The synergy of *Helicobacter pylori* and lipid metabolic disorders in induction of Th17-related cytokines in human gastric cancer

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**ABSTRACT**

**Aim:** To study the impact of *Helicobacter pylori (H. pylori)* and lipid metabolic disorder on the expression of Th17-related cytokines in gastric cancer (GC).

**Methods:** GC specimens were randomly collected from 42 patients, of whom 15 had *H. pylori* infection and 27 were without. Tumor RNA was extracted for reverse transcription quantitative polymerase chain reaction quantification of gene expression.

**Results:** The mRNA levels of interleukin (IL)-6 and leptin, which are known to regulate Th17 differentiation, were upregulated by 20 and 6 folds, respectively, in *H. pylori*-infected compared to uninfected patients. IL-17A and granulocyte-macrophage colony-stimulating factor, two cytokines produced by Th17 cells, were 5- and 6-fold higher in tumors with *H. pylori* infection, respectively. Consistently, RORγt, a transcription factor regulating Th17 differentiation, was increased 6-fold in *H. pylori*-positive vs. negative tumors. Further elevation of RORγt was seen in advanced *H. pylori*-associated tumors. In addition, *H. pylori* infection was also associated with enhanced expression of CXCL1 (5 folds), chemotactic factor capable of driving bone marrow-derived immature myeloid cells. Interestingly, we observed that *H. pylori*-associated increase of IL-17A was enhanced in the group with higher plasma triglycerides.

**Conclusion:** The findings demonstrate a cross-talk and synergistic role of *H. pylori* infection and abnormal lipid metabolism in GC development, at least partly via cooperative induction of Th17 differentiation and activation.
INTRODUCTION

Gastric cancer (GC) is currently the third leading cause of cancer-related death worldwide due to the highly metastatic property and poor prognosis.\(^1,2\) The overall 5-year survival rate of GC patients is only between 15 to 35%.\(^3\) Epidemiological studies show that persistent Helicobacter pylori (H. pylori) infection accounts for approximate 75% of confound risk factors for GC.\(^4-6\) Understanding the underlying mechanism of GC development associated with H. pylori infection will be important for developing novel therapeutic methods.

H. pylori, a gram-negative spiral-shaped pathogenic bacterium, specifically colonizes and induces damage to the gastric epithelium leading to chronic gastritis, ulcers and even cancer.\(^2,7,8\) Considerable studies have demonstrated that a mixed response of Th1 and Th17 cells plays a critical role in H. pylori-induced inflammatory gastric diseases and cancer.\(^9,10\) The phenotypes of T helper subsets are determined by the local cytokine milieu and their lineage-specific transcription factors.\(^11-13\) H. pylori elicits Th1 response to produce interferon-γ and tumor necrosis factor-α causing chronic gastritis and ulcers.\(^9,13\) Th17 cells are also frequently recruited by H. pylori to the gastric mucosa, and are characterized by expression of interleukin (IL)-17A/F, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, IL-22 and IL-23, and the transcription factor of ROR\(_\gamma\)T.\(^11,14\) While activation of Th17 cells contributes to bacterial eradication,\(^15\) Th17-mediated immune-response can be detrimental to gastric epithelium during gastritis.\(^9,14\) Th17 cells can be further activated in tumor microenvironment due to involvement of IL-6 and transforming growth factor-β.\(^16,17\) Although activation of Th17 cells might have antitumor activity by facilitating the recruitment of other effector immune cells,\(^18\) Th17-derived IL-17A favors angiogenesis and tumor growth through inducing IL-6 that in turn activates STAT3 signaling to promote tumor survival and angiogenesis.\(^19,20\)

It has been reported that obesity and diabetes can worsen the process of Helicobacter-associated GC.\(^19,21\) However, the cross-talk between Helicobacter infection and metabolic disorders in the gastric carcinogenesis remains not completely understood. We recently demonstrated that high fat diet (HFD) and obesity could strongly enhance H. felis-induced GC in mice.\(^19\) We observed that H. felis infection potently stimulates stomach Th17 recruitment and development, and enhanced mobilization of bone-marrow derived IMCs via CXCL1 expression. Interestingly, the local Th17-associated gastric inflammation results in increased IL-17A in blood and causes adipose inflammation in HFD-fed obese mice. In turn, fat-derived IL-6 and leptin can promote gastric Th17 expansion, thus forming a positive loop in Th17 activation. These findings suggest that Th17 and IL-17A play a critical role in the synergy of Helicobacter infection and metabolic abnormalities in accelerating GC progression. In present study, we used clinical GC specimens to document Th17-related cytokines and explore the roles of H. pylori infection and lipid metabolic disorders in GC development. Our results suggest that dysregulated lipid metabolism may synergize with H. pylori to promote GC development.

