Induction and prevention of gastric cancer with combined *Helicobacter pylori* and capsaicin administration and DFMO treatment, respectively

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Research

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Abstract

Background: Gastric cancer risk evolves over time due to environmental, dietary, and lifestyle changes including *Helicobacter pylori* (*H. pylori*) infection and consumption of hot peppers (i.e. capsaicin). *H. pylori* infection promotes gastric mucosal injury in the early phase of capsaicin exposure. In addition, capsaicin consumption is reported to suppress immune function and increase host susceptibility to microbial infection. This relationship suggests a need to investigate the mechanism of how both *H. pylori* infection and capsaicin contribute to gastric inflammation and lead to gastric cancer. No previous experimental animal models have been developed to study this dual association. Here we developed a series of mouse models that progress from chronic gastritis to gastric cancer. C57-Balb/c mice were infected with the *H. pylori* (SS1) strain and then fed capsaicin (0.05% or 0.2g/kg/day) or not. Consequently, we investigated the association between *H. pylori* infection and capsaicin consumption during the initiation of gastric inflammation and the later development of gastric cancer. Tumor size and phenotype were analyzed to determine the molecular mechanism driving the shift from gastritis to stomach cancer. Gastric carcinogenesis was also prevented in these models using the ornithine decarboxylase inhibitor DFMO (2-difluoromethylornithine).

Results: This study provides evidence showing that a combination of *H. pylori* infection and capsaicin consumption leads to gastric carcinogenesis. The transition from chronic gastritis to gastric cancer is mediated through interleukin-6 (IL-6) stimulation with an incidence rate of 50%. However, this progression can be prevented by treating with anti-inflammatory agents. In particular, we used DFMO to prevent gastric tumorigenesis by reducing inflammation and promoting recovery of disease-free stasis. The anti-inflammatory role of DFMO highlights the injurious effect of inflammation in gastric cancer development and the need to reduce gastric inflammation for cancer prevention.

Conclusions: Overall, these mouse models provide reliable systems for analyzing the molecular mechanisms and synergistic effects of *H. pylori* and capsaicin on human cancer etiology. Accordingly, preventive measures like reduced capsaicin consumption, *H. pylori* clearance, and DFMO treatment can lessen gastric cancer incidence. Lastly, anti-inflammatory agents like DFMO can play important roles in prevention of inflammation-associated gastric cancer.

Introduction

The Centers for Disease Control and Prevention estimates that roughly two-thirds of adults harbor *Helicobacter pylori* (*H. pylori*), a bacterium that colonizes the stomach and duodenum. Without treatment, *H. pylori* infection is a known risk factor for gastrointestinal illnesses like chronic gastritis, peptic ulcers, and stomach cancer. In 2001, an epidemiologic study demonstrated that patients infected with *H. pylori* were nearly six times more likely to develop gastric cancer compared to uninfected people¹. Other gastric cancer risk factors include dietary and lifestyle practices including capsaicin consumption, stress levels,

and ingestion of inflammatory foods or beverages. In summary, the etiology of gastric cancer is comprised of bacterial variability, along with host genetic and environmental factors; however, the molecular mechanisms governing these factors have not been fully elucidated^{2,3,4}.

Evidence suggests that *H. pylori* infection impacts the development and progression of gastric mucosal injury during early capsaicin exposure from hot pepper consumption⁵. Capsaicin reportedly suppresses immune function and increases host susceptibility to infection^{6,7,8}. Other data suggest an epidemiological link between stomach cancer incidence and chili pepper-rich diets^{5,9}. Ultimately, capsaicin's role in carcinogenesis remains controversial. Additionally, most studies have not considered *H. pylori* infection to be a coinciding or modifying component during gastric cancer development. This unidentified interplay between *H. pylori* infection and other agents may be critical to the initiation and persistence of gastric inflammation, as well as the development of gastric cancer. Here we explore the combined effect of *H. pylori* infection and capsaicin exposure on the development of gastritis and gastric carcinogenesis, as well as possible anti-inflammatory regimens for disease prevention. The selected anti-inflammatory agent, DFMO (2-difluoromethylornithine), reportedly alters immune populations within the tumor microenvironment and inhibits tumor growth by increasing CD8⁺ infiltration^{10,11}. Despite its chemopreventive potential, the anti-inflammatory and anti-cancer effects of DFMO against gastritis and gastric cancer are unknown.

Previous studies have not adequately addressed *H. pylori* infection as a concurring or modifying factor for gastric cancer risk. Additionally, the role of capsaicin consumption in the development of gastritis and gastric cancer has not been experimentally elucidated before now. Here, we explore the combined effect of *H. pylori* infection and capsaicin consumption on the development of gastritis and later gastric cancer. Lastly, we highlight DFMO as an anti-inflammatory treatment against gastritis and as a preventative measure against gastric carcinogenesis.

