2.761) | 0.839 |
| SRQ-D | 0.993 (0.919–1.074) | 0.897 |
| Heartburn | 0.955 (0.899–1.016) | 0.146 |
| PDS-like* | 1.957 (0.639–5.992) | 0.239 |
| EPS-like† | 0.796 (0.208–3.046) | 0.739 |
| *H. pylori* | 1.821 (0.273–3.787) | 0.271 |
| GNβ3 (CC; CT/TT) | 1.400 (0.436–4.496) | 0.577 |
| 5-HT$_{1A}$ (GG; GC/CC) | 0.791 (0.310–2.017) | 0.624 |
| 5-HT$_{2A}$ (CC; CT/TT) | 1.957 (0.639–5.992) | 0.239 |
| 5-HT$_{3A}$ (CC; CT/TT) | 1.916 (0.591–6.214) | 0.280 |
| 5-HT$_{4A}$ (AA; GA/GG) | 2.027 (0.206–4.928) | 0.418 |

FD, functional dyspepsia; PDS, postprandial distress syndrome; EPS, epigastric pain syndrome.
*Most bothersome symptom based on physician interview was early satiety.
†Most bothersome symptom based on physician interview was upper abdominal pain.

**Table 4** Association between GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ polymorphism and gastric emptying in PDS patients

| Genotypes | $T_{max}$ > 60 min | $T_{max}$ < 60 min | OR (95% CI) | $P$ value |
|---|---|---|---|---|
| GNβ3 CC | 6 | 5 | 2.00 (0.519–7.7703) | 0.501 |
| CT/TT | 15 | 25 | | |
| 5-HT$_{1A}$ GG | 17 | 17 | 3.25 (0.879–12.00) | 0.131 |
| GC/CC | 4 | 13 | | |
| 5-HT$_{2A}$ CC | 6 | 7 | 1.31 (0.368–4.663) | 0.924 |
| TT/CT | 15 | 23 | | |
| 5-HT$_{3A}$ CC | 18 | 22 | 2.18 (0.503–9.442) | 0.490 |
| CT/TT | 3 | 8 | | |
| 5-HT$_{4A}$ AA | 2 | 4 | 0.37 (0.061–2.232) | 0.390 |
| GA/GG | 26 | 19 | | |

PDS, postprandial distress syndrome.

**Table 5** Association between GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ polymorphism and gastric emptying in PDS patients without reflux

| Genotypes | $T_{max}$ > 60 min | $T_{max}$ < 60 min | OR (95% CI) | $P$ value |
|---|---|---|---|---|
| GNβ3 CC | 6 | 3 | 5.71 (1.117–2.918) | 0.045 |
| CT/TT | 7 | 20 | | |
| 5-HT$_{1A}$ GG | 8 | 13 | 1.6 (0.399–6.414) | 0.953 |
| GC/CC | 5 | 10 | | |
| 5-HT$_{2A}$ CC | 5 | 6 | 0.63 (0.147–2.697) | 0.690 |
| TT/CT | 8 | 17 | | |
| 5-HT$_{3A}$ CC | 12 | 16 | 5.25 (0.567–48.58) | 0.213 |
| CT/TT | 1 | 7 | | |
| 5-HT$_{4A}$ AA | 1 | 3 | 0.43 (0.040–4.593) | 0.625 |
| GA/GG | 14 | 18 | | |

PDS, postprandial distress syndrome.

## GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes in PDS patients without gastro-esophageal reflux symptom

Moreover, to investigate whether there is any significant relationship between five genotypes and $T_{max}$ value in PDS patients without gastro-esophageal reflux symptom, we compared five genotypes with PDS patients without gastro-esophageal reflux symptom with or without impaired gastric emptying in similar way. We confirmed that there was a significant relationship ($P$ = 0.045) between GNβ3 825CC genotype and PDS patients without gastro-esophageal reflux symptom accompanying impaired gastric emptying (Table 5). In contrast, there were no significant relationship between 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes and PDS patients without gastro-esophageal reflux symptom accompanying with impaired gastric emptying ($P$ = 0.953; $P$ = 0.690; $P$ = 0.213; $P$ = 0.625) (Table 5).

## Association between GNβ3, 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{3A}$, and 5-HT$_{4A}$ genotypes and clinical symptoms in FD patients

As GNβ3 genotype has been previously reported to be linked to clinical symptoms in FD patients, we also determined whether five genotypes including GNβ3 are associated with several clinical symptoms in Japanese FD patients based on Rome III classification. In our FD populations, GNβ3 825CC genotype in FD patients is significantly ($P$ = 0.0485) associated with the feeling of hunger compared with GNβ3 825CT and TT genotypes (Fig. 1).

As ghrelin levels have been reported to be associated with appetite, we tried to determine whether plasma ghrelin levels are linked to GNβ3 825CC genotype in FD patients. We measured both plasma acylated ghrelin (7.08 ± 0.63 fmol mL$^{-1}$) and des-acylated ghrelin levels (74.7 ± 6.22 fmol mL$^{-1}$) in FD patients. There was no significant difference ($P$ = 0.269) in acylated ghrelin levels in GNβ3 825CC and GNβ3 825CT/TT genotypes.

In contrast, there are not significant differences between 5-HT$_{1A}$ GG, 5-HT$_{2A}$ CC, 5-HT$_{3A}$ CC, and 5-HT$_{4A}$ AA genotypes and clinical symptoms compared with other genotypes, respectively (Fig. 1).

## DISCUSSION

The major findings of this study are (i) There was a significant relationship between GNβ3 825CC genotype and PDS patients without gastro-esophageal reflux

