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The effect of trimethylamine *N*-oxide on *Helicobacter pylori*-induced changes of immunoinflammatory genes expression in gastric epithelial cells

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ABSTRACT

Colonization of *Helicobacter pylori* (*H. pylori*) induces immune and inflammatory response in gastric mucosa. Trimethylamine *N*-oxide (TMAO), from diet and metabolite through the action of gut microbiota, has been linked to inflammatory diseases. To investigate the effects of TMAO and *H. pylori* infection on gene expression in gastric epithelial cells, Human gene chip Affymetrix HTA 2.0 was used in this study. 1312 genes were identified as differentially expressed genes in GES-1 cells with *H. pylori* and TMAO co-treatment compared to the control. GO and KEGG analyses indicated that the functions of these differentially expressed genes were related closely with immune inflammation. GO-network showed that Toll-like receptor signaling pathway was the most important biological processes and 49 up-regulated genes related to immune inflammation were obtained. The synergistic effects of *H. pylori* and TMAO enhanced the genes expression of IL-6, CXCL1, CXCL2, FOS and C3 related to immune inflammation in comparison with those of non-infected control cells, *H. pylori*-infected cells, and TMAO-stimulated cells. RT-PCR verified the expression levels of IL-6, CXCL1. Additionally, expression levels of 2053 genes were altered and 52 immunoinflammatory genes were upregulated in comparison with *H. pylori*-infected cells. This study suggested that TMAO altered the expression levels of immunoinflammatory genes induced by *H. pylori* infection, and the synergistic effects of *H. pylori* and TMAO provided novel insights into the development of chronic gastritis, gastric ulcer and gastric cancer.

1. Introduction

Helicobacter pylori (*H. pylori*), a spiral-shaped Gram-negative bacterium, colonizes the stomach for over half of all humans and the ensuing gastric inflammatory response is the main risk factor for chronic gastritis, peptic ulcers and gastric malignancies [1,2]. *H. pylori* infection not only damages the gastric epithelial cell directly, but also amplifies the inflammatory response by increasing inflammatory cytokine production, which leads to disorder of epithelial cell proliferation and apoptosis [3,4]. That *H. pylori* infection leads to chronic inflammation is the major cause of gastric cancer. Yet the majority of *H. pylori*-colonized individuals remain asymptomatic in the early stages of infection. That long term colonization of *H. pylori* in human gut remains undetected is mainly due to its ability to modify and subvert the activated immune response as well as adaptive immunity by modulation of effector T cell functions [5,6]. Nozaki et al. [7] reported that synergism of high-salt diet and *H. pylori* promote the development of gastric cancer through the effects of high-salt diet and *H. pylori* on gastric cancer in Mongolian Gerbils. Therefore, it is a necessity to combine other pathogenic factors with *H. pylori* as a systematic research sample rather than single risk factors for prediction and therapy of chronic gastritis, peptic ulcers and gastric malignancies.

Trimethylamine *N*-oxide (TMAO) is derived primarily from metabolite through the action of gut microbiota, and also partly comes from dietary including meat, egg, dairy products and salt water fish [8–10]. Missailidis et al. [11] demonstrated that TMAO level correlated with promoting systemic inflammation and is an independent predictor of mortality in Chronic Kidney Disease. Studies have also shown a striking association between TMAO levels and atherosclerosis and cardiovascular disease risks in all kinds of people, which are inflammatory diseases mostly developed by chronic inflammation [12–14]. Seldin et al. [15] have discovered that TMAO promoted vascular inflammation through NF-k B signaling pathway, which was involved in controlling the cytokine-induced genes expression of immunoinflammatory response. Many studies have shown that the NF-k B signaling pathway was

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Table 1
Primer information for quantitative real-time PCR.

Gene symbol	Primer sequence(5′-3′)	Amplicon length
IL-6	Forward: CTTCGGTCCAGTTGCCTTCT Reverse: TGGAATCTTCTCCTGGGGGT	86 bp
CXCL1	Forward: ATTTCTGAGGAGCCTGCAAC Reverse: CACATACATTCCCCTGCCTT	100 bp
GAPDH	Forward: TGCACCACCAAC TGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG	87 bp

activated through recognition of *H. pylori* by TLR-induced pro-inflammatory cytokines, including IL-6, IL-8 and IL-1b, and activated inflammatory factors such ascyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [16,17].

Consequently, TMAO seems likely to have an effect on the genes expression in *H. pylori*-infected tissues. However, there is rare reportment that the effect of TMAO on *H. pylori*-induced immune inflammatory response in vivo. To this end, in this study, the effects of *H. pylori* and TMAO on genes expression in gastric epithelial cells were analyzed by microarray system. Furthermore, biological processes and signaling pathways containing differentially expressed genes were assayed through bioinformatics methods. In addition, we also assessed the synergistic effect of *H. pylori* and TMAO on gene expression related to the immune inflammatory in GES-1 cells compared to those of non-infected cells, *H. pylori*-infected cells, and TMAO-stimulated cells.