Results

Capsaicin and *pylori* infection induce gastritis leading to gastric cancer

To investigate the role of capsaicin consumption in the progression of *H. pylori*-associated gastric cancer, we developed a mouse model treated with a combination of capsaicin and *H. pylori* infection to induce gastritis, leading to gastric cancer (Fig. 1a). Macroscopic morphometric analysis revealed that capsaicin consumption induced gastric inflammation (at 32 weeks) as the initiating process, which led to tumor development (at 52 weeks) in the stomach of mice with *H. pylori* infection (Fig. 1b). Tumor growth in mice treated with both capsaicin and *H. pylori* confirmed the role of a combination of capsaicin and *H. pylori* in gastric tumorigenesis. Macroscopic analysis of tumor area revealed development of tumors only in mice treated with both *H. pylori* and capsaicin ($30.26 \pm 7.017 \text{ mm}^2$; $p < 0.0001$; Fig. 1c). Mice treated with a combination of *H. pylori* and capsaicin showed significantly lower body weight compared to

untreated mice (Fig. 1d). Overall, these results indicated that *H. pylori* and capsaicin together caused a progressive shift from gastritis to gastric cancer. These results provided experimental animal evidence showing the combined effect of capsaicin and *H. pylori* infection on the development of gastric cancer. Histological analysis also showed multifocal elongation of the gastric pits, glandular atrophy, and a significant reduction in the glandular zone in atrophic foci. These changes were significantly less in mice treated with capsaicin and infected with *H. pylori* exhibiting tumors (0.2 ± 0.2 ; $p < 0.0001$), compared to mice treated with only capsaicin (2.60 ± 0.24 ; $p = 0.02$) or infected with *H. pylori* only (1.0 ± 0.316 ; $p = 0.0002$; Supplementary Fig. 1a-d).

Mice treated with capsaicin and *pylori* exhibit increased gastric atrophy and accelerated tumor growth

Histologic analysis revealed significant glandular atrophy in the fundus of mice at 32 weeks of a combination treatment of capsaicin and *H. pylori* compared with untreated mice or mice treated only with capsaicin or *H. pylori* (Fig. 2). Dual treatment resulted in reduction of parietal cell components, especially parietal and chief cells, which were replaced by gastric mucus-producing cells. Pepsinogen I and II, gastrin, somatostatin (gastric endocrine regulators), and H⁺ /K⁺ ATPase (parietal and chief cells) showed substantially lowered expression in mice exhibiting gastric tumorigenesis compared with untreated mice or mice treated singularly (0.264 ± 0.09 ; $p < 0.0001$; Fig. 2a-f). Gastrin, somatostatin, and H⁺/K⁺ ATPase are required for normal gastric mucosal development and parietal cell activation^{12,13}. In mice exhibiting carcinogenesis, these biomolecules were almost completely ablated. As a result, a combination of capsaicin and *H. pylori* directly accelerated loss of differentiated epithelial cell types, leading to chronic atrophic gastritis and eventually to gastric tumorigenesis. The combination treatment shifts gastric tissue to a tumor phenotype with increased numbers of inflammatory cells found in the tumor gastric mucosa. This observation suggests that *H. pylori* or capsaicin alone is not enough to cause gastric tumorigenesis and that a combination is required to trigger the formation of submucosal glands and invasion of tumor cells.

Diminished expression of tumor suppressor genes coincides with accelerated tumorigenesis

The effect of a combination of capsaicin and *H. pylori* is associated with increased tumor progression due to substantially reduced expression of several gastric tumor suppressor genes (TSGs), including *Tff1* by *qPCR* (0.273 ± 0.120), *Tff2* (0.126 ± 0.078), *Gkn1* (0.143 ± 0.045) and *Gkn2* (0.172 ± 0.060) in the group of mice that developed tumors compared to other groups ($p < 0.0001$; Fig. 3a-d). Loss of these gastric-specific TSGs promotes tumorigenesis^{14,15,16}. *Tff1* and *Tff2* genes are upstream regulators of *gastrokine* (*Gkn*) gene expression. Therefore, loss of *Tff1* or *Tff2* expression could lead to tumor growth¹⁷. These genes were substantially downregulated in mice exhibiting gastric tumors, and the

reduction was associated with the combined treatment of capsaicin and *H. pylori* infection. Overall, inhibition of *Tff1*, *Tff2*, *Gkn1*, and *Gkn2* is known to be associated with tumorigenesis^{17,18}.

Mice administered capsaicin and *pylori* display increased gastric tissue damage

The extent of gastric tissue damage was evaluated as well. We found that mice treated simultaneously with capsaicin and *H. pylori* exhibited tumor development. These mice displayed significantly higher activities of malondialdehyde (MDA; 401.100 ± 45.33 mg/mL; $p < 0.01$), myeloperoxidases (MPO; 0.402 ± 0.050 U/L; $p < 0.0001$), catalase (30.47 ± 2.645 U/mL; $p = 0.018$), carbonyl (220.4 ± 37.35 nmol/mg; $p < 0.001$), and lipoperoxidase (LPO; 601.6 ± 31.61 μ mol/L; $p < 0.0001$) compared to mice treated with combination of *H. pylori* and capsaicin (Fig. 4a-e). A combination of capsaicin and *H. pylori* infection increases peroxidation processes and leads to the production of superoxide radicals, such as MDA, MPO, and hydrogen peroxide, which can form adducts with DNA and proteins. Subsequently, we proposed that *H. pylori* infection and capsaicin metabolites, including vanillylamine and vanillic acid and peroxides damage DNA, DNA repair enzymes, and immune cell protein cytokines, resulting in gastric tissue damage^{19,20,21}.

Mouse-derived xenograft model (MDX) confirms the efficacy of the *pylori*- and capsaicin-associated gastric cancer model

To further elucidate the effect of capsaicin intake and *H. pylori* infection on the development of gastric cancer, we used gastric tumors from mice treated with both *H. pylori* and capsaicin to develop a mouse xenograft model (MDX). We successfully developed the MDX model with tumors at the first (M1 = 100%) and second (M2 = 100%) passages (Fig. 5a-d). Histological analysis further confirmed the tumorigenicity and gastric origin of this combination by showing high expression levels of cytokeratin (PCK-26; 0.797 ± 0.026 , 0.682 ± 0.025 , 0.761 ± 0.072), CD-34 (0.730 ± 0.067 , 0.722 ± 0.030 , 0.749 ± 0.035) and low expression levels of SMA- α (0.092 ± 0.017 s, 0.046 ± 0.013 , 0.099 ± 0.017) in M0, M1, and M2 tumor tissues, respectively ($p < 0.001$; Fig. 5).