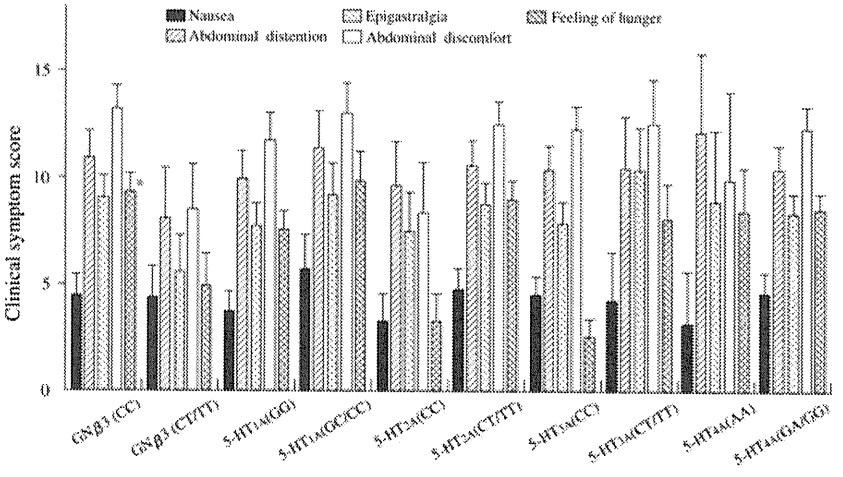


**Figure 1** Association between GN$\beta$3, 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{3A}$, and 5-$HT_{4A}$ genotypes and gastrointestinal symptoms in FD patients. There was no significant relationship between 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{3A}$, 5-$HT_{4A}$, and various clinical symptoms. In Japanese, GN$\beta$3 825CC genotype in FD patients is significantly ($P = 0.0485$) associated with the feeling of hunger compared with GN$\beta$3 825CT and TT genotypes. GN$\beta$3: 14CC, 60CT/TT; 5-$HT_{1A}$: 44GG, 30GC/CC; 5-$HT_{2A}$: 17CC, 57CT/TT; 5-$HT_{3A}$: 58CC, 16CT/TT; 5-$HT_{4A}$: 7AA, 67GA/GG. * *vs* GN$\beta$3 825CT/TT genotypes. FD, functional dyspepsia.

symptoms with impaired gastric emptying, (ii) GN$\beta$3 825CC genotype in FD patients was significantly associated with the feeling of hunger symptom compared with GN$\beta$3 825CT and TT genotypes.

There is increasing evidence that susceptibility to functional GI disorders is also influenced by hereditary factors.[27–29] In this study, we have first reported that GN$\beta$3 CC variant was significantly associated with disturbance of gastric emptying in PDS patients without gastro-esophageal reflux symptoms. Previous studies have reported that there is reasonable evidence that the GN$\beta$3 status is associated with depression,[30] increased immune cell activation,[11] and altered activation of $\alpha$2-adrenoreceptors.[31] In addition, Holtmann *et al.* have reported that the homozygous GN$\beta$3 825CC was associated with upper abdominal symptoms unrelated to meals in Germany,[12] while previous studies have shown that the homozygous GN$\beta$3 825CC or 825TT was also associated with meal-unrelated dyspepsia in people randomly selected from the US community and EPS patients in Japan.[13,32] Recent study has reported that there was no significant relationship between gastric emptying and GN$\beta$3 genotype in Rome II-based FD patients.[33] In this study, we investigated the relationship between gastric emptying and the GN$\beta$3 subunit 825 genotype among FD, PDS, and healthy volunteers based on Rome III classification. We have previously reported that the $T_{max}$ value as a marker of gastric emptying in PDS patients was significantly greater compared with that of healthy volunteers.[3] Therefore, in our study, we focused on the GN$\beta$3 genotype in PDS patients with or without impaired gastric emptying and found a significant relationship between GN$\beta$3 825CC genotype and impaired gastric emptying in PDS patients without gastro-esophageal reflux symptoms. In addition, because of small number of subjects of Rome III subgroups, type II error could not be excluded. Thus, our result should be treated carefully with caution until replicated.

The age of onset of gastro-esophageal reflux disease (GERD) is variable and many individuals develop the disease during childhood. Gastro-esophageal reflux disease is the most common esophageal disorder of children, affecting about 11% of all infants during their first year of life.[34] Epidemiological data justify theory formation about a genetic component in the pathophysiology of GERD. The disease etiology is further complicated by a substantial genetic contribution as shown by familial clustering,[35] autosomal dominant familial transmission of disease[36,37] as well as twin studies.[38] As Vries *et al.* have reported that GERD is associated with the GN$\beta$3 825CT genotype,[39] we investigated whether the GN$\beta$3 825CC variant were associated with disturbance of gastric emptying in PDS patients without gastro-esophageal reflux symptoms in this study. Further studies are needed to clarify whether the reduction of threshold of 5-HTs receptors is associated with reflux symptom through protein kinase-mediated signaling pathways induced by impairment of G-protein-coupled receptors (GPCRs) via GN$\beta$3 825CT variant in these patients.

Considering that there were the discrepancy about clinical symptoms and gastric motility for GN$\beta$3 825 alleles of FD patients in several countries,[12,13,19,32,33] it seems to be a very important factor that Japanese patients with *H. pylori*-infected gastritis have low levels of acid secretion compared with Europeans and Americans.[40] It is very critical issue about the relationship between gastric acidity and gastric motility because Lee *et al.* and Schwartz *et al.* have reported that intraduodenally administrated acid affects gastroduodenal motility as well as visceral hypersensitivity.[41,42] Considering these previous studies, high

prevalence of *H. pylori* infection may be considered to play an important role in the etiology of certain FD patients in Japan.[43] Saito *et al.* have reported that acceleration of gastric emptying was observed in *H. pylori*-infected animal model.[44] In our study, the proportion of *H. pylori*-infected PDS patients was 37%. In our previous study, *H. pylori* infection reduced ghrelin-producing cell numbers which are linked to gastric emptying.[45] Therefore, in the future study, we should investigate whether GNβ3 825 genotype may be linked to gastric emptying among *H. pylori*-negative subjects. Our findings thus needed to be replicated in different populations and other races. On the other hand, the allele distribution in controls was very similar to allele distributions that have been observed in previous studies of Japanese.[32,46] Oshima *et al.* and Tahara *et al.* have reported GNβ3 825TT genotype is associated with EPS-like dyspepsia or dyspepsia, respectively. [32,46] This discrepancy between these reports and our results may have occurred in sample selection, such as patient's age, psychological condition (SRQ-D score is high), Rome III-categorized patients, and visiting care centers. The novel 5-$HT_{1A}$ agonist R137696 has been reported to affect the proximal gastric function[16] as well as previous studies.[47,48] 5-$HT_{3A}$ receptors also seem to be involved both in the transmission of the sensation that arises from the stomach and in the process of gastric emptying and accommodation.[49] However, we have first compared genotypes of 5-HTs with gastric emptying in Rome III-based FD patients. We could not find any significant relationship between genotypes of 5-HTs such as 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{3A}$, and 5-$HT_{4A}$ and gastric emptying in these FD patients.