METHODS

Clinical specimens

Forty-two GC specimens were randomly collected from Fujian Provincial Cancer Hospital in China. H. pylori infection was clinically diagnosed and confirmed with the expression of CagA, VacA or both.\(^22,23\) The patients were also divided into high and normal lipid groups with a diagnostic cut-off of 1.7 mmol/L of plasma triglyceride (TG).\(^24\) All tumors were histological diagnosed according to the World Health Organization classification. The pathological TNM stage and clinical stages were also recorded.\(^25\)

Extraction of RNA and quantitative real-time PCR

Total RNA was extracted using Triazol kit (Invitrogen Company, USA) with slight modifications of protocol. The RNA was reverse transcribed using Hifair™ III 1st Strand cDNA Synthesis Kit (Yesen Company, China). The cDNAs were then used in quantitative polymerase chain reaction (qPCR) quantitative analysis of IL-6, leptin, IL-17A, GM-CSF, CXCL1 and ROR\(_\gamma\)T mRNA expression levels in ABI 7500 system (Applied Biosystems, Foster, CA) by using Hieff™ qPCR SYBR® Green Master Mix (Yesen Company, China). Their relative levels were normalized to β-actin expression. Specific primers used in this study were listed in Table 1.

Statistical analysis

Data analysis was conducted by using Graph pad 6.0 Software. After log transformation, normal distribution was analyzed. Comparison between the two groups was done using t-test and Spearman analysis of correlation was performed between the groups. The contingency were analyzed by using Chi-square testing.
RESULTS

The effects of metabolic milieu on *H. pylori*-induced IL-17A expression
Since obesity was suggested to play an important role in *H. felis*-induced GC by stimulating Th17 response in mice,[19] we determined the effects of *H. pylori* infection and lipid metabolic disorders on Th17-related cytokines in human GC. Our results showed that IL-17A was 5-fold higher in *H. pylori*-infected than uninfected patients [Figure 1A], while approximately 1.8-fold increase in IL-17A levels was seen in patients with high TG (> 1.7 mmol/L) comparing to those with normal TG [Figure 1B]. However, abnormal TG could not alone induce IL-17A expression as evidenced in *H. pylori*-negative patients [Figure 1C]. *H. pylori* could increase IL-17A expression by 3.2 folds in patients with normal plasma TG, but could further increase IL-17A expression (5.5 folds) in the milieu of high TG content [Figure 1C]. However, the contingency analysis showed that there was no significant correlation between *H. pylori* infection and metabolic factors including plasma glucose, cholesterol, low density lipoprotein, high density lipoprotein and TG [Table 2]. Taken together, the data demonstrated that *H. pylori* infection and lipid metabolic disorders could synergistically increase IL-17A expression.

*H. pylori* infection contributes to Th17 differentiation and response
IL-6 and leptin have been reported to promote Th17 differentiation and play roles in tumor progression.[19,26] Consistently, our results showed that the levels of IL-6 and leptin expression were increased 20 and 6 folds, respectively, in *H. pylori*-infected vs. uninfected tumors [Figure 2A and B]. This data suggested that *H. pylori* infection promoted the expression of factors that regulate Th17 differentiation.

GM-CSF is an additional cytokine released by Th17 cells, and importantly, has been strongly linked to pathogenicity of Th17 cells in other disease states.[27] Thus, we next examined whether GM-CSF was

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**Figure 1:** IL-17A expression in *H. pylori*-associated GC was enhanced in abnormal lipid milieu. (A) *H. pylori* induced IL-17A expression. RNA was extracted from GC specimens. The levels of IL-17A were quantitated with RT-qPCR and compared between HP (+) and HP (-) groups; (B) the effect of high TG content on *H. pylori*-induced IL-17A expression. The GC patients were divided into groups with high TG (> 1.7 mmol/L) and normal TG (≤ 1.7 mmol/L). IL-17A levels were compared between these two groups; (C) the synergistic effects of *H. pylori* and aberrant lipid metabolism on IL-17A induction. Regression analysis was employed. ***P < 0.001, HP (+) vs. HP (-); *P < 0.05, high TG vs. normal TG. *H. pylori*: Helicobacter pylori; GC: gastric cancer; IL: interleukin; RT-qPCR: reverse transcription quantitative polymerase chain reaction; TG: triglyceride
associated with *H. pylori* infection in human HC. Indeed, higher levels of gastric GM-CSF were seen in *H. pylori*-positive vs. negative tumors [Figure 3A]. Further, we observed that *H. pylori*-induced GM-CSF expression was closely associated with enhanced IL-17A expression [Figure 3B]. Our results suggest that *H. pylori* infection may activate Th17 responses as evidenced by the induced expression of IL-17A and GM-CSF.

