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Alterations in metabolic pathways in stomach of mice infected with *Helicobacter pylori*

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ABSTRACT

Numerous studies of *Helicobacter pylori* (*H. pylori*) have been performed, but few studies have evaluated the effects of *H. pylori* infections using metabolome analysis, which involves the comprehensive study of low molecular weight metabolites. In this study, the metabolites in the stomach tissue of mice that had been infected with *H. pylori* SS1 for 1, 3, or 6 months were analyzed, and then evaluations of various metabolic pathways were performed to gain novel understandings of *H. pylori* infections. As a result, it was found that the glycolytic pathway, the tricarboxylic acid cycle, and the choline pathway tended to be upregulated at 1 month after the *H. pylori* SS1 infection. The urea cycle tended to be downregulated at 6 months after the infection. High levels of some amino acids were observed in the stomach tissue of the *H. pylori* SS1-infected mice at 1 month after the infection, whereas low levels of many amino acids were detected at 3 and 6 months after the infection. These results suggest that *H. pylori* infection causes various metabolic alterations at lesional sites, and these alterations might be linked to the crosstalk between *H. pylori* and the host leading to transition of disease conditions.

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1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative microaerophilic bacterium that chronically colonizes the gastric epithelium of more than 50% of the world's population. This bacterium plays an important role in the development of several gastrointestinal diseases including non-symptomatic chronic gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma [1–3]. Furthermore, it has been categorized as a group I carcinogen in humans [4]. The outcomes of such diseases depend on multiple factors like host immune gene polymorphisms and the amount of gastric acid present in the stomach. *H. pylori* virulence factors, such as the cytotoxin-associated gene pathogenicity island-encoded protein CagA and the vacuolating cytotoxin VacA, are also important, e.g., these cytotoxins appear to modulate the host's immune system [5].

In this study, we explored the pathogenesis of *H. pylori* infections using metabolomics/metabolome analysis. Metabolomics/metabolome analysis involves the comprehensive study of low molecular weight metabolites; i.e., the levels of such metabolites are assessed in order to determine the cellular processes that occur in a particular cell type, tissue, organ, or organism. The metabolome represents the endpoint of the omics cascade so it is also the closest point in the cascade to the phenotype. The genome, which is found in the upstream part of the omics cascade and contains numerous genes, is basically not affected by exogenous factors, such as environmental and dietary factors. Even if a certain gene contains a mutation, the host's body might remain unchanged due to the effects of homeostatic processes. In addition to variations in DNA, mRNA, and protein expression, the metabolome is also affected by the enzymatic activities of various proteins, and alterations in the levels of metabolites can also be caused by exogenous factors; therefore, the metabolomic profiles can be located in a summary of the other upstream omics profiles. Thus, the metabolome analysis might be able to express subtle alterations in metabolic pathways

Abbreviations: *H. pylori*, *Helicobacter pylori*; MALT, mucosa-associated lymphoid tissue; LC/MS, liquid chromatography/mass spectrometry; GC/MS, gas chromatography/mass spectrometry; TCA, tricarboxylic acid; IDO, indoleamine 2,3-dioxygenase.

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and deviations from homeostasis before phenotypic changes arise [6,7], and hence, could be useful for *H. pylori*-related researches.

In this study, C57BL/6J mice were orally infected with *H. pylori* SS1, a mouse-adapted strain, and their stomach tissue was collected at 1, 3, or 6 months after the infection. The metabolites in the stomach tissues were analyzed using liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Evaluations based on the glycolytic pathway, tricarboxylic acid (TCA) cycle, choline pathway, urea cycle, glutathione cycle, purine pathway, pyrimidine pathway, and amino acid