Capsaicin dose in mice resembles human capsaicin consumption and mimics stage-dependent gastric cancer development

To apply our observations to humans, we optimized the mice's intake to reflect human intake to determine the dose-dependent (0.01-0.5% or 0.05-2.6g/kg/day) effect of capsaicin. Mice treated with both *H. pylori*

and capsaicin developed gastritis, leading eventually to cancer. To confirm this relationship in mice and to establish its relevance in humans, we conducted toxicity experiments in mice and optimized the capsaicin amount to determine its dose-dependent (0.01-0.5% or 0.05-2.6g/kg/day) effect. Mice treated with a combination of *H. pylori* infection and capsaicin (at a dose greater than 0.06% or 0.312g/kg/day but less than 0.05% or 0.26g/kg/day), which is relevance to human dose of capsaicin (0.021-0.025-g/kg/day) induced a non-toxic effect. Notably, a dose greater than 0.05% or 0.26g/kg/day induced significant toxicity and severe gastric inflammation. In contrast, a dose less than 0.05% or 0.26g/kg/day did not cause any toxic effect or any significant gastric inflammation in mice (Supplementary Tables 1 and 2). As a result, we selected a non-toxic, dose of 0.05% or 0.26g/kg/day². These results suggest that capsaicin dosage contributes considerably to the development of gastric inflammation and carcinogenesis.

We also examined capsaicin intake, metabolism, and excretion. We found a high blood assimilation of capsaicin in mice treated with capsaicin and *H. pylori* (0.002 ± 0.0004 g/kg/day) compared to mice treated only with capsaicin (0.002 ± 0.0002 g/kg/day). This observation corresponded with low urine dissimulation of capsaicin in the gastric tumor group (0.0003 ± 0.00001 g/kg/day) compared to the group treated with only capsaicin (0.0006 ± 0.0001 g/kg/day; Fig. 6a). Similar results were found in human samples; gastric cancer patients (0.003 ± 0.0002 g/kg/day) and chronic gastritis patients (0.001 ± 0.0002 g/kg/day) showed high levels of capsaicin blood assimilation compared to healthy subjects (0.0005 ± 0.0001 g/kg/day; $p < 0.0001$; Fig. 6b). These results also confirmed the role of capsaicin consumption in the development of physiological abnormalities and gastric cancer. High levels of capsaicin metabolites in urine following capsaicin consumption may be a sign of gastric tumor development as well. The risk for capsaicin-associated cancer is influenced by the expression of capsaicin metabolizing enzymes, such as 16-hydroxycapsaicin, 17-hydroxycapsaicin, and 16, 17-dehydrocapsaicin, vanillylamine, and vanillin, which increase the risk for cancers. As a result, propose that capsaicin is a potential carcinogen^{22 23}. Ultimately, we developed a mouse model that utilizes a capsaicin dosage relevant to human capsaicin intake. With this model, gastric tumorigenesis is induced without toxicity in peripheral organs. As a result, this mouse model was used to study the effect of capsaicin and its metabolites on the development of gastric cancer.

Capsaicin-treated and *pylori*-infected mouse tumors show increased pro-inflammatory cell infiltration

Gastric inflammation leads to gastric tumor development. In our mouse model, the number of pro-inflammatory cell types was significantly increased (i.e., leukocyte infiltration), as observed by the numbers of activated macrophages (F4/80 positive) and neutrophils (myeloperoxidases, MPO) in mice exhibiting gastric tumors (0.746 ± 0.041 and 0.721 ± 0.064 , respectively), compared to untreated mice or mice treated with only capsaicin or *H. pylori* ($p < 0.0001$; Supplementary Fig. 2a-b). Due to increased immunocyte infiltration, we analyzed several inflammatory-related chemokines and cytokines at the

mRNA and protein (secretion) levels by using a customized multiplex magnetic bead array. In mice treated with capsaicin and *H. pylori*, gastric tumorigenesis was associated with augmented Th1 (IFN- γ , IL-1 β , and GM-CSF) and Th2 (IL-10, IL-6, IL-13) cytokine expression ($p < 0.001$; Fig. 7a-l). In particular, the pro-inflammatory cytokines IL-6 and IFN- γ showed high and low expression in mice with gastric cancer induced by treatment with capsaicin and *H. pylori*, respectively. Overall, *H. pylori* infection and capsaicin consumption produced great sensitivity and specificity towards IL-6 and IFN- γ expression. As a result, IL-6 and IFN- γ cytokines may play a critical role in the development of gastric inflammation and later gastric cancer.