In the present data, there was a significant relationship between the feeling of hunger and GNβ3 825CC genotype in Rome III-based FD patients. In contrast, in Japanese patients, Tahara *et al.* have reported that the homozygous 825T allele of the GNβ3 protein influences the susceptibility of Japanese to dyspepsia.[47] In our data, we could not find any significant relationship between the GNβ3 825CC genotype and Rome III-based symptoms, such as abdominal distention, epigastralgia, and abdominal discomfort. However, we investigated that there was a significant relationship between the feeling of hunger and the GNβ3 825CC genotype in FD patients. We could not find a significant relationship between disturbed $T_{max}$ value and the feeling of hunger ($P = 0.608$) or early satiety ($P = 0.239$) in FD patients using multiple logistic analysis. In contrast, Stanghellini *et al.* have reported that disturbed gastric emptying is associated with satiation and impaired food intake.[7] We have previously reported that there was a significant relationship between low level of acylated ghrelin linked to appetite and $T_{max}$ value.[3] However, in our data, the score for feeling of hunger was not significantly ($P = 0.473$) associated with acylated-ghrelin levels. In addition, there was also no significant difference ($P = 0.269$) in acylated ghrelin levels in GNβ3 825CC and GNβ3 825CT/TT genotypes. Further studies are needed to clarify the mechanism by which the GNβ3 825CC genotype is associated with the feeling of hunger in *H. pylori*-negative FD patients.

Taken together, in this study, we determined that there was a significant relationship between impairment of gastric emptying and the GNβ3 825CC genotype in Rome III-based PDS patients without gastro-esophageal reflux symptoms. Further studies are needed to clarify whether the GNβ3 825CC genotype are linked to disturbance of gastric emptying and feeling of hunger via diminished transduction responses.

## REFERENCES


1 Tack J, Talley NJ, Camilleri M *et al.* Functional gastroduodenal disorders. *Gastroenterology* 2006; **130**: 1466–79.
2 Quigley EM. Gastric emptying in functional gastrointestinal disorders. *Aliment Pharmacol Ther* 2004; **20**(suppl 7): 56–60.
3 Shindo T, Futagami S, Hiratsuka T *et al.* Comparison of gastric emptying and plasma ghrelin levels in patients with functional dyspepsia and non-erosive reflux disease. *Digestion* 2009; **79**: 65–72.
4 Futagami S, Iwakiri K, Shindo T *et al.* The prokinetic effect of mosapride citrate combined with omeprazole therapy improves clinical symptoms and gastric emptying in PPI-resistant NERD patients with delayed gastric emptying. *J Gastroenterol* 2010; **45**: 413–21.
5 Hamm HE. The many faces of G protein sihnaling. *J Biol Chem* 1998; **273**: 669–72.
6 Holtmann G, Gschossmann J, Neufang-Hüber J, Gerken G, Talley NJ. Differences in gastric mechanosensory function after repeated ramp distensions in non-consulters with dyspepsia and healthy controls. *Gut* 2000; **47**: 332–6.
7 Stanghellini V, Tosetti C, Patermico A *et al.* Risk indicators of delayed gastric emptying of solids in patients with functional dyspepsia. *Gastroenterology* 1996; **110**: 1036–42.
8 Wilmer A, Van Cutsem E, Andrioli T, Tack J, Coremans G, Janssens J. Ambulatory gastro-jejunal manometry in severe motility-like dyspepsia: lack of correlation between dysmotility, symptoms, and gastric emptying. *Gut* 1999; **42**: 235–42.
9 Greydanus MP, Vassallo M, Camilleri M, Nelson DK, Hanson RB, Thomforde GM. Neurohormonal factors in functional dyspepsia: insights on pathophysiological mechanisms. *Gastroenterology* 1991; **100**: 1311–8.

10 Drossman DA, McKee DC, Sandler RS *et al.* Psychological factors in the irritable bowel syndrome. A multivariate study of patients and nonpatients with irritable bowel syndrome. *Gastroenterology* 1988; **95**: 701–8.

11 Baumgart D, Naber C, Haude M *et al.* G protein beta 3 subunit 825 T allele and enhanced coronary vasoconstriction on alpha(2)-adrenoceptor activation. *Cir Res* 1999; **85**: 965–9.

12 Holtman G, Siffert W, Haag S *et al.* G-protein beta 3 subunit 825 CC genotype is associated with unexplained (functional) dyspepsia. *Gastroenterology* 2004; **126**: 971–9.

13 Camilleri CE, Carlson PJ, Camilleri M *et al.* A study of candidate genotypes associated with dyspepsia in a U.S. community. *Am J Gastroenterol* 2006; **101**: 581–92.

14 Locke GR 3rd, Zinsmeister AR, Talley NJ *et al.* Familial association in adults with functional gastrointestinal disorders. *Mayo Clin Proc* 2000; **75**: 907–12.

15 Van Oudenhove L, Kindt S, Vos R, Coulie B, Tack J. Influence of buspirone on gastric sensorimotor function in man. *Aliment Pharmacol Ther* 2008; **28**: 1326–33.

16 Boeckxstaens GE, Tygat GN, Wajs E *et al.* The influence of the novel 5-HT1A agonist R137696 on the proximal stomach function in healthy volunteers. *Neurogastroenterol Motil* 2006; **18**: 919–26.

17 Graf S, Sarna SK. 5-HT-induced jejunal motor activity: enteric locus of action and receptor subtypes. *Am J Physiol* 1996; **270**: G992–1000.

18 Grider JR, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine4 receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* 1998; **115**: 370–80.

19 Van Lelyveld N, Linde JT, Schipper M, Samsom M. Candidate genotypes associated with functional dyspepsia. *Neurogastroenterol Motil* 2008; **20**: 767–73.

20 Drossman DA. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006; **130**: 1377–90.

21 McColl K, Murray L, El-Omar E *et al.* Symptomatic benefit from eradicating *Helicobacter pylori* infection in patients with nonulcer dyspepsia. *N Engl J Med* 1998; **339**: 1869–74.

22 Portincasa P, Altomare DF, Moschetta A *et al.* The effect of acute oral erythromycin on gallbladder motility and on upper gastrointestinal symptoms in gastrectomized patients with and without gallstones: a randomized, placebo-controlled ultrasonographic study. *Am J Gastroenterol* 2000; **95**: 3444–51.

23 Hellmig S, Von Schoning F, Gadow C *et al.* Gastric emptying time of fluids and solids in healthy subjects determined by $^{13}C$ breath tests: influence of age, sex and body mass index. *J Gastroenterol Hepatol* 2006; **21**: 1832–8.

24 Rockliff BW. A brief self-rating questionnaire for depression (SRQ-D). *Psychosomatics* 1969; **10**: 236–43.

25 El-Omar EM, Banerjee S, Wirz A, McColl KE. The Glasgow dyspepsia severity score-a tool for the global measurement of dyspepsia. *Eur J Gastroenterol Hepatol* 1996; **8**: 967–71.