CXCL1 has been demonstrated to be secreted by inflamed stomach and adipose associated with *H. pylori* infection,\(^\text{[19]}\) acting as a potent mobilizer of bone marrow-derived IMCs. Consistently, our present results showed that the expression of CXCL1 was significantly increased in *H. pylori*-positive vs. negative patients [Figure 3C], and a close correlation of CXCL1 with IL-17A expression was established [Figure 3D].

### Table 1: The primers used in RT-qPCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5' to 3')</th>
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<tr>
<td>IL-6</td>
<td>AGACAGCCACTCACCTCTTCT</td>
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<tr>
<td></td>
<td>TTTCCACAGGCAAGTCTCCT</td>
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<tr>
<td>IL-17A</td>
<td>AATCTCCACCGCAATGGAGGA</td>
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<tr>
<td></td>
<td>ACCAGTATTTCTCCACGCGG</td>
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<td>CXCL1</td>
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</tr>
<tr>
<td></td>
<td>AGCCCCCTGCTTCATAACGCA</td>
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<tr>
<td>GM-CSF</td>
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<tr>
<td></td>
<td>CCCTCCTGGCTGAACAGAG</td>
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<tr>
<td>CagA</td>
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<td></td>
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<td>VacA</td>
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<td>β-actin</td>
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<td>CCACGTCACCACCTCATGATGG</td>
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RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin; CXCL1: chemokine (C-X-C motif) ligand 1; GM-CSF: granulocyte-macrophage colony-stimulating factor

### Table 2: Altered glycolipid metabolic factors and *H. pylori* infection

<table>
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</tr>
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<td>14</td>
<td>0.53</td>
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<td>9</td>
<td>13</td>
<td></td>
</tr>
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<td>CHO</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
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<td>10</td>
<td>0.74</td>
</tr>
<tr>
<td>Normal</td>
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The contingency was analyzed by using Chi-square testing. The cut-offs for diagnosis of metabolic abnormality were: GLU > 6.1 mmol/L, CHO > 5.7 mmol/L, LDL > 3.07 mmol/L, HDL < 0.9 mmol/L, TG > 1.7 mmol/L, according to clinical criteria. *H. pylori*: *Helicobacter pylori*; GLU: glucose; CHO: cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein; TG: triglyceride

**Figure 2:** *H. pylori* induced IL-6 and leptin expression. (A) The levels of IL-6 expression was quantitated by RT-qPCR and compared between HP (+) and HP (-) GCs; (B) leptin was similarly assayed to determine the effects of *H. pylori* infection. ***P < 0.001, HP (+) vs. HP (-). *H. pylori*: *Helicobacter pylori*; GC: gastric cancer; IL: interleukin; RT-qPCR: reverse transcription quantitative polymerase chain reaction
uninfected individuals did so [Figure 4B].

RORγt is the most important transcription factor for the differentiation and activation of Th17 cells.[30] We thus analyzed RORγt expression in *H. pylori*-associated GC and explored its potential role in tumor progression. We found that an overall 6-fold increase of RORγt in *H. pylori*-infected vs. uninfected GC [Figure 4C], and that *H. pylori*-associated expression of RORγt and IL-17A were positively correlated [Figure 4D]. In the absence of *H. pylori* infection, the levels of RORγt were not different between early and advanced tumors [Figure 4E and F]. In contrast, RORγt expression was further enhanced in *H. pylori*-associated tumor progression, with higher expression in larger tumors (> 4 cm) [Figure 4E] and those with more metastatic capability (T3/T4 stages) [Figure 4F].

**DISCUSSION**

It has been long recognized that unresolved inflammation induced by *H. pylori* will favor gastric carcinogenesis. Eradication of *H. pylori* has been shown to be beneficial in preventing GC development.[31,32] Obesity and diabetes have become a great problem in modern societies, which profoundly increase the frequencies of malignant neoplasms, including GC.[21,33,34] Although *H. pylori* infection and metabolic disorders can independently promote tumor progression, there are considerable evidences showing that they can also exert a synergistic effect on tumorogenesis.[19] However, the molecular mechanisms behind this synergy remain elusive. We previously reported that *H. felis*-induced GC in obese mice can be influenced by the gastric homing and activation of Th17 cells, which trigger a series of inflammatory responses in both stomach and adipose tissues through releasing IL-17A.[19] Our current results further the concept that chronic *H. pylori* infection and aberrant lipid metabolism can interact to activate Th17 responses and facilitate GC.