DFMO prevents *pylori* and capsaicin-induced gastric cancer

We determined the role of IL-6 and IFN- γ in gastric cancer prevention using the anti-inflammatory agent DFMO (scheme illustrated in Fig. 8h). We administered capsaicin and *H. pylori* to mice followed by DFMO treatment. Macroscopic morphometric analysis revealed that DFMO (100 and 200 mg/kg) inhibited gastric inflammation in a dose-dependent manner and prevented gastric tumorigenesis (Fig. 8a, b). The anti-inflammatory potential of DFMO against gastritis and therefore against gastric cancer was evaluated by analyzing IL-6 and IFN- γ status. We observed a significant dose-dependent decrease and increase in the pro-inflammatory cytokine IL-6 and IFN- γ level for DFMO-treated mice compared to untreated mice exhibiting gastritis and to *H. pylori*-eradicated mice ($p < 0.0001$; Fig. 8c, d), respectively. In contrast, COX-1 and COX-2 expression was also substantially reduced ($p < 0.001$; Fig. 8e, f). Histological analysis also showed multifocal elongation of the gastric pits, glandular atrophy, and a significant reduction in the glandular zone in atrophic foci. These changes were significant in mice treated with capsaicin and infected with *H. pylori* exhibiting gastric inflammation (2.800 ± 0.200), compared to mice treated with DFMO (200 mg/kg; 1.400 ± 0.244) or untreated (0.6 ± 0.245 ; $p = 0.002$; Supplementary Fig. 3a-d). DFMO-treated mice (100 mg/kg or 200 mg/kg) showed significantly higher body weight compared to mice treated with a combination of capsaicin and *H. pylori* infection (Fig. 2g). This result suggests that DFMO may prevent gastric tumorigenesis induced by a combination of capsaicin consumption and *H. pylori* infection. DFMO appears to act as an anti-inflammatory agent to prevent capsaicin-associated gastric cancer development by targeting COX-1 and COX-2 and by regulating IL-6 and IFN- γ expression.

Discussion

Previous epidemiological and meta-analysis studies demonstrated a dose-dependent relationship between capsaicin consumption (30–80 g/day) and an increased risk of developing certain cancers, including stomach cancer^{24 25 26}. Our study provides a biological rationale explaining high gastric cancer incidence in regions with high capsaicin consumption and *H. pylori* infection. Our mouse model confirms that capsaicin contributes to the induction of gastric inflammation leading to gastric cancer in the presence of *H. pylori* infection (Fig. 1).

Normal gastric mucosal development requires gastrin, somatostatin, and H + K + ATPase; however, all three are greatly diminished in mice with gastritis and tumorigenesis (Fig. 2). These ablations affect gastric mucosal health and parietal cell activation. Moreover, treatment with a combination of capsaicin and *H. pylori* infection leads to increased peroxidation. This observation suggests that stimulation of gastric inflammation and gastric tumorigenesis involves production of inflammation-inducing proteins and cytokines (Fig. 4). Thus, capsaicin and *H. pylori* synergistically and directly contribute to accelerated loss of differentiated epithelial cell types, leading to chronic atrophic gastritis and gastric tumorigenesis.

We propose that *H. pylori* regulates pro-inflammatory cytokine production and release of IL-6, IFN- γ , IL-17A, IL-1 β , IL-27, and TNF- α . This cytokine release causes gastric tissue damage leading to activation of inflammatory cancer signaling pathways (Fig. 7). The outcome of IL-6 stimulation suggests that pre-existing gastric immunopathology accelerates the loss of differentiated epithelial cell types, leading to profound glandular atrophy and gastric tumorigenesis. In fact, IL-6 stimulation with IFN- γ inhibition may promote the shift from gastric inflammation to gastric carcinogenesis in mice administered both capsaicin and *H. pylori* (Fig. 7). As a result, IL-6 inhibitors can serve as a therapy against gastric cancer, in addition to lowered capsaicin intake and pathogen clearance. Gastritis patients with high IL-6 levels could also benefit from IL-6 inhibition as a preventive measure against gastric carcinogenesis (Fig. 7).

Tumorigenesis in C57Balb/c mice is attributed to IL-6 and IFN- γ deregulation in capsaicin-treated and *H. pylori*-infected mice. This deregulation leads to reduced expression of Tff1 and Tff2, along with reduced expression of putative tumor suppressor genes, Gkn1 and Gkn2. Tff1 and Tff2 are important endogenous regulators of gastric homeostasis, and they show profound loss of expression during capsaicin exposure and *H. pylori* infection^{14,16}. Accordingly, capsaicin and *H. pylori* are critical regulators of Tff2 and upstream gastrophilic gene expression. Tff2 inhibition likely promotes tumor growth due to loss of anti-proliferative gastrophilic and Tff1 genes. Additionally, the human GKN2 protein predominantly exists as a heterodimer with Tff1¹⁷; attenuated expression of either protein may potentiate their combined loss of function. Downregulation of both Tff1 and Tff2 expression also frequently occurs in gastric cancers displaying tumor suppressor function. However, loss of Tff1 and Tff2 promotes increased gastric inflammation and accelerates fundic atrophy with significant loss of parietal and chief cell lineages (Fig. 3). This finding provides a mechanism for capsaicin-associated gastric cancer cell proliferation¹⁸.

Our data suggest that combined capsaicin exposure and *H. pylori* infection modulate and imbalance Th1 and Th2 cytokine levels, leading to mucosal inflammation, inhibition of gastric acid secretion, and induction of fundic atrophy. Tumorigenesis in capsaicin- and *H. pylori*-treated mice was associated with augmented Th1 cytokine release, particularly IL-6, along with attenuated Th2 cytokine expression. Th1 cytokines like IL-6 promote chronic atrophic gastritis and predisposition to tumorigenesis through inhibition of gastrin and somatostatin, as indicated earlier (Fig. 7). In combination, capsaicin and *H. pylori* provide a gastric cancer development mechanism in which local immune responses modulate and decrease essential gastric hormones.

Here, the selected anti-inflammatory agent, DFMO (2-difluoromethylornithine) inhibits ornithine decarboxylase (ODC) activity, an enzyme catalyzing a rate-limiting step of polyamine biosynthesis. ODC is necessary for entry into and progression through the cell cycle; additionally, Selvakumaran et al. demonstrate that ODC is a transcriptional target of the c-myc oncogene (35). In our mouse model, DFMO acts by inhibiting IL-6 and promoting IFN- γ cytokine release (Fig. 2), plus it enhances antitumor CD8⁺ T cell infiltration and augments adoptive T cell therapy. Furthermore, DFMO impairs the suppressive activity of myeloid-derived suppressor cells (MDSC), reverses tumor-induced immunosuppressive mechanisms, and invokes a measurable antitumor immune response^{27 28 29}. Consequently, DFMO treatment can serve as a preventive measure against gastric tumorigenesis induced by capsaicin consumption and *H. pylori* infection.