26 Futagami S, Shindo T, Kawagoe T *et al.* Migration of eosinophils and CCR2-/CD68-double positive cells into the duodenal mucosa of patients with postinfectious functional dyspepsia. *Am J Gastroenterol* 2010; **105**: 1835–42.

27 Morris-Yates A, Talley NJ, Boyce PM, Nandurkar S, Andrews G. Evidence of a genetic contribution to functional bowel disorders. *Am J Gastroenterol* 1998; **8**: 1311–7.

28 Levy RL, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel synfrome in twins; heredity and social learning both contribute to etiology. *Gastroenterology* 2001; **121**: 799–804.

29 Kalantar JS, Locke GR 3rd, Zinsmeister AR, Beighley CM, Talley NJ. Familial aggregation of irritable bowel syndrome: a prospective study. *Gut* 2003; **52**: 1703–7.

30 Zill P, Baghai TC, Zwanzger P *et al.* Evidence for an association between a G-protein beta3-gene variant with depression and response to antidepressant treatment. *Neuroreport* 2000; **11**: 1893–7.

31 Lidemann M, Virchow S, Ramann F *et al.* The G protein beta 3 subunit 825T allele is a genetic marker for enhanced T cell response. *FEBS Lett* 2001; **495**: 82–6.

32 Oshima T, Nakajima S, Yokoyama T *et al.* The G-protein beta3 subunit 825 TT genotype is associated with epigastric pain syndrome-like dyspepsia. *BMC Med Genet* 2010; **11**: 13.

33 Grudell ABM, Camilleri M, Carlson P *et al.* An exploratory study of the association of adrenergic and serotonergic genotype and gastrointestinal motor functions. *Neurogastroenterol Motil* 2008; **20**: 213–9.

34 Costa AJ, Silva GA, Gouveia PA, Pereira Filho EM. Prevalence of pathologic gastroesophageal reflux in regurgitant infants. *J Pediatr (Rio J)* 2004; **80**: 291–5.

35 Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. *Am J Gastroenterol* 1999; **94**: 1172–8.

36 Hu FZ, Preston RA, Post JC *et al.* Mapping of a gene for severe pediatric gastroesophageal reflux to chromosome 13q14. *JAMA* 2000; **284**: 325–34.

37 Orenstein SR, Shalaby TM, Finch R *et al.* Autosomal dominant infantile gastroesophageal reflux disease: exclusion of a 13q14 locus in five well characterized families. *Am J Gastroenterol* 2002; **97**: 2725–32.

38 Mohammed I, Cherkas LF, Riley SA, Spector TD, Trudgill NJ. Genetic influences in gastro-oesophageal reflux disease: a twin study. *Gut* 2003; **52**: 1085–9.

39 Vries DR, Linde JJ, Herwaarden MA, Smout AJ, Samsom M. Gastroesophageal reflux disease is associated with the C825T polymorphism in the G-protein $\beta 3$ subunit gene (GN$\beta 3$). *Am J Gastroenterol* 2009; **104**: 281–5.

40 Kapadia CR. Gastric atrophy, metaplasia, and dysplasia: a clinical perspective. *J Clin Gastroenterol* 2003; **36** (Suppl 5): S29–36.

41 Lee KJ, Vos R, Janssens J, Tack J. Influence of duodenal acidification on the sensorimotor function of the proximal stomach in humans. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G278–84.

42 Schwartz MP, Samsom M, Smout AJ. Chemospecific alterations in duodenal perception and motor response in functional dyspepsia. *Am J Gastroenterol* 2001; **96**: 2596–602.

43 Suzuki H, Hibi T, Marshall BJ. *Helicobcater pylori*: present status and future prospects in Japan. *J Gastroenterol* 2007; **42**: 1–15.

44 Saito Y, Suzuki H, Tsugawa H *et al.* Dysfunctional gastric emptying with down-regulation of muscle-specific microRNAs in *Helicobacter pylori*-infected mice. *Gastroenterology* 2011; **140**: 189–98.

45 Tatsuguchi A, Miyake K, Gudis K *et al.* Effect of *Helicobacter pylori* infection on ghrelin expression in

human gastric mucosa. *Am J Gastroenterol* 2004; **99**: 2121–7.
46 Tahara T, Arisawa T, Shibata T *et al.* Homozygous 825T allele of the GNB3 protein influences the susceptibility of Japanese to dyspepsia. *Dig Dis Sci* 2008; **53**: 642–6.
47 Coulie B, Tack J, Sifrim D, Andrioli A, Janssens J. Role of nitric oxide in fasting gastric fundus tone and in 5-HT1 receptor-mediated relaxation of gastric fundus. *Am J Physiol* 1999; **276**: G373–7.
48 Tack J, Coulie B, Wilmer A, Andrioli A, Janssens J. Influence of sumatriptan on gastric fundus tone and on the perception of gastric distension in man. *Gut* 2000; **46**: 468–73.
49 Tack J, Sarnelli G. Serotonergic modulation of visceral sensation: upper gastrointestinal tract. *Gut* 2002; **51**: 77–80.

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CASE REPORT

# Eosinophilic esophagitis: a case report with a review of the literature

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**Abstract** Eosinophilic esophagitis (EE) is an allergic inflammatory condition of the esophagus and is characterized by dense eosinophilic infiltration of the esophagus. There has been a dramatic increase in the diagnosis of EE in Western countries in recent years; however, in Japan, there are very few reports of EE. We present a rare case of EE in a 70-year-old Japanese woman, who had dysphasia for 2 years, but which worsened over a 6-month period. Laboratory examinations showed peripheral eosinophilia (1279/μl). Significant thickening of the esophageal wall was observed on computed tomography scan and many circular rings appeared when an esophagogastroduodenoscopy was carried out. From these circular rings, EE was suspected and a biopsy was then taken from the esophagus. As the histologic findings from the esophageal biopsy showed that >25 eosinophils existed per high-power field, the patient was diagnosed with EE. Oral corticosteroid (prednisolone 30 mg/day) therapy was administered and after 3 days of treatment her symptoms almost disappeared. EE needs to be considered as a differential diagnosis if patients with non-erosive reflux disease have dysphagia but do not respond to high-dose proton pump inhibitor therapy.

**Keywords** Eosinophilc esophagitis · Gastroesophageal reflux disease · Dysphagia

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## Introduction

Eosinophilic esophagitis (EE) is a newly-recognized disease, which over the past decade has been increasingly diagnosed in Western countries [1]. It is an allergic inflammatory condition of the esophagus and is characterized by dense eosinophilic infiltration of the esophagus [2]. Patients with EE suffer from gastroesophageal reflux symptoms, such as dysphagia, food impaction and heartburn and in the past, EE has been confused with eosinophilic gastroenteritis, food allergy, and gastroesophageal reflux disease (GERD) [3].

