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Suppressed *Helicobacter pylori*-associated gastric tumorigenesis in *Fat-1* transgenic mice producing endogenous ω-3 polyunsaturated fatty acids

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ABSTRACT

Dietary approaches to preventing *Helicobacter pylori* (*H. pylori*)-associated gastric carcinogenesis are widely accepted because surrounding break-up mechanisms are mandatory for cancer prevention, however, eradication alone has been proven to be insufficient. Among these dietary interventions, omega-3-polyunsaturated-fatty acids (ω-3 PUFAs) are often the first candidate selected. However, there was no trial of fatty acids in preventing *H. pylori*-associated carcinogenesis and inconclusive results have been reported, likely based on inconsistent dietary administration. In this study, we developed an *H. pylori* initiated-, high salt diet promoted-gastric tumorigenesis model and conducted a comparison between wild-type (WT) and *Fat-1*-transgenic (TG)-mice. Gross and pathological lesions in mouse stomachs were evaluated at 16, 24, 32, and 45 weeks after *H. pylori* infection, and the underlying molecular changes to explain the cancer preventive effects were investigated. Significant changes in: i) ameliorated gastric inflammations at 16 weeks of *H. pylori* infection, ii) decreased angiogenic growth factors at 24 weeks, iii) attenuated atrophic gastritis and tumorigenesis at 32 weeks, and iv) decreased gastric cancer at 45 weeks were all noted in *Fat-1*-TG-mice compared to WT-mice. While an increase in the expression of Cyclooxygenase (COX)-2, and reduced expression of the tumor suppressive 15-PGDH were observed in WT-mice throughout the experimental periods, the expression of Hydroxyprostaglandin dehydrogenase (15-PGDH) was preserved in *Fat-1*-TG-mice. Using a comparative protein array, attenuated expressions of proteins implicated in proliferation and inflammation were observed in *Fat-1*-TG-mice compared to WT-mice. Conclusively, long-term administration of ω-3 PUFAs can suppress *H. pylori*-induced gastric tumorigenesis through a dampening of inflammation and reduced proliferation in accordance with afforded rejuvenation.

INTRODUCTION

The International Agency for Research on Cancer defined *Helicobacter pylori* (*H. pylori*) as a class I carcinogen [1] as *H. pylori* caused gastric carcinogenesis [2] and the eradication of *H. pylori* prevented metachronous gastric cancer (MGC) after endoscopic resection of early gastric cancer [3]. During carcinogenesis, *H. pylori* provoked gastric inflammation, oxidative stress, and several harmful events including genetic and epigenetic pathways [4], after which its eradication can be a solution for prevention. However, in intervention trials studying gastric cancer prevention, *H. pylori* eradication did not prevent MGC in patients undergoing endoscopic submucosal dissection, have yielded rather disappointing results [5]. Therefore, non-microbial dietary intervention has been considered...
as an alternate eradication method or as a provision of surrounding break up to either remove mutagenic inflammation leading to cancer progression [6–9].

Generally, strategies to reduce the occurrence of gastric cancer, include improvement of sanitation, high intake of fresh fruits, safe food-preservation methods, and avoidance of smoking [10]. Additionally, mitigating the chronic inflammatory response associated with infectious disease has been recommended [11], including non-microbial dietary intervention or supplementation with phytoceuticals with the hope that these approaches may be an effective way of preventing cancer through long-term control of gastric inflammation [12]. Increased consumption of fatty fish or fish oil supplements containing anti-inflammatory ω-3 PUFAs is also an intriguing intervention since ω-3 PUFAs has been shown to have a therapeutic role in inflammatory diseases such as rheumatoidarthritis, inflammatory bowel disease, asthma, cardiovascular, and neurodegenerative diseases [13–15] and various cancers by reducing the level of AA-derived eicosanoids and inflammatory cytokines, (including Interleukin (IL)-1, IL-2, IL-6, IL-8, Interferon (IFN)-γ, and Leukotriene B4) and Tumor necrosis factor-α, promoting anti-inflammatory activities [16, 17]. In spite of these achievements, the expected clinical impact of ω-3 PUFAs has been reduced, perhaps because applying ω-3 PUFAs-containing diet scan bring considerable variations between the experimental groups [18, 19], resulting from an in consistent intake and uncertain purity of ω-3 PUFAs.