Conclusion.

This study describes the mechanisms governing the induction and progression of chronic gastritis to gastric cancer under the inflammatory triggers of *H. pylori* infection and capsaicin exposure. This study also proposes DFMO treatment to prevent gastric cancer through pro-inflammatory IL-6 cytokine inhibition, resulting in diminished gastric inflammation. This study is the first to provide experimental animal evidence showing gastric cancer prevention by DFMO treatment in mice with capsaicin consumption and *H. pylori* infection. In the future, these mouse models can also facilitate further studies into the molecular mechanisms of chronic gastritis and gastric cancer.

Methods

Preparation of *H. pylori* strains

Helicobacter pylori (*H. pylori*, Sydney strain or SS1) was provided by Professor Young-Joon Surh (Seoul National University, Seoul, South Korea). *H. pylori* SS1 was grown on Columbia Agar (Oxoid, Basingstoke, Hampshire, England, Cat number CM0331) plates for 48 h at 37 °C under microaerophilic conditions on 7% lysed horse blood (Solarbio, Beijing Solarbio Science & Technology, Beijing, China Cat number S9050) agar and antibiotics, including amphotericin B (1.5 μ g/ml; Solarbio, Cat number: A8250), trimethoprim (1.25 μ g/ml) (Tichea Chemical Industry Development Co, Shanghai, China, Product code T2286), and vancomycin (2.5 μ g/ml) (Solarbio, Cat number: V8050). Identification was performed by Gram staining (Solarbio, Cat number G1060) and testing for urease (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Cat number C013-2), catalase (Nanjing Jiancheng Bioengineering Institute, Cat number A007-1), and molecular markers.

Animal model experimental design

Mice were eight-week-old male C57-BL/6J-219 mice (18–20 g; Beijing Vital River Laboratory, Beijing, China) that were free of *Helicobacter* spp, *Citrobacter rodentium* and *Salmonella* spp. Mice were maintained under specific pathogen-free conditions and fed sterilized commercial pellet diets (Beijing

HFK Bioscience, Beijing, China) and sterile water ad-libitum and housed in an air-conditioned biohazard room at a controlled temperature of 24 °C, under a 12 h light/dark cycle. The experiments were performed after protocol approval by the ethics committee of China-US Hormel Cancer Institute, Henan, China and were conducted in accordance with the current guidelines for laboratory animal care. C57-BL/6J-219 (Access No: CUHCI2015011) were randomly assigned to 5 groups of 10 animals each. After one week of acclimation, mice were administered capsaicin (0.05% or 0.2 g/kg/day) in dietary food for two weeks prior to infection with *H. pylori*, and capsaicin administration was continued throughout the experiment. To facilitate *H. pylori* colonization, pantoprazole (20 mg/kg) was administered by gavage 3 times to lower gastric acidity. Each mouse was administered a suspension of the *H. pylori* SS1 strain containing 10^8 CFUs/mL by gavage 3 times per week. Mice (n = 5) were euthanized on consecutive weeks of 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 and 52 weeks. A control group was maintained without any treatment and were euthanized at same time as experimental. In another mice model, doses of capsaicin ranging from 0.5–5.0 g/kg/day were administered to mice (n = 5) to determine the optimal capsaicin dose to induce gastric tumorigenesis. In addition, after confirmation of gastritis development at 32 weeks after *H. pylori* infection, a DFMO-treated gastritis mouse model was developed (Access No: CUHCI2016015). *H. pylori* was eradicated by administering by gavage a triple regimen of omeprazole (400 µmol/kg/day; Jiangsu Pengyao Pharmaceutical Co, Yinxing, Jiangsu, China), metronidazole oral suspension (14.2 mg/kg/day; Guizhou Feiyuling Pharmaceutical Co, Guizhou, China), and clarithromycin granules in oral suspension (7.15 mg/kg/day; Xiuzheng Pharmaceutical Co. Changchun, Jilin, China) twice daily for 7 days with a 30–60 min interval between omeprazole and antibiotics. Persistence of gastric inflammation after successful eradication of *H. pylori* was analyzed for two months (41 weeks). C57-BL/6J-219 mice that still had *H. pylori* after the eradication therapy were excluded from analysis. *H. pylori*-eradicated mice were divided into two groups based on DFMO dose (100 and 200 mg/kg groups; 49 weeks) (Sigma Aldrich, St. Louis, MO, USA, Cat number 17378). Mice were administered a suspension of DFMO by gavage 3 times a week for 2 months. A toxicity assay was performed to optimize the amount for consumption. Mice (n = 10) were administered different amounts of capsaicin (0.01–0.5% or 0.05–2.6 g/kg/day) up to 3 weeks and mouse survival rate was analyzed.

Gastric sample preparation

Mice were euthanized and their stomachs excised and gently rinsed with cold saline. For gastric tumor phenotype analyses, stomachs were opened along the greater curvature and spread out on a polypropylene sheet (Jiangsu Jiangyin Jinfeng Textile Co., Jiangsu, China). The area (mm²) of the mucosal erosive lesions was measured and tumors were macroscopically identified in the gastric mucosae by micro-dissection of gastric tumor tissue. The anterior wall of the pyloric antrum contains the pyloric glands and gastric epithelial cells were cut into several linear strips for quantitative enzyme activity, DNA, RNA, protein, and histological analyses. Other peripheral organs were also collected, including brain, lung, liver, kidney, spleen, colon, and heart, in order to analyze by H&E staining the toxic effect of capsaicin consumption.