There are only a few reports of EE from Japan and other Asian countries [4–6]. We present a rare case of EE in Japan.

## Case report

We report on a 70-year-old Japanese woman who had dysphasia for 2 years, but which worsened over a 6-month period, and during this time a sore throat and heartburn also appeared. She visited the general hospital, where her upper gastrointestinal tract was examined using endoscopy but no abnormal findings were apparent and she was diagnosed with non-erosive reflux disease (NERD). She then took a double-dose of proton pump inhibitor (PPI) for 4 weeks but her symptoms did not improve. She also visited the

Department of Otolaryngology in the general hospital, where she was evaluated using a laryngoscope and she also underwent a cervical esophagography. The laryngoscope showed no abnormal findings but the cervical esophagography showed a transient esophageal narrowing in the middle to lower esophagus (Fig. 1). The otolaryngologist thought that the esophageal motor abnormality was the cause of this esophageal narrowing and the patient was referred to our hospital. At this time, she was unable to eat a solid meal because of having severe dysphagia.

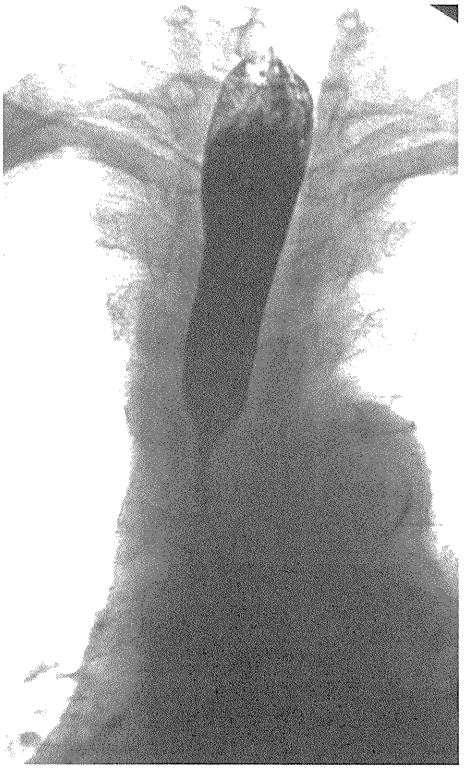

**Fig. 1** Barium cervical esophagogram showing narrowing of the esophageal lumen in the middle to lower esophagus

The patient's medical history included diabetes mellitus, which she has had since the age of 50 and which has been treated with insulin [Inolet 30R (12–12–12 unit)] and bronchial asthma, which she has had since childhood, but which was stable from taking theophylline 200 mg (p.o.) and budesonide air inhalation twice per day. The patient did not suffer from any other allergic disease. With regard to the physical examination, mild edema of the lower limbs was observed but there were no other abnormal findings.

Laboratory examinations on the first day of admission showed that peripheral eosinophilia (1279/μl), HbA1C (7.1%), and glucose (175 mg/dl) during a fasting period were high. The immunoglobulin E (IgE) level was normal and 57 types of the antigen-specific IgE and parasitic worm eggs were examined, all of which were negative.

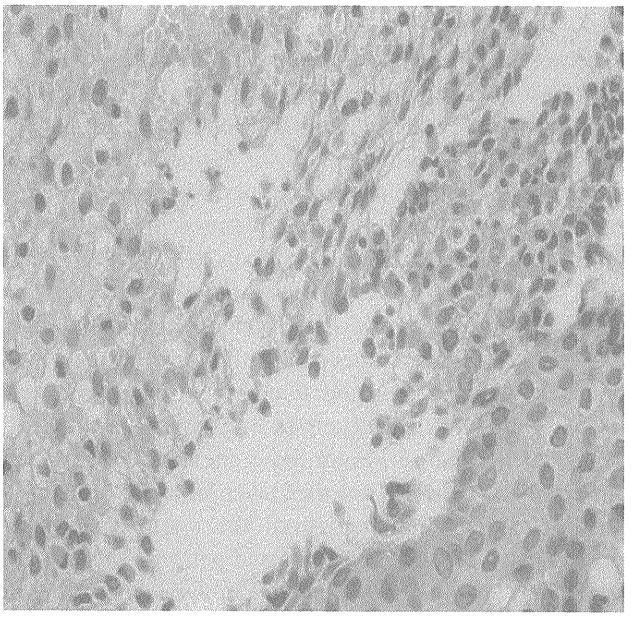

**Fig. 3** Histologic findings from the esophageal biopsy. Pathologic features of the esophagus showing >25 eosinophils in the esophageal mucosa (H&E, ×400)

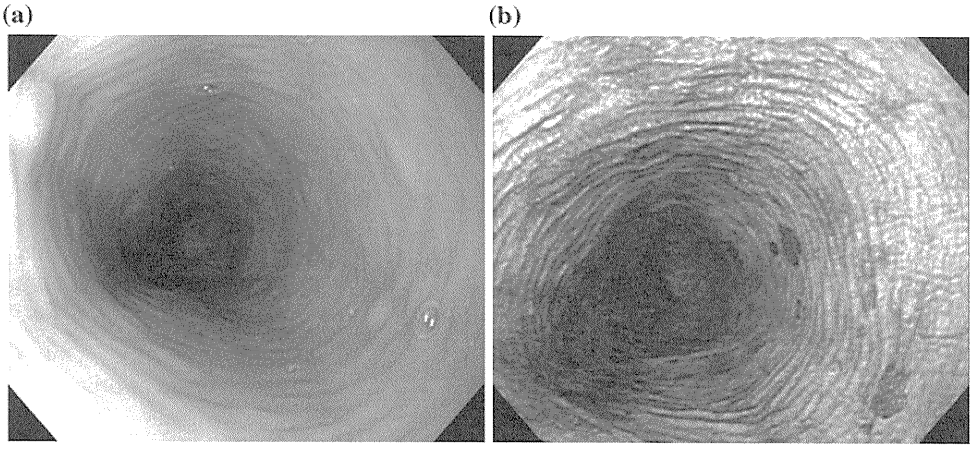


**Fig. 2** Esophageal endoscopic findings. **a** Conventional endoscopic finding, **b** dye esophageal endoscopic finding by iodine staining. Circular rings and edematous esophageal mucosa were observed

An esophagogastroduodenoscopy (EGD) was carried out the day after admission and many circular rings appeared immediately after the endoscope was inserted into the esophagus (Fig. 2a). These circular rings disappeared quickly but the esophageal mucosa was edematous. Except for the circular rings and the edematous esophageal mucosa, there were no abnormal findings in the esophagus, stomach or duodenum. Figure 2b shows esophageal endoscopic findings using iodine staining dye where, after spraying with iodine, many circular rings immediately appeared but then quickly disappeared. From these circular rings, EE was suspected and a biopsy was then taken from the middle esophagus. Histologic findings from the esophageal biopsy showed that >25 eosinophils existed per high-power field (HPF) (Fig. 3).