2. Materials and methods

2.1. Bacteria strain and culture conditions

H. pylori strain, a clinical strain from Sichuan Provincial People's Hospital, was isolated from a patient with gastric ulcer and moderate gastritis in this study. *H. pylori* strain was grown in 3% (w/v) Columbia blood agar base (Oxoid, UK) mixed with 1.2% (w/v) Brain heart infusion (Oxoid, UK) containing 7% (v/v) sheep blood, 10 μg/mL vancomycin, 10 μg/mL amphotericin, 2500 U/L polymyxin B sulfate salt, 5 μg/mL trimethoprim, 10 μg/mL nalidixic acid (all purchased from Sigma, USA), under microaerophilic conditions (5%–6% O_2, 8%–10% CO_2, 85% N_2) at 37°Cfor 5 days.

2.2. Cell culture

Human gastric epithelial cell line GES-1, stored in Key Laboratory of Biological Resource and Ecological Environment of Chinese Education Ministry, was maintained in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, AUS) and was incubated in 6-well plates (0.25–1 × 10^6 cells/well) overnight at 37 °C with 5% CO_2. When the cultures were 70–80% confluent, the medium was replaced with a freshly prepared serum-free medium containing no antibiotics. *H. pylori*, suspended in DMEM, was added to cells at a bacterium/cells ratio of 100:1. The cells were treated by TMAO (5 mmol/L, Sigma, USA) treatment for 24 h after stimulating by *H. pylori* for 3 h as first experimental group. The GES-1 cells were stimulated with *H. pylori* for 24 h as the second experimental group. And the GES-1 cells were treated with TMAO for 24 h as the third experimental group. The cells weren't treated as a negative control group.

2.3. RNA extraction and reverse transcription

Total RNA was isolated from GES-1 cells in 6-well plates using Trizol (Invitrogen, USA) according to the manufacturer's instructions. A total of 1 μg RNA was used as the template for cDNA synthesis by utilizing the Prime script™ RT reagent kit with gDNA eraser (Ta Ka Ra, Japan).

2.4. Microarray expression profiling and data analysis

Human gene chip Affymetrix HTA 2.0 was conducted for this study, which contains more than six million probes and covers 44,699 coding genes (including 245,349 transcripts, 560,472 exons and 296,058 exon clusters) and 22,829 non-coding genes. Screening of differentially expressed genes by multiple differential method (Fold change = $2^{\text{experiment group\_NS} - \text{control group\_NS}}$) based on the standard fold change (FC) ≥ 2, or fold change ≤ 0.5 ($P < 0.05$). Gene Ontology annotations and enrichment analysis were performed to clarify the function of differentially expressed genes [18,19]. Degree value was used to evaluate the importance of the GO terms. We performed GO-network further analyses for the differential genes to classify the differences of the GO terms and obtain key GO terms. To better understand the biological pathway of differentially expressed genes, enrichment analysis of KEGG pathways was conducted in the KEGG database [20].

The *P*-value was used to determine the significance of the enrichment, and the false discovery rate (FDR) was used to evaluate the significance of the *P*-value. Significant GO terms and pathways were filtered in accordance with $P < 0.05$ and FDR < 0.05. The GO terms and pathways were filtered by the enrichment scores (−Lg (*P*)) in differentially expressed genes.

2.5. RT-PCR

Real time PCR was performed on iQ5 Real Time PCR detection system (Bio-Rad Laboratories, USA) with SYBR® Pre-mix Ex Taq™ II (Ta Ka Ra, Japan), programmed for 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10 s. The results were expressed as the mean ratio of a specific m RNA compared with that for GAPDH. Primers of selected genes for RT-PCR designed through Primer-BLAST were in Table 1.

2.6. ELISA

Supernatants from the treated cells were collected and cytokine analysis for IL-6 and CXCL1 was performed according to the manufacturer's instructions using a human IL-6 and CXCL1 ELISA Development Kit (Mlbio, Shanghai, China). Optical density measurements were taken at 450 nm. The absolute concentration of IL-6 and CXCL1 in the culture medium was calculated from the standard curve.

2.7. Statistical analysis

All experiments were conducted in triplicate and the results were expressed as mean value ± standard deviation (SD). Data were analyzed with SPSS 17.0 software (SPSS® Inc. USA) using one way analysis of variance (ANOVA), and differences between groups were determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Table 2
The number of differentially expressed genes in GES-1 cells with *H. pylori* and TMAO co-treatment in comparison with non-infected control cells, *H. pylori*-infected cells and TMAO-stimulated cells. The data based on fold change ≥ 2.0 or ≤0.5 is calculated.

Sample	The total number of the differentially expressed genes	Up-regulation and percentage	Down-regulation and percentage
H. pylori vs. Control	2880	1271 (44%)	1609 (56%)
TMAO vs. Control	513	220 (43%)	293 (57%)
H. pylori + TMAO vs. Control	1312	419 (32%)	893 (68%)
H. pylori + TMAO vs. *H. pylori*	2053	1074 (52%)	979 (48%)
H. pylori + TMAO vs. TMAO	993	382 (38%)	611 (62%)