Patients And Gastroendoscopy

All gastric patients were subjected to gastroendoscopy and examination in the Second Affiliated Zhengzhou University Hospital and Henan Cancer Hospital (Zhengzhou, Henan, China). Blood samples and tissue biopsies were obtained from consenting patients from the antral and corpus portions of the stomach during gastrointestinal endoscopy and gastric surgery. The patients who donated the primary tumors were completely informed and provided written consent (Access No: CUHCl2015009).

Morphometric and histological analysis

Morphometric analysis was performed using Image-J software (<http://rsb.info.nih.gov/ij/index.html>); National Institutes of Health, Bethesda, MD). For the histological analysis, tissues examined consisted of a section of stomach taken from the greater curvature and cut from the fundus to the proximal end of the corpus. Longitudinally bisected half stomachs were retained in buffered formaldehyde sections of 6 mm and stained with H&E. These samples were scored depending on the severity of (i) superficial regional disruption of the gastric gland with epithelial cell loss, (ii) neutrophil infiltration & mononuclear cell infiltration (inflammatory cells), (iii) glandular zone reduction, and (iv) functional atrophy (loss of parietal and chief cells), using a scale ranging from 0 to 4 (0: none; 1: weak; 2: mild; 3: moderate; and 4: severe) for each criterion. The sections were assessed by an experienced pathologist without knowledge of the treatments. The presence of mononuclear cell infiltration and poly morphonuclear (PMN) cell infiltration indicated chronic gastritis. The length of the glandular zone, primarily composed of parietal cells, was measured as a proportion of total mucosal thickness. Antral tumor tissues were analyzed with measurement of tumor volume and compared with the controls.

Immunohistochemistry (ihc)

Paraffin-embedded gastric tissues were analyzed by immunohistochemistry (IHC). Serial sections (4–6 mm each) were deparaffinized in xylene and rehydrated in an capsaicin concentration gradient and evaluated with antibodies to detect *H. pylori* (1:100), pepsinogen I and II (1:100 each), MPO (1:100), F4/80 (1:100), CD-34 (1:100), PCK-26 (1:100), and SMA- α (1:100). Sections were subsequently incubated with their respective secondary antibodies for 30 minutes at room temperature. The signal was visualized with peroxidase-labeled streptavidin complexes by DAB, and the sections were briefly counterstained with hematoxylin. The immunohistochemical localization pattern was also recorded by digital imaging (Nikon Ti-DS, Japan). The ImageScope (11.1.1.752) software program was used and the labeling index was calculated as a percentage of positive cells relative to the total number of counted cells.

Quantitative Enzyme And Activity Assays

Quantitative enzyme assays included measurement of malondialdehyde (MDA; Cat number A003-1), myeloperoxidase (MPO; Cat number A044), carbonyl (Cat number A087), lipoperoxidase (LPO), catalase

(Cat number A007-1), and capsaicin dehydrogenase (ADH; Cat number A083-1) and were performed according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Real-time RT-PCR

Total RNA was extracted using a commercial RNA extraction kit (Ambion by Life Technologies, Van Allen Way Carsbad, USA, 92008), and cDNA was synthesized by amfiRivert cDNA synthesis platinum master mix (GenDEPOT, Katy, TX, USA, Cat number R5600-200). Real-time PCR (qRT-PCR) was conducted using a 7500 FastDX (Applied Biosystems, MA, USA) and the power SYBR green PCR master mix (Applied biosystem, Warrington WA1 4SR, UK, Cat number 4367659). Primer IDs and sequences are shown in Supplementary Tables 3.

Cytokine and chemokine protein measurement using a multiplex bead array

Cytokine and chemokine protein levels in mouse (Cat number 740446) and human (Cat number 740118) serum were measured using a multiplex magnetic bead array kit (customized by BioLegend LEGENDplex, San Diego, CA, USA). The multiplex bead arrays were performed according to the manufacturer's instructions with a minimum detectable concentration varying from 0.96–11.27 pg/mL. The Legendplex (version: 7.0) software program was used to analyze the FACS data and the cytokine concentrations were calculated in pg/mL against standard values.

Urine collection and measurement (metabolic cage experiments)

Capsaicin metabolism and urinary flow rate were determined by placing mice individually in metabolic cages. Mice were allowed a 3-day habituation period to adapt to the environment. Later, food and water intake, urinary flow rate, and body weight were recorded every day. Subsequently, a 12 h collection (9 p.m. to 9 a.m.) of urine was performed to obtain the urinary parameters, including volume, pH, and color. Data were analyzed for capsaicin intake, metabolism, and excretion. Capsaicin content in the serum and urine samples from mice and humans was measured according to the Capsaicin ELISA Commercial kit's instructions (Shanghai Jianglai Biotechnology, Shanghai, P.R.C, Cat number JL22882).