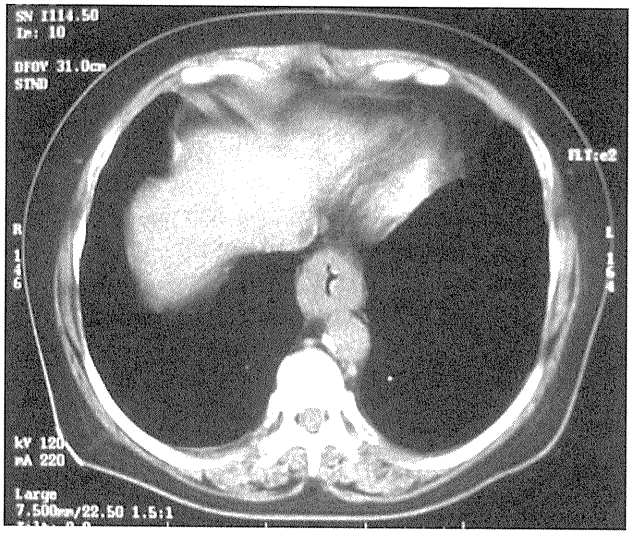


**Fig. 4** CT scan of the chest demonstrating the thickened wall of the esophagus

Significant thickening of the esophageal wall was observed on the computed tomography (CT) scan of the chest 3 days after admission (Fig. 4). Except for this, no abnormal findings were observed on the CT scan of the chest or abdomen. Figure 5 shows esophageal contractions after water swallowing (5 days after admission and prior to treatment), where ineffective esophageal peristalsis was observed.

Three elements need to be present for a diagnosis of EE: (a) clinical symptoms of esophageal dysfunction, (b) >15 eosinophils in 1 high-power field, and (c) lack of responsiveness to high-dose PPI or normal pH monitoring of the distal esophagus. All 3 elements were present in this case, therefore the patient was diagnosed with EE.

After the diagnosis of EE, oral corticosteroid (prednisolone 30 mg per day) was administered to the patient and after 3 days of treatment her symptoms had almost disappeared. After 7 days, the symptoms had completely disappeared and the prednisolone was then gradually tapered off. She now takes 2.5 mg prednisolone per day and there has been no recurrence of the symptoms. Figure 6 shows the clinical course of the symptoms, eosinophil and prednisolone treatment.

An esophagogastroduodenoscopy was carried out 4 weeks after the prednisolone treatment. The circular rings had disappeared and it was possible to see the vessels in the esophageal mucosa (Fig. 7). The histologic findings from the esophageal biopsy 4 weeks after the prednisolone treatment, showed only a few eosinophils in the biopsy specimen (Fig. 8).

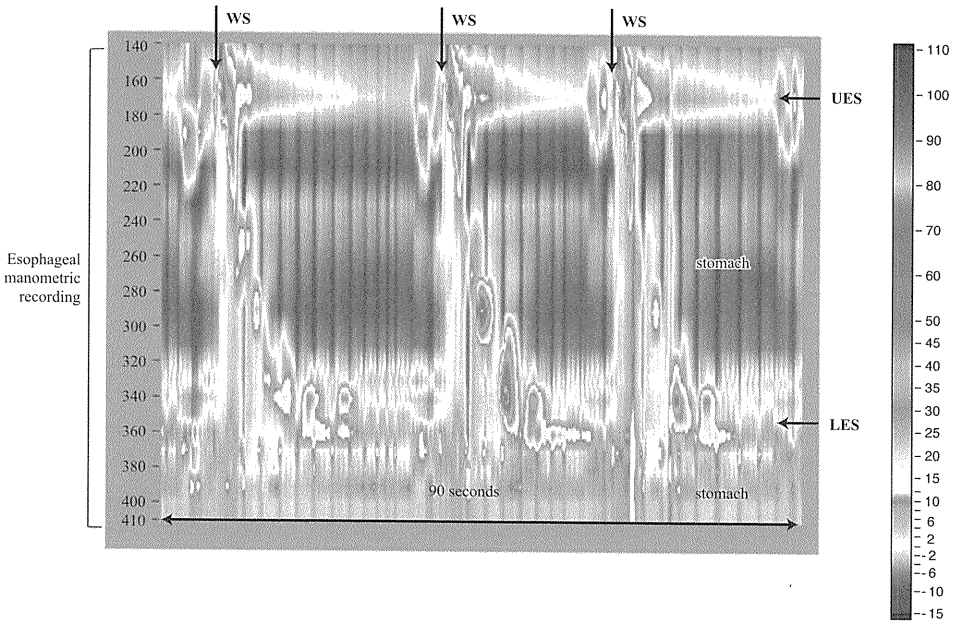


**Fig. 5** High-resolution 21-channel perfused manometric recordings in a patient with eosinophilic esophagitis. Time is on the *x*-axis and distance from nares is on the *y*-axis. A computer program was used to code and record pressures over time for each channel, as outlined in vertical color coding on the *right-hand side* of the figure. Anatomical landmarks and motor events are labeled on the figure. Multiple ineffective esophageal contractions after swallowing 5 ml water can be seen. *LES* lower esophageal sphincter, *UES* upper esophageal sphincter, *WS* water swallowing

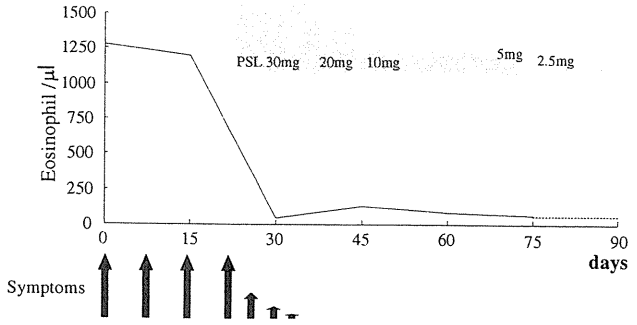


**Fig. 6** Clinical course of symptoms, eosinophils and prednisolone treatment

## Discussion

EE is a chronic inflammatory disease characterized by an elevated count of eosinophils in the esophagus. Although EE had not been previously been thought to affect adults, in recent times, adult cases have been reported [7]. In fact, there has been a dramatic increase in the diagnosis of EE in Western countries over the last few years, due to both an increased awareness of this disorder as well as an actual increase in prevalence [8, 9]. In Japan, however, to date there are only a few reports [4–6].