We demonstrate that *H. pylori* infection is associated with striking elevation of IL-17A content in GC [Figure 1A]. The expression of IL-17A is also increased in the patients with abnormal high plasma

![Figure 3](https://example.com/figure3.png)

*Figure 3: H. pylori induced GM-CSF and CXCL1 expression. (A and C) The quantitative assays of GM-CSF (A) and CXCL1 (C) were performed by RT-qPCR. ***P < 0.001, HP (+) vs. HP (-); (B and D) The correlation of GM-CSF (B) and CXCL1 (D) with IL-17A expression was analyzed. H. pylori: Helicobacter pylori; RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin; CXCL1: chemokine (C-X-C motif) ligand 1; GM-CSF: granulocyte-macrophage colony-stimulating factor*
levels of TG, though to a lesser extent compared to that of *H. pylori* infection [Figure 1B]. We consistently observe a significant synergy between *H. pylori* infection and abnormal lipid metabolism in producing IL-17A [Figure 1C]. CD4+ T-cells are widely observed in *Helicobacter*-associated GC, and of these, IL-17A is predominantly produced by the Th17 subset. However, future studies are needed to understand whether IL-17A is also produced from other sources, such as CD8+ T-cells and/or innate lymphoid cells (ILCs). Nevertheless, given the observation that other Th17-related cytokines, such as IL-6 and GM-CSF, are also increased, our results suggest that *H. pylori* infection and altered TG metabolism cooperate in enhancing the Th17 response.

As a pro-inflammatory subset of T cells, it is possible that Th17 cells can also activate antitumor immunity. Myeloid-derived suppressor cells (MDSCs) are known to home to the site of tumors and facilitate their avoidance of cytotoxic T cells. Thus, the production of CXCL1 and GM-CSF may be critical members of the cytokine milieu, as these have been reported to promote the recruitment and function of MDSCs, respectively. In agreement with previous observations, we found that both GM-CSF and CXCL1 were greatly increased in *H. pylori* positive tumors [Figure 3A and C] and their upregulation was coincident with elevated IL-17A [Figure 3B and D].

Figure 4: Roles of *H. pylori* and RORγt in tumor progression. (A and B) The association of *H. pylori* infection with tumor sizes (A) and stages (B); (C) RORγt was enhanced by *H. pylori*. RORγt was assayed via RT-qPCR; (D) the correlation of RORγt with IL-17A; (E and F) the expression levels of RORγt were compared in *H. pylori*-related and unrelated tumors. The levels of RORγt were also compared in tumors with different sizes and different stages. HP (+) vs. HP (-): ***P < 0.001; ≤ 4 cm vs. > 4 cm: *P < 0.05; T1/T2 vs. T3/T4: **P < 0.01. *H. pylori*: Helicobacter pylori; RT-qPCR: reverse transcription quantitative polymerase chain reaction; IL: interleukin.
and triglyceride is extensively induced in \( \gamma \)-infected patients \( ROR_{\gamma} \)[19,39,40,41], and the expression is further enhanced in advanced tumors [Figure 4E and F]. \( ROR_{\gamma} \) has been widely investigated in Th17 cells but seldom in malignant diseases. Thus, future studies should include analysis of the important association of \( ROR_{\gamma} \) and \( H. \) pylori infection.

In summary, we demonstrate that \( H. \) pylori infection and abnormal lipid metabolism can exert a synergistic role in Th17 activation and response to promote GC development. These observations are important to understand GC pathogenesis and can be of therapeutic significance.

DEclarations

Acknowledgments

We are grateful to all the patients who have contributed to the data of this work.

Authors’ contributions

Conceived and designed the study: J.Z. Zeng, W. Han, A.S.T. Wong, R.E. Ericksen Performed the study: H. Wang, G. Chen, J. Liu, M. Yang, Z.X. Wu Prepared the manuscript: J.Z. Zeng Revised the manuscript: W. Han, R.E. Ericksen

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Informed consents were signed by patients.

Ethics approval

Our experimental protocols were approved by The Hospital Ethics Committee and The Ethics Committee of Xiamen University, China.

REFERENCES

Th17 activation by *H. pylori* and triglyceride