Fat-1 transgenic (Fat-1 TG) mice are capable of producing ω-3 fatty acids from ω-6 type fatty acids because of the transgenic over-expression of n-3 desaturase lead to abundant ω-3 PUFAs with reduced levels of ω-6 fatty acids in their organs and tissues without a dietary n-3 supply [20]. Here we used these mice to explore the effect of ω-3 PUFAs, especially the exact effect of ω-3 PUFAs on H. pylori associated gastric lesions, something that has never been studied before. We hypothesize that Fat-1 TG mice may be conferred a chemo-preventive benefit through anti-inflammatory or anti-mutagenic actions of ω-3 PUFAs. Using H. pylori infected mice, our study can provide important preclinical evidence that ω-3 PUFAs are efficient in attenuating H. pylori-associated gastritis and preventing gastric tumors and reveal molecular insights for the use of dietary ω-3 PUFAs in chemoprevention of H. pylori-associated gastric cancer.

RESULTS

Attenuated gastric inflammation in Fat-1 TG mice compared to WT mice at 16 weeks after H. pylori infection

After 16 weeks of H. pylori infection, there were no significant changes on gross lesions of resected stomachs of H. pylori-infected WT and Fat-1 TG mice (see Supplementary Figure S1). However, the gastric mucosa of WT mice were thicker than Fat-1 TG mice and this thickening was accompanied with intense infiltrations of inflammatory cells in sub-mucosal and mucosal layers. Compared to the non-infected group, marked inflammatory cells were observed in the sub-mucosa and mucosa in the stomachs of H. pylori-infected WT mice (Figure 1B), whereas these inflammatory cell infiltrations were significantly decreased in Fat-1 TG mice after H. pylori infection (P < 0.05, Figure 1C). Therefore, we compared the expression levels of inflammatory mediators between H. pylori-infected WT and Fat-1 TG mice. As seen in Figure 1D, the expression of cytokines known to be increasingly expressed at H. pylori-infected gastritis including Vascular endothelial growth factor (VEGF), Cyclooxygenase (Cox)-2, IL-1β, IL-8, INF-γ, and IL-6, [21], were all significantly increased in H. pylori-infected WT compared to non-infected vehicle control group (P < 0.001). However, their expression levels were all significantly decreased in H. pylori-infected Fat-1 TG mice (P < 0.01). Since the sources of these inflammatory cytokines are macrophages and monocytes, we tested for macrophages using F4/80 immunohistochemical staining. As seen in Figure 1E, F4/80 expressions were significantly increased after H. pylori infection in WT mice, but significantly decreased in Fat-1 TG mice (P < 0.05).

Mitigated chronic atrophic gastritis in Fat-1 TG mice compared to WT mice at 24 weeks after H. pylori infection

Contrary to gross lesions at 16 weeks, H. pylori infection lead to significant gross changes at 24 weeks, as seen in Figure 2A and Supplementary Figure S2; portions of the gastric mucosa had a nodular and granular appearance, while the remaining portion of stomach looked thin and transparent. Instead, the gross lesions of H. pylori-infected Fat-1 TG mice were not changed compared to non-infected WT or Fat-1 TG mice even at 24 weeks. Some mice administered only HSD without H. pylori infection showed some mass-like lesion on gross observation (see Supplementary Figure 2A), whereas none were observed in HSD-administered Fat-1 TG mice. Almost all H. pylori-infected WT mice showed multiple nodular lesions accompanied by a thin surrounding gastric wall, whereas no significant changes were noted in Fat-1 TG mice at 24 weeks (see Supplementary Figure 2A and 2B). On pathological analysis, gastritis cysticaprumfunda, disappearance of paretal cells, and profuse inflammatory cell infiltrations on submucosa and mucosa were noted at H. pylori-infected WT. As seen in Figure 2A, there were no prominent changes in H. pylori-infected Fat-1 TG mice, only mild gastritis and focal erosive changes were observed. The pathological scoring between H. pylori-infected WT and Fat-1 TG mice was differed significantly at 24 weeks (P < 0.05, Figure 2B). Since there were significant differences in inflammatory activities,
A

WT (vehicle control) Group
Wild-type littermates

WT (H. pylori infected) Group

Fat-1 TG (vehicle control) group
Fat-1 transgenic mice

Fat-1 TG (H. pylori infected) Group

-0.5  0  1  16  24  32  45 (weeks)

- PPI (Proton pump inhibitor): Pantoprazol 20mg/kg, i.p injection
- H. pylori: SS1 strain, 1x10^8 CFU/mouse, gavage administration
- WT and Fat-1 transgenic mice on C57BL/6 background; 4-5 week-old age, n= 40 (each group)
- Diet start; AIN-46A pellet diet containing 7.5% NaCl
- Kill; 16, 24, 32, and 45 weeks, respectively (n=10)

B  16 weeks

<table>
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<th>WT</th>
<th>Fat-1</th>
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C

![Graph showing total pathologic score (M±SD) for WT and Fat-1 groups under HP conditions.](image)

\[ P < 0.05 \]
proliferative status, and angiogenic activities between WT and Fat-1 TG mice at 24 weeks of *H. pylori* infection (Figure 2B), we had measured the expression levels of the inflammatory mediators Cox-2 and IL-1β and angiogenic growth factors VEGF and Platelet-derived growth factor (PDGF); there were significant differences between WT and Fat-1 TG mice (Figure 2C). Further evaluation of COX-2 and Prostaglandin dehydrogenase (PDGH) was done by Western blots, COX-2 levels were significantly increased in WT mice after *H. pylori* infection (*P* < 0.05), but not in Fat-1 TG mice. The expression of 15-PGDH was significantly lower in *H. pylori*-infected WT mice at 24 weeks (*P* < 0.001), but significantly preserved at Fat-1 TG mice (*P* < 0.05, Figure 2D). In order to compare the angiogenic activities, we performed immunohistochemical staining with Cluster of differentiation (CD)31 endothelial antibody. As seen in Figure 2E, *H. pylori* infection led to a significant increase of CD31 in gastric mucosa (*P* < 0.001), but these expressions were not increased in *H. pylori*-infected Fat-1 TG mice. Though the expression of CD31 was higher in Fat-1 TG mice compared to WT, there was no statistical significance. In order to screen further changes in molecular events between groups, we performed protein array analysis. As seen in Figure 2F,
H. pylori infection in WT mice led to significant increases in IL-1α, endothelin-1, amphiregulin, and Fibroblast growth factor (FGF) \((P < 0.01)\), however, no significant changes were observed in Fat-1 TG mice, suggesting that the ischemic and proliferative conditions in H. pylori-infected WT mice were significantly relieved in H. pylori-infected Fat-1 TG mice.

Increased H. pylori-induced tumorigeneis in WT mice, but not in Fat-1TG mice observed at 32 weeks

Our model of H. pylori infection followed with 7.5% high salt diet provoked significant tumorigenesis after 32 weeks of H. pylori infection on gross observation
Long-term (45 weeks) preventive effects of ω-3 PUFA-producing Fat-1 TG mice against H. pylori-induced gastric carcinogenesis

We maintained our model up to 45 weeks after H. pylori infection to compare the chemo-preventive effects of ω-3 PUFAs. As seen in Figure 4A and Supplementary Figure S4A, all H. pylori-infected mice had developed variable sized gastric tumors, whereas only 30% of Fat-1
Figure 3: Significantly attenuated tumorigenesis in Fat-1 TG mice at 32 weeks of H. pylori infection. (A) Current model of H. pylori infection followed with 7.5% high salt diet provoked significant tumorigenesis after 32 weeks of H. pylori infection on gross observation. There were multiple ulcerated tumors on the stomach accompanied with several nodules, but very thin gastric wall, transparently looking. However, no significant changes in gross appearance were noted in H. pylori-infected Fat-1 TG mice except small sized nodular lesions in 30% Fat-1 TG mice. Pathological findings in H. pylori-infected WT mice at 32 weeks showed gastric adenoma and chronic atrophic gastritis (H&E stain, Magnification, × 40). (B) Pathological scoring between groups showed significant differences in pathological scores between WT and Fat-1 TG mice (P < 0.05). (C–D) RT-PCR for inflammatory and angiogenic growth factors and immunohistochemical staining for CD31 were done. There were significant increases in Cox-2, IL-1β, IL-6, VEGF, PDGF mRNA and CD31 in H. pylori-infected WT mice, but significantly decreased in Fat-1 TG mice. (D) Western blots for COX-2, 15-PGDH, and VEGF according to group. The expressions of 15-PGDH were significantly decreased in H. pylori-infected WT mice (P < 0.005), while COX-2 expressions were significantly increased in WT mice at 32 weeks of H. pylori infection. On the other hand, the expressions of tumor suppressive 15-PGDH were significantly preserved in H. pylori-infected Fat-1 TG mice (P < 0.005). Just like COX-2, there were significant decrements in VEGF expressions in Fat-1 TG mice compared to WT mice.
TG mice developed gastric tumors and those were smaller than those in WT mice (see Supplementary Figure S4B). When reviewing the gross and pathological appearance, some tumors were shown to be more invasive and larger in size in WT mice compared to Fat-1 TG mice (Figure 4A and 4B). As seen in Figure 4C, 1:1 mounting view of whole stomach of WT – H. pylori group showed moderately-differentiated adenocarcinoma, by which there was significant difference in tumorigenesis between WT and Fat-1 TG mice at 45 weeks after H. pylori infection. The expression of important inflammatory and angiogenic growth factors, including Cox-2, IL-1β, IL-6, and PDGF mRNA are presented in Figure 4D and were significantly increased in H. pylori-infected WT, but not Fat-1 TG mice at 45 weeks. The protein expressions of COX-2 and VEGF were noted in similar way. Explaining the significant differences in tumorigenesis between groups, proliferative activities were measured. β-catenin, Cyclin-dependent kinase (CDK)4, phosphorylated Protein kinase B (PKB or AKT) (Figure 4E), CD26, Heparin-binding EGF-like growth factor (HB-EGF), Insulin-like growth factor-binding protein (IGFBP)3 (Figure 4F), Bromodeoxyuridine (BrdU), and Ki-67 (Figure 4G) were included in these measurements reflecting H. pylori-associated proliferative activities. As seen in Figure 4E, 4F, and 4G, H. pylori infection was associated with significant increases in β-catenin, CDK4, Akt activation, Ki-67 as well as BrdU incorporation, whereas these proliferative markers were not significantly different from the non-infected Fat-1 TG mice (Figure 4E and 4G). At this stage, we also conducted protein arrays to identify proteins whose expression are significantly different between WT and Fat-1 TG mice. As seen in Figure 4F, CD26, FGF1 (HB-EGF), and IGFBP3 were significantly increased in H. pylori-infected WT, but not changed in Fat-1 TG mice.

Dose of exogenous ω-3 PUFAs showing similar lipid profiles of stomach as seen in Fat-1 TG mice

Though the current experiment investigated the exact role of ω-3 PUFAs against H. pylori infection by using Fat-1 TG mice generating ω-3 PUFAs in the stomach after being fed ω-6 PUFAs rich diets, we tested how much exogenous ω-3 PUFAs is required to achieve the above protection from H. pylori infection. We administered various doses of ω-3 PUFAs (0.5 g/60 kg to 10 g/60 kg) through oral feeding and compared the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in the stomach. As seen in Figure 5A and 5B, ω-3 PUFAs more than 0.5 g/60 kg showed similar patterns in Fat-1 TG mice because the ratio of ω-6 PUFAs/ω-3 PUFAs was noted in mice fed more than 0.5 g/60 kg ω-3 PUFAs (Figure 5B).

DISCUSSION

In the current experiment, ω-3PUFAs attenuated H. pylori-associated gastric inflammation, rejuvenated H. pylori-induced CAG, and prevented H. pylori-associated gastric tumorigenesis in Fat-1 TG mice. In terms of cancer prevention, concerted mechanisms including COX-2 inhibition, 15-PGDH preservation, regulation of proliferation, and mitigated tumorigenic angiogenesis were revealed. Through measuring real levels of lipid profiles in the stomach after exogenous administration of ω-3 PUFAs, we found that dietary intake of more than 0.5 g/60 kg ω-PUFAs could achieve similar lipid profile as seen in Fat-1 TG mice. To our knowledge, this might be the first study demonstrating the inhibitory effect of ω-3PUFAs on H. pylori-induced inflammation.
Figure 4: Long-term efficacy of ω-3 PUFA on *H. pylori*-induced gastric tumorigenesis at 45 weeks. (A) All of *H. pylori*-infected mice had developed variable sized gastric tumors, whereas only 30% mice developed gastric tumors in Fat-1 TG mice, smaller in size than WT mice (H&E stain, Magnification, x 40). (B–C) The mean pathological scoring was significantly decreased in Fat-1 TG mice compared to WT mice (*P* < 0.01). 1:1 mounting view of whole stomach of WT - *H. pylori* group showed moderately-differentiated adenocarcinoma, by which there was significant difference in tumorigenesis between WT and Fat-1 TG mice at 45 weeks after *H. pylori* infection. (D) Measuring the expressions of Cox-2, IL-1β, IL-6, and PDGF mRNA between groups, *H. pylori*-infected WT showed significantly increased expressions, but significantly attenuated at Fat-1 TG mice. (E) Western blots for β-catenin, CDK4, phosphorylated-Akt, and total Akt according to group. (F) Protein array. (G) Immunohistochemical staining for BrdU and ki-67. All of figure E, F, and G consistently suggested *H. pylori* infection for 45 weeks led to significantly increased proliferative actions in WT mice, but these mucosal proliferative mechanisms were significantly decreased in Fat-1 TG mice (*P* < 0.001, Magnification × 100). The stomachs were stained for BrdU and Ki67, the number of positive cells was represented as mean ± SD.
and gastric tumorigenesis, signifying the future potential of ω-3 PUFAs to ameliorate either H. pylori-associated CAG or gastric cancer through dietary intervention in the clinic.

Under the same H. pylori infection conditions, the expression of inflammatory and angiogenic growth factors were significantly reduced in Fat-1 TG mice compared to WT (Figure 1D). This suggests that ω-3 PUFAs were capable of suppressing the main inflammatory mechanism and providing an efficient gastro-protection against H. pylori infection beyond inhibitory actions on H. pylori colonization. However, since several in vitro and in vivo studies showed that ω-3 PUFAs could inhibit H. pylori growth in vitro and its colonization in the gastric mucosa of mice in vivo [22, 23], we have investigated the possibility of inferior colonization or bactericidal effects due to ω-3 PUFAs synthesized in Fat-1 TG mice. Similar colonization statuses as measured using direct culture, rapid urease test, e.g., CLO test, and Giemsa staining were observed in WT and TG mice up to 8 weeks of colonization. To avoid reduced bacterial colonization in Fat-1 TG mice, we administered the proton pump inhibitor, pantoprazole, before H. pylori inoculation to enhance their colonization rates followed with thrice administration of H. pylori cultures, and following this protocol, we did not find any differences in H. pylori colonization between WT and Fat-1 TG mice. Therefore, we could rule out that the cancer prevention outcome was not related with lowered colonization in Fat-1 TG mice, even though an in vitro study showed some bacterostatic effects of EPA or DHA.

![Graph A](image1.png)

![Graph B](image2.png)

Figure 5: The comparison of concentration of DHA, EPA and ratio of ω-6 and ω-3 PUFAs; between WT mice following administration of exogenous ω-3 PUFA and endogenous ω-3 generated within Fat-1 TG mice. (A–B) The DHA and EPA were extracted from stomach of Fat-1 TG mice and WT mice administrated with ω-3 dose dependently and analyzed by LC/MS/MS as described in Materials and Methods.
To determine the molecular mechanisms of the ω-3 PUFAs-mediated decrease in *H. pylori*-associated inflammation 16 to 24 weeks after *H. pylori* infection, we first looked at inflammatory mediators and angiogenic growth factors. Since the increased expression of inflammatory mediators such as COX-2, IL-1β, IL-6, IFN-γ, and IL-8 were one of core pathogenic mechanisms in *H. pylori*-associated gastritis [24, 25] and these inflammatory responses are thought to be one of the core processes involved in gastric carcinogenesis [26, 27], we have focused on to the serial changes of COX-2-Prostaglandin E2 (PGE2) pathway [28]. As seen in Figures 1, 2, and 3, these inflammatory mediators were significantly decreased in *Fat-1* TG mice compared WT mice. Both ω-6 and ω-3 PUFAs are precursors of potent lipid mediators termed eicosanoids, eicosanoids derived from ω-6 PUFAs have pro-inflammatory and immune-active functions, whereas eicosanoids derived from ω-3 PUFAs have anti-inflammatory properties [13, 29]. This is why the increased intake of ω-3 PUFAs led to a decrease in the risk of many chronic inflammation-based diseases including arthritis, diabetes, obesity, cardiovascular diseases, inflammation, and cancer [30, 31]. COX can generate anti-inflammatory mediators from ω-3 PUFAs [32] including: i) eletrophilicoxo-derivatives (EFOX), a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist that mostly transduces the beneficial anti-inflammatory effects of DHA, ii) COX inhibitor, iii) aspirin and iv) ω-3 PUFAs-derived lipid autacoids termed resolvins and protectins [33]. Similarly, even though we have published the cancer-preventive effects of the non-steroidal anti-inflammatory drugs (NSAIDs) nimesulide and celecoxib [34] and multiple case-control cohort randomized control trial (RCT), a pooled analysis, and meta-analyses also suggest a preventive effect of aspirin or NSAIDs on the development of non-cardia gastric cancer [35]. The adverse effects associated with the use of NSAIDs may lead to poor adherence as chemo-preventive agents and lead to other harmful effects on the kidney and cardiovascular system. Therefore, it should be emphasized that ω-3 PUFAs raises low toxicological constraints.

Secondly, the observation that at 24 weeks, the preservation of 15-PGDH—a PG degrading enzyme—accompanied with attenuated COX-2, VEGF, PDGF, and CD31 expression in *Fat-1* TG mice compared WT mice strongly explained the cancer-preventive actions of ω-3 PUFAs [36]. Since 15-PGDH may function as a tumor suppressor through antagonizing oncogenic action of COX-2, 15-PGDH has been found to be down-regulated and a contributor to elevated levels of PGE2 in most tumors, as seen in our *H. pylori*-associated gastric carcinogenesis model. Since the expression of 15-PGDH and COX-2 appears to be regulated reciprocally in cancer cells, up-regulation of 15-PGDH can be either down-regulated by transcriptional repressors or the attenuation of enzymatic turnover [37]. From our study, significant down-regulation was noted in WT mice after *H. pylori* infection, but preserved or elevated expression levels of 15-PGDH were observed in *Fat-1* TG mice. We documented that ω-3 PUFAs significantly up-regulated 15-PGDH to prevent tumorigenesis relevant to intestinal polyposis [38]. Regarding the changes of decreased 15-PGDH following *H. pylori* infection [39], the decreased expression of 15-PGDH were reversed with successful *H. pylori* eradication, in which suppressed 15-PGDH-associated gastritis [24, 25] and these expression of 15-PGDH and COX-2 appears to be regulated to elevated levels of PGE2 in most tumors, as seen in our study, additional mechanisms of ω-3 PUFAs-mediated chemoprevention include its role as a structural component providing the optimal function of cellular membranes including: i) membrane fluidity, ii) enzyme...
activity, iii) balanced ω-6:ω-3 PUFAs production and gene expressions, suggesting that increasing the amount of ω-3 PUFAs consumed at the population level may be a possible potential health benefit; increased intake of ω-3 PUFAs result in prevention of chronic diseases and reduction in the burdening of the health care. From our study, we also investigated why a number of ω-3 PUFAs-containing dietary supplements have shown on the market claiming to protect against the development of a variety of conditions including cancer, but meta-analysis did not provide results of evidence based medicine to suggest a significant association between ω-3 PUFAs and cancer incidence [54]. In the current study, we have compared the gastric levels of ω-3 PUFAs after administering several dose of ω-3 PUFAs (Figure 5) and found that intake more than 0.5 g/60 kg resulted in a similar ω-6/ω-3 PUFAs ratio in the stomach. Our results support the use of dietary ω-3PUFAs in H. pylori-infected patients as a safe prophylactic/preventive strategy before stepping into irreversible condition. Clinical trials to document the chemo-preventive effects of ω-3 PUFAs have mostly focused on colorectal polyps; Higurashi T et al. [55] performed a double blind, placebo-controlled RCT to explore the effects of EPA against colorectal aberrant crypt foci (ACF) and highlighted the suppressive effect of 2.7g EPA/day on the formation of ACF and Hull MA et al. [56], after their SeaFOod trials, The seafood Polyp Prevention Trial, concluded that EPA can prevent polyp formation.

Lastly, we have checked microbiota changes in WT and Fat-1 TG mice during 24 and 32 weeks through 454 pyrosequencing measurement and found significant changes between WT and Fat-1 TG mice (data not shown). These significant changes in fecal microbiota in Fat-1 TG mice might come from the following two possibilities: i) the direct influence of ω-3 PUFAs on microbiota change or ii) changes reflecting mitigated conditions of H. pylori-induced chronic atrophic gastritis by fatty acid produced in Fat-1 TG mice. Conclusively, our findings suggest that he increased abundance of ω-3PUFAs in tissue significantly reduced gastric inflammation and tumorigenesis in Fat-1 mice. Clinical trials to document the effects of exogenous administration of more than 0.5 g/60 kg high purity ω-3 PUFAs in chronic H. pylori infection should be conducted to determine the benefit of the use of ω-3 PUFAs in chemo-preventive strategies for H. pylori-induced atrophic gastric disease.

MATERIALS AND METHODS

H. pylori-infected mice model

Animals

Five-week-old male C57BL/6 mice (WT mice) were purchased from Orient (Seoul, Korea) and Fat-1 transgenic (Fat-1 TG) mice were provided kindly from Dr. Jing X. Kang (Boston, MA). They were housed in a cage maintained at 23°C in a 12 h/12 h light/dark cycle under specific pathogen-free conditions (n = 160). We divided four groups; 1) WT mice as vehicle control group 2) WT mice as H. pylori-infected group 3) Fat-1 mice as vehicle control group 4) Fat-1 mice as H. pylori-infected group. First, all groups were given intraperitoneal (i.p.) injections of proton pump inhibitors (PPIs, pantoprazole, 20 mg/kg; Amore-Pacific Pharma) three times per week to facilitate H. pylori colonization through lowered gastric acidity. And then, each mouse was intragastrically inoculated with a suspension of H. pylori containing 10^7 CFUs/mL or with an equal volume (0.1 mL) of clean TS broth using gastric intubation needles. The H. pylori-infected mice were fed a special pellet diet based on AIN-46A containing 7.5% NaCl (high salt diet, Biogenomics, Seongnam, Korea) for 45 weeks (Figure 1A) to promote H. pylori-induced carcinogenic process in all infected animals [57]. And randomized groups of mice (n = 10) sacrificed at 16, 24, 32 and 45 weeks post H. pylori infection, respectively. The stomachs of mice were opened along the greater curvature and washed with ice cold PBS. The numbers of either erosions/ulcers or protruded nodule/mass were determined under the magnified photographs (see Supplementary Figures S1–S4). Stomachs were isolated and subjected to a histologic examination, ELISA, Western blotting, and RT-PCR. All animal studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University Cancer Institute after IRB approval.

Gross lesion index

After sacrificing the mice, the isolated stomachs were open along the greater curvature and washed in ice-cold saline. To investigate the degree of gross mucosal pathology, the mucosal sides of the stomachs were photographed using a digital camera and part of the mucosa was immediately fixed with 10% formalin solution. The gross damage of the gastric mucosa was assessed by three gastroenterologists, who were blinded to the treatments, using a gross ulcer index [58]. All the gross photographs were displayed in Supplementary Figures and tumorous lesion was depicted with white arrow.

Index of histopathologic injury

For histopathological analysis, the stomach were fixed in 10% neutralized buffered formalin, processing using the standard method and embedded in paraffin. Sections of 4 μm thickness were then stained with hematoxylin and eosin [59]. The glandular mucosae of corpus and antrum were examined histologically. The pathological changes of H. pylori-infection, such as inflammatory cells infiltration, erosive lesions, ulceration, dysplasia, adenoma formation (precancerous lesion), were graded by three gastroenterologists, who were blinded to

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the group, using an index of histologic injury defined [60].
In this study, inflammation was defined as grade the
infiltration of inflammatory cells, 0: none, 1: under
the lamina propria, 2: half of mucosa 3: until the epithelial
gland layer (all mucosa). The erosion was defined as
proportion of erosive lesion, 0: none, 1: loss of epithelial
gland layer (1/3 proportion), 2: two-three portion of
mucosa (2/3 proportion) 3: all mucosa (3/3 proportion)

**BrdU staining for assessing mucosal proliferation**

To estimate the rate of proliferation that is increased
during carcinogenesis, we injected BrdU before sacrifice,
and performed immunohistochemical analysis with anti-
BrdU antibody.

**Immunohistochemical staining**

Immunohistochemistry was performed on replicate
sections of mouse gastric tissues. After deparaffinization
were dewaxed and rehydrated with graded alcohol, and
boiled three times in 100 mM Tris buffered saline (pH 6)
with 5% urea in an 850 W microwave oven for 5 min
each. And then cooling in water for 15 min and washed in
PBS, and slides were incubated overnight with the primary
antibody at 4°C. Antibodies: F4/80 (1:500; eBioscience,
San Diego, CA) or CD31 (1:300; Dako, Santa Clara,
CA) or Ki-67 (1:300; Santa cruz, Santa Cruz, CA) in the
presence of 1.0% bovine serum albumin respectively.
Slides incubated with secondary antibody (1:300) for 1 h
at room temperature, and then with 40-6-
diamidino-2-phenylindole (DAPI, 100 ng/ml) for 1 min at room
temperature. And finally the slides were counterstained
with hematoxylin (Sigma-Aldrich).

**RT-PCR**

Total RNA was isolated using the Trizol (Invitrogen,
Carlsbad, CA). Trizol was added to 1.5 ml tube, which
were then incubated 10 min at 4°C and gently mixed with
100 µl chloroform (Merck, Rahway, NJ). After incubation
for 10 min in ice, samples were centrifuged at 10,000 g
for 30 min. Supernatants were extracted and mixed with
200 µl isopropanol (Merck), and mixtures were incubated
at 4°C for 1 h. After centrifuging at 13,000 g for 30 min,
pellets were washed with 70% (v/v) ethanol. After
allowing the ethanol to evaporate completely, pellets were
dissolved in 40 µl diethylene pyrocarbonate-treated water
(Invitrogen Life Technologies). cDNA was prepared using
reverse transcriptase originating from Murine-Moloney
leukemia virus (Promega, Madison, WI), according to
the manufacturer’s instructions. The polymerase chain
reaction (PCR) was performed over 25 cycles of: 94°C
for 20 s, 58.5 for 30 s, and 72°C for 45 s. Oligonucleotide
primers were purchased from Bioneer (Daejeon, Korea).

Oligonucleotide primers were as follows; for VEGF, sense
5’-GAA GCT ACT GCC GTC GA-3’ and antisense
5’-TCC TCT TCC ATG TCA GGC-3’, for COX-
2, sense 5’-GAA ATG GCT GCA GAG TTG AA-3’ and
antisense 5’-TCA TCT AGT CGT GAG TGG GA-3’, for
PDGF, sense 5’-ACG TCA TGT TAC GGC TTC CT-3’
and antisense 5’-CAG TGT GAC TGT GTC TCC CC-3’,
for IL-1β, sense 5’-CAG CCT CCG AGA TGA ACA ACA
AAA-3’ and antisense 5’-TGG GGA ACT CGT ACT
CAA ACT-3’, for IL-8, sense 5’-GGG GCT TTG CCG
TGC AAT AA-3’ and antisense 5’-GCA GAG GCT GCA
GCC AAA A-3’, for IFN-γ, sense 5’-ACA ATG AAC GCT
ACA CAC TG-3’ and antisense 5’-TCA AAC TTG GCA
ATA CTC AT-3’, for IL-6, sense 5’-AAG AGA CTT CCA
GCC AGT TG-3’ and antisense 5’-TGG ATG TTC TTG
GTC CTT AG-3’, and for GAPDH, sense 5’-GCT GCT
GAT TCT GTG GA-3’ and antisense 5’-TCC AGC
TCT GGG ATG ACC TT-3’.

**Western blotting**

Cells were harvested and lysed in lysis buffer
(Cell signaling Technology) containing 1 mM
phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich St.
Louis, MO). After 30 min of incubation, samples were
centrifuged at 12,000 g for 15 min 4°C. The supernatants
were then collected and protein quantification was carried
out with a Bio-Rad protein assay. Equal amounts soluble
protein (30 µg) were denaturated by heating at 100°C for
3 minutes. Proteins were separated by sodium dodecyl
sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)
and transferred to polyvinylidene fluoride membranes. The
membranes were blocked in 5% BSA in PBST for 30 min.
And then, the membranes probed initially with specific
primary antibody, washed, incubated with peroxidase-
conjugated secondary antibodies, and rewashed. The
protein bands were detected by chemiluminescence
(Supersignal, Pierce) exposure on chemiluminescence
system (GE Healthcare, Buckinghamshire, UK). The
general procedure for Western blot analysis of cultured
mouse gastric mucosal cells was similar to the procedures
described above. Antibodies used in the current study
were COX-2, purchased from Thermo and β-actin,
VEGF, β-catenin, purchased from Santa Cruz and
CDK4, phospho-Akt, Akt, purchased from cell Signaling
Technology and 15-PGDH, purchased from Cayman.

**Cytokine protein array**

Cytokine protein array was performed using Mouse
Cytokine Antibody Array 3 (4 membrane arrays) with
Accessories, for simultaneous detection of 54 cytokines
related proteins in 2 samples from R&D systems
(Minneapolis, MN). After blocking the array membranes
for 30 minutes, the membranes were incubated with 1 ml
of serum at room temperature for 2 hours. After washing
with buffer, we added primary biotin-conjugated antibody to each membrane, for incubation at room temperature for 2 hours. After washing with buffer and addition of horseradish peroxidase-conjugated streptavidin to each membrane, we exposed them to detection buffer, using a luminescent image analyzer system (LAS-4000, Fuji Film; Tokyo, Japan). Density was expressed as the percentage of the detected value from the sample versus the background result, using a gelpro32 program (Media Cybernetics, Rockville, MD).

**Gas chromatography for measuring lipid profiles in the stomach**

Fatty acid profiles were analyzed using gas chromatography as described previously [19]. Briefly, 1 cm of mice tails (in order to perform the phenotyping of mice) or blocks of colon tissue (5 × 5 mm) were grounded to powder under liquid nitrogen. Samples were then subjected to extraction of total lipids and fatty acid methylation by heating at 100°C for 1 h under 14% boron trifluoride (BF3)-methanol reagent (Sigma, St. Louis, MO) and hexane (Sigma). Fatty acid methyl esters were analysed by gas chromatography using a fully automated 6890 N Network GC System (Agilent Technologies) equipped with a flame-ionization detector. Peaks of resolved fatty acids were identified by comparison with fatty acid standards (Nu-chek-Prep), and area percentage for all resolved peaks was analysed using GC ChemStation Software (Agilent Technologies, Santa Clara, CA) [61].

**Statistical analyses**

The data are presented as means ± standard deviations (S.D.). The data were analyzed by 1-WAY ANOVA, and the statistical significance between groups was determined by Student *t* test. Statistical significance was accepted at *P* < 0.05.

**Abbreviations**

*H. pylori*, Helicobacter pylori; ω-3 PUFAs, Omega-3-polyunsaturated-fatty acids; WT, Wild-type; TG, Transgenic; COX, Cyclooxygenase; PGDH, Prostaglandin dehydrogenase; 15-PGDH, Hydroxyprostaglandin dehydrogenase; MGC, Metachronous gastric cancer; IL, Interleukin; IFN, Interferon; VEGF, Vascular endothelial growth factor; PDGF, Platelet-derived growth factor; CD, Cluster of differentiation; FGF, Fibroblast growth factor; CDK, Cyclin-dependent kinase; AKT, Protein kinase B; HB-EGF, Heparin-binding EGF-like growth factor; IGFBP, Insulin-like growth factor-binding protein; BrdU, Bromodeoxyuridine; PGE2, Prostaglandin E2; RCT, Randomized control trial.

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**CONFLICTS OF INTEREST**

No conflicts of interest.

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