The first reported case of EE was by Landres and colleagues in 1978 [10]. Many patients with EE have asthma or other atopic conditions and, in addition, 5–58% of patients with EE are reported to show eosinophilia [11–14]. In 40–73% of patients with EE, increased serum IgE levels, a positive RAST result and a positive skin prick test result have also been reported [11, 12, 14, 15]. Our patient showed increased blood eosinophils but the serum IgE level was normal and she had a history of bronchial asthma. The allergen that causes EE is not known but the antigens which trigger EE may include various ingested food allergens as well as inhaled aeroallergens. It is thought that eosinophils cause mucosal damage through the release of cytotoxic granule proteins, reactive oxygen intermediates and lipid mediators [8]. Cytokines and chemokines such as interleukin (IL)-5 and eotaxin, play an important role in the migration and accumulation of eosinophils and T cells and mast cells may also be involved in the inflammatory response [8, 16].

It has been reported that the predominant symptoms of EE in adults are dysphagia and food impaction, and severe dysphagia and heartburn were also the predominant symptoms in our case. Management of these symptoms in patients with EE is difficult as EE is persistently resistant to PPI therapy. A diagnosis of EE is made more difficult because the symptoms of GERD and EE are often similar, and in the past, patients with EE have been incorrectly diagnosed as suffering from GERD.

EE is a primary esophageal disorder in which there are at least 15 eosinophils per HPF on microscopy [17], without associated eosinophilic infiltration of the stomach or intestine. The essential examination, therefore, in order to correctly diagnose EE, is an upper endoscopy with biopsies. One biopsy has a sensitivity of 50%, while five biopsies increase the sensitivity to 100% [18]. GERD patients may also show an elevated eosinophil count, but this elevation is much lower than that of patients with EE. The eosinophilic changes in GERD are limited to the distal esophagus only, where the density is <5–10 eosinophils per HPF. In the diagnostic guidelines of EE, three criteria need to be present for a diagnosis of EE: (a) clinical symptoms of esophageal dysfunction, (b) >15 eosinophils in 1 HPF, and (c) lack of responsiveness to high-dose PPI or normal pH monitoring of the distal esophagus [17]. Histologic findings from the esophageal biopsy in our case showed that >25 eosinophils existed per HPF. As all 3 criteria were present in our case a diagnosis of EE was made.

An esophagogastroduodenoscopy has been reported to reveal abnormalities in the majority of EE patients, such as absent vascular markings, ridges, furrows, vertical lines, corrugations, rings and adherent whitish plaques [7, 19, 20]. In our case, many circular rings appeared immediately after the endoscope was inserted into the esophagus, but they disappeared quickly. Also in cases with EE, the esophageal mucosa has been reported as being fragile and mucosal tears have been observed in the affected esophagus, which have been reported as occurring spontaneously during endoscopic observation or having been induced by minor trauma during biopsy procedures. In our case, the esophageal mucosa was edematous, but esophageal mucosa fragility or mucosal tears were not clearly evident.

In cases with EE, esophageal motility disturbance has been reported by several investigators and many studies have demonstrated a high degree of ineffective esophageal peristalsis [21]. In our case, ineffective esophageal peristalsis was similarly observed using high resolution manometry. The possibility of EE should therefore be considered when diagnosing patients with abnormal esophageal motor function.

Treatment strategies for EE should include dietary modification and medical therapy [22]. The initial approach to the disorder is often an allergy evaluation in order to identify the allergens which may be present in the diet or in the environment [23]. In our case, however, 57 types of the antigen-specific IgE and parasitic worm eggs were examined, all of which were negative. Systematic and topical corticosteroids effectively resolve acute clinicopathologic features of EE but in our case, topical corticosteroid had already been administered as therapy for bronchial asthma. This therapy was effective for bronchial

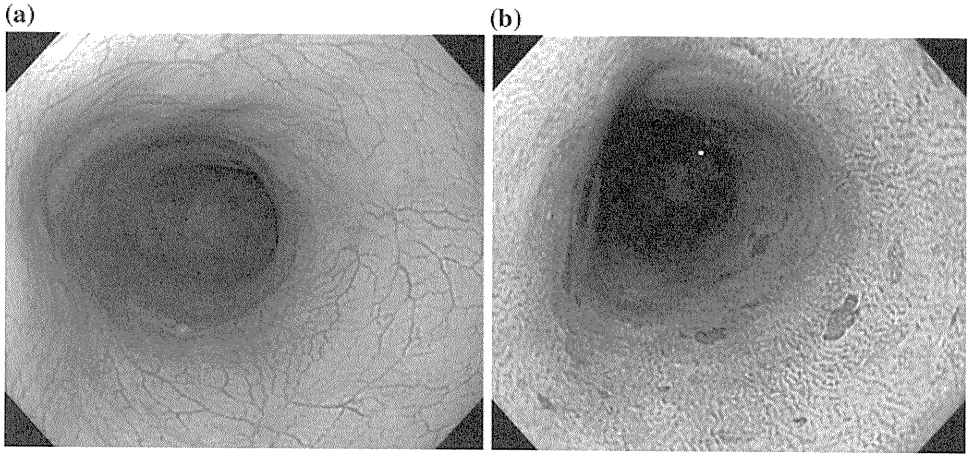


**Fig. 7** Esophageal endoscopic findings 4 weeks after prednisolone treatment. **a** Conventional endoscopic findings, **b** dye endoscopic findings by iodine staining. Circular rings disappeared and it was possible to see the vessels in the esophageal mucosa