Mouse-derived Xenograft Model

A mouse-derived xenograft model was developed from gastric tumor tissue of mice treated with capsaicin (Access No: CUHCI2016024). Tissue samples were placed in a Petri plate containing phosphate buffered saline (PBS) with 0.1 ml of penicillin (1×10^5 U/ml; North China Pharmaceutical, Hebei, China), gentamycin (8×10^3 U/ml; Kaifeng Pharmaceutical Co., Henan, China), and streptomycin

(1×10^5 U/ml; North China Pharmaceutical, Hebei, China). The tissues were divided into 3 parts. One portion was implanted into CB17 SCID mice purchased from Beijing Vital River Laboratory (Beijing, China). The second portion was fixed in 10% formalin, and the third portion was used for protein extraction. The mice were anesthetized using 0.3 ml of 0.4% (w/v) pentobarbital sodium (150 μ l/10 g; Sinopharm Chemical Reagent, Guangdong, China) for every 20 g of body weight. Then mice were subcutaneously implanted with tissues weighing 0.10—0.12 g and measuring ~ 3 mm. Animals were monitored periodically for their weight and tumor growth. A second passage was performed and the same protocol was followed as described above.

Statistical analysis

The experiments were randomized and investigators were blinded to histological examination during all experiments. All statistical analyses were performed using Graphpad Prism 5.0 software (San Diego, CA, USA), with differences between groups considered significant with a p value < 0.001. Data are presented as mean values $\pm$ S.E.M. Histopathological scores and all other experimental data were compared using a t-test (two-sided) or one-way analysis of variance (ANOVA) followed by (post hoc) Newman-Keuls multiple and Turkey multiple comparison tests. The discriminatory ability of markers for gastric cancer was evaluated by Receiver Operating Characteristics Curve (ROC) providing the Area Under the Curve (AUC). All tests were two-sided and p-values $\leq$ 0.01 were considered statistically significant. Statistical software IBM SPSS 20.0 (SPSS Inc., Chicago, IL) and R program package (Wirtschafts Universität, Wien, Austria) were used to perform these analyses.

Abbreviations

Gkns, Gastrokines; H. pylori, Helicobacter pylori; IARC, International Agency of Research on Cancer; IFN, interferon; IL, interleukin; LPO, lipoperoxidase; MDA, malondialdehyde; MDX, mouse xenograft model; MPO, myeloperoxidases; SMA- α , smooth muscle antigen; Tff1, Trefoil factor1; Tff2, Trefoil factor2; TNF, tumor necrosis factor; TSGs, tumor suppressor genes; WHO, World Health Organization.

Declarations

Ethics approval and consent to participate

The institutional review board at China-US (Henan) Cancer institute approved the study. Subjects provided informed consent to be included

Consent for publication

All experiments were approved by the ethics committee of The Affiliated Cancer Hospital Affiliated to Zhengzhou University, Henan, P. R. China. Written informed consent was got from every patient before the start of our research.

Data availability

All data needed to evaluate the conclusions of this paper are present in the paper and/or Supplementary Materials. The source data underlying Figs 1c–d, 2a–f, 3a–d, 4a–e, 5b–e, 6a–b, 7a–l and 8c–g and Supplementary Figs S1a–d, S2a–b and S3a–d, are provided as Source Data files. All other data are available from the corresponding author (Z.D.) by request.

Competing interests

None of the authors declare any competing interests.

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Author Contributions

F.A. designed and performed the experiments, derived the models and analyzed the data; F.A. and Z.D. conceived the idea. Z.D. supervised the project. F.A., M. X. and J. M. analyzed the data and interpretate the results. F.A. and K. J. designed the animal protocol and performed animal experiment. F. A., Y.G and Q. W. performed the pathological analysis of mouse tissue samples. F.A, wrote and A.M.B. edited the manuscript.

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Supplemental Legends

Supplementary Fig. 1. Gastric histology scores in gastric disease mice models. Individual lesion scores of every mouse were compared for **a)** glandular zone, **b)** epithelial defects, **c)** atrophy, and **d)** infiltration of inflammatory cells; (n = 5/cohort). Mice exhibiting gastric tumorigenesis induced by combined *H. pylori* and capsaicin treatment showed a significantly higher score (greater severity) compared to mice exhibiting gastritis with no tumor. Mice treated with only capsaicin or only *H. pylori* showed milder pathology, and the untreated group had normal histology.

Supplementary Fig. 2. Analysis of inflammatory mediators in tissues from the gastric disease mouse models. Immunohistochemical analysis of MPO and F4/80 expression in gastric tissues of the various mouse models. Quantitative immunohistochemistry to detect **a)** MPO and **b)** F4/80 expression revealed a higher score in tumor tissue compared to tissues from mice treated with capsaicin or *H. pylori* only. Staining intensity was weaker in untreated gastric tissue compared to gastric tumor tissue. The scale bars represent 100 μ m (10x), and the scale bar in the inset images represents 20 μ m (40x). A score of 1 denotes the highest staining or expression levels, whereas a score of 0 indicates negative staining.

Supplementary Fig. 3. Gastric histology scores in gastric disease mice models. Individual lesion scores of every mouse were compared for **a)** glandular zone, **b)** epithelial defects, **c)** infiltration of inflammatory cells, and **d)** atrophy; (n = 5/cohort). Mice exhibiting gastric tumorigenesis induced by combined *H. pylori* and capsaicin treatment showed a significantly higher score (greater severity) compared to mice treated with DFMO exhibiting low gastric inflammation gastritis with no tumor. Mice eradicated with *H. pylori* showed milder pathology, and the untreated group had normal histology.

Figures

Figure 1

Development of mouse models for capsaicin- and *H. pylori*-induced progression of gastritis to gastric cancer. a) Schematic drawing showing the effect of capsaicin consumption and *H. pylori* infection in the development and progression of gastritis to gastric tumorigenesis mediated through the stimulation of IL-6. b) Macroscopic (top) and histologic (bottom) analyses of gastric mucosae ($n = 5/\text{cohort}$). Images of tumors at 52 weeks from mice infected with *H. pylori* and treated with capsaicin; tumor areas are outlined with dotted circles. Hematoxylin and eosin (H&E) stained cross sections and cut along the fundus to the proximal end of the corpus as shown by the straight dotted line. Gastric pathology was substantially changed after 32 or 52 weeks of combination treatment with *H. pylori* and capsaicin. The panels are magnified 10x (bar, 100 μ m) and 40x (bar, 20 μ m). Arrow and G = glandular zone, I = inflammatory cells, IN = muscular mucosae invasion. c) The size of gastric tumors from mice treated with capsaicin and infected with *H. pylori* compared to mice treated with only capsaicin or *H. pylori* is shown. d) Effect of capsaicin, *H. pylori*, or a combination of capsaicin with *H. pylori* infection on mouse body weights. Body weights are shown as treated/untreated. e) The chemical structural of capsaicin. Data are expressed as mean values $\pm$ S.E.M., ($n = 5/\text{cohort}$).

Figure 2

Immunohistochemical and qPCR analysis of gastric disease mouse models. Immunohistochemical detection of pepsinogen I and II and *H. pylori* expression in gastric tissues from mice treated with both capsaicin and *H. pylori*. Paraffin-embedded gastric tissues were stained to detect *H. pylori*, pepsinogen I, or pepsinogen II, respectively. Quantitative immunohistochemical detection of a) *H. pylori*, b) pepsinogen I, or c) pepsinogen II expression. A strong intensity was observed in untreated gastritis tissue compared to gastric cancer tissues. Scale bars represent 100 μ m (10x), and the scale bar in the inset images

represents 20 μm (40x). A score of 1 denotes the highest staining, whereas a score of 0 indicates negative staining. mRNA analysis of d) gastrin, e) H+K+ ATPase, and f) somatostatin by qPCR. Average fold change in mRNA relative to untreated control (n = 5/cohort).

Figure 3

Gastric tumor suppressor gene expression generally decreases in mice treated with both capsaicin and *H. pylori*. mRNA analysis of a) *Tff1*, b) *Tff2*, c) *Gkn1*, and d) *Gkn2* by qPCR. Histograms show the average fold change in mRNA expression compared to untreated mice (n = 5/cohort).

Figure 4

The level of tissue damage in gastric mucosa from gastric disease mouse models. Biochemical activity of a) LPO, b) carbonyl, c) catalase, d) MPO, and e) MDA in mice. Mice exhibiting gastritis or tumorigenesis show higher biochemical activity of these parameters compared to mice treated with capsaicin or *H. pylori* only (n = 5/cohort).

Figure 5

Mouse-derived xenograft model (MDX) to study the effect of combined treatment with capsaicin and *H. pylori*. a) Macroscopic analyses of tumor tissue. Tumor images of first (M1) and second passage (M2) tumors showed a high growth rate in SCID mice. Tumors from mice treated with capsaicin and infected with *H. pylori* are represented as M0. The M1 and M2 tumor tissues were similar to M0 in b) weight and c) volume (mm³). d) Body weight in grams measured per week. Mice were weighed before treatment (week 0) and then twice per week for 7 weeks. Mice with M0, M1, and M2 tumors lost weight compared to untreated control mice (n = 5/cohort). e) Histological analyses of tumor tissues from mice treated with a

combination of capsaicin and *H. pylori* (M0) with first (M1) and second passage (M2) xenografts. Histochemical analysis of PCK-26 and CD-34 in mouse tumor tissue (MDX) showed strong staining compared to the weak staining of SMA- α . A score of 1 denotes the highest staining and expression, whereas a score of 0 indicates negative staining or no expression. Quantitative immunohistochemistry of PCK-26 and CD-34 exhibits high scores in tumor tissues compared to a low score for SMA- α .

Figure 6

Capsaicin consumption in the development of gastric tumorigenesis. Association of capsaicin intake, metabolism, and excretion in mouse and human gastric disease samples. Capsaicin concentration in a) mouse and b) human (n = 10) serum and urine samples was used to calculate capsaicin intake, absorption (serum), and excretion (urine). Analysis of urine samples from the gastric disease mouse models.

Figure 7

Analysis of cytokines in serum from gastric disease mouse models. Secretory profiles of pro-inflammatory cytokines a) IL-27, b) IFN- β , c) IL-22P70, d) IL-1 α , e) IL-23, f) IL-10, g) TNF- α , h) IL-17A, i) IL-6, j) IL-1 β , k) IFN- γ , and l) MCP-1 in mouse serum samples measured by multiplex magnetic bead array. Graphs show secreted protein expression relative to serum samples from untreated mice (n = 5/cohort).

Figure 8

DFMO preventive gastritis mouse model. a) The gastric tissues from mice exhibiting gastritis induced by capsaicin and *H. pylori* or mice with *H. pylori* that was eradicated and treated with DFMO (100 or 200 mg/kg) did not show tumor development (n = 5/cohort). Histological sections were stained with H&E. The scale bars represent 100 μ m ($\times 10$) and the scale bar in the inset images in each panel represents 20 μ m ($\times 40$). b) The chemical structural of DFMO. c) IL-6, d) IFN- γ secretory protein levels in mouse serum samples were measured by a multiplex magnetic bead array (n = 5). Quantitative immunohistochemistry of e) COX-1 and f) COX-2 expression in gastric tumors and gastritis tissue. g) Effect of DFMO (100mg/kg) and (200mg/kg) on mouse body weights. Body weights are shown as treated/untreated. Data are expressed as mean values $\pm$ S.E.M., (n = 5/cohort). h) A schematic drawing showing the proposed mechanism of capsaicin consumption combined with *H. pylori* infection in the development and progression of gastritis to gastric tumorigenesis.

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