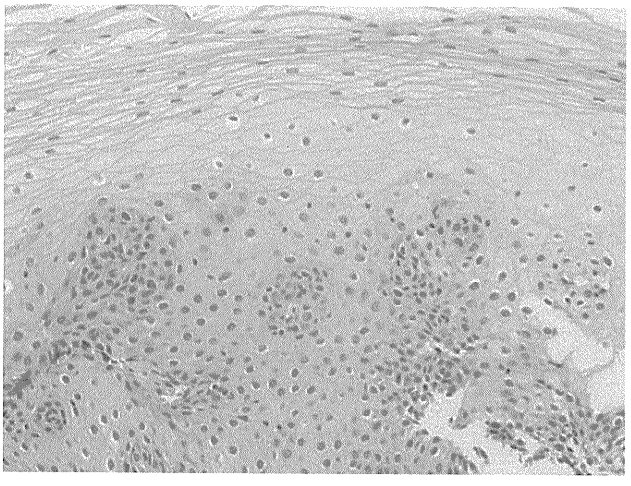

**Fig. 8** Histologic findings 4 weeks after prednisolone treatment. Few eosinophils existed (H&E, ×400)

asthma but was not effective for EE. Our case was an emergent case because the patient had severe dysphagia and was not able to eat a solid meal at the time of visiting our hospital. In emergent cases, systematic corticosteroid therapy is recommended [17] and was therefore commenced. The patient started taking prednisolone 30 mg/day and after 3 days of treatment her symptoms had almost disappeared; after 7 days of treatment they had completely disappeared. We then began to gradually taper off the prednisolone. She is now taking 2.5 mg of prednisolone per day with no recurrence of the symptoms.

The prognosis for patients with EE is generally very good. Symptoms usually respond to treatment from corticosteroids, although the need for re-treatment is common. To date, no evidence for any malignant potential of this condition has been identified [12].

Thus we have presented a rare case of EE in Japan. There have been many cases of EE reported in Western countries, but because EE is still rare in Japan, and is a disorder which doctors seldom encounter, its diagnosis is often delayed, sometimes for years. If patients with NERD have dysphagia and heartburn but do not respond to high-dose PPI therapy, EE needs to be considered as a differential diagnosis.

## References

1. Nielsen RG, Husby S. Eosinophilic oesophagitis: epidemiology, clinical aspects, and association to allergy. J Pediatr Gastroenterol Nutr. 2007;45:281–9.
2. Blanchard C, Rothenberg ME. Basic pathogenesis of eosinophilic esophagitis. Gastrointest Endosc Clin N Am. 2008;18:133–43.
3. Kelly JK, Lazenby AJ, Rowe PC, Yardley JH, Perman JA, Sampson HA, et al. Eosinophilic esophagitis attributed to gastroesophageal reflux: improvement with an amino acid-based formula. Gastroenterology. 1995;109:1503–12.
4. Furuta K, Adachi K, Kowari K, Mishima Y, Imaoka H, Kadota C, et al. A Japanese case of eosinophilic esophagitis. J Gastroenterol. 2006;41:706–10.
5. Kamimura K, Oosaki A, Sugahara S, Mori S, Morita T, Kimura K. Eosinophilic esophagitis: a case report. Effective treatment with systemic corticosteroids for the relapse of the disease. Clin J Gastroenterol. 2008;1:46–51.
6. Fujiwara H, Morita A, Kobayashi H, Hamano K, Fujiwara Y, Hirai K, et al. Infiltrating eosinophils and eotaxin: their association with idiopathic eosinophilic esophagitis. Ann Allergy Asthma Immunol. 2002;89:429–32.
7. Potter JW, Saeian K, Staff D, Massey BT, Komorowski RA, Shaker R, et al. Eosinophilic esophagitis in adults: an emerging problem with unique esophageal features. Gatrointest Endosc. 2004;59:355–61.
8. Arora AS, Yamazaki K. Eosinophilic esophagitis: asthma of the esophagus? Clin Gastroenterol Hepatol. 2004;2:523–30.
9. Straumann A, Beglinger C. Eosinophilic esophagitis: the endoscopist's enigma. Gastrointest Endosc. 2006;63:13–5.
10. Landres RT, Kuster GGR, Strum WB. Eosinophilic esophagitis in a patient with vigorous achalasia. Gastroenterology. 1978;74:1298–301.
11. Teitelbaum JE, Fox VL, Twarog FJ, Nurko S, Antonioli D, Gleich G, et al. Eosinophilic esophagitis in children: immunopathological analysis and response to fluticasone propionate. Gastroenterology. 2002;122:1216–25.

12. Straumann A, Spichtin HP, Grize L, Bucher KA, Beglinger C, Simon HU. Natural history of primary eosinophilic esophagitis: a follow up of 30 adult patients for up to 11.5 years. Gastroenterology. 2003;125:1660–9.
13. Arora AS, Perrault J, Smyrk TC. Topical corticosteroid treatment of dysphagia due to eosinophilic esophagitis in adults. Mayo Clin Proc. 2003;78:830–5.
14. Croese J, Fairley SK, Massom JW, Chong AK, Whitaker DA, Kanowski PA, et al. Clinical and endoscopic features of eosinophilic esophagitis in adults. Gastrointest Endosc. 2003;58:516–22.
15. Spergel JM, Beausoleil JL, Mascarenhas M, Liacouras CA. The use of skin prick tests and patch tests to identify causative foods in eosinophilic esophagitis. J Allergy Clin Immunol. 2002; 109:363–8.
16. Blanchard C, Wang N, Stringer KF, Mishra A, Fulkerson PC, Abonia JP, et al. Eotaxin-3 and a uniquely conserved gene expression profile in eosinophilic esophagitis. J Clin Invest. 2006;116:536–47.
17. Furuta GT, Liacouras CA, Collins MH, Gupta SK, Justinich C, Putnam PE, et al. Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. Gastroenterology. 2007;133:1342–63.
18. Gonsalves N, Policarpio-Nicolas M, Zhang Q, Rao MS, Hirano I. Histopathologic variability and endoscopic correlates in adults with eosinophilic esophagitis. Gastroint Endosc. 2006;64:313–9.
19. Zimmerman SL, Levine MS, Rubesin SE, Mitre MC, Furth EE, Laufer I, et al. Idiopathic eosinophilic esophagitis in adults: the ringed esophagus. Radiology. 2005;236:159–65.
20. Remedios M, Campbell C, Jones DM, Kerlin P. Eosinophilic esophagitis in adults: clinical, endoscopic, histologic findings, and response to treatment with fluticasone propionate. Gastrointest Endosc. 2006;63:3–12.
21. Martin LM, Vaquero CS, Prudencio SS, Perona JC, Gisbert JP, Otero RM. Eosinophilic esophagitis in the adult-clinical, endoscopic, pH-metric, and manometric findings. Rev Esp Enferm Dig. 2008;100:476–80.
22. Gupte AR, Draganov PV. Eosinophilic esophagitis. World J Gastroenterol. 2009;15:17–24.
23. Kagalwalla AF, Sentongo TA, Ritz S, Hess T, Nelson SP, Emerick KM, et al. Effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. Clin Gastroenterol Hepatol. 2006;4:1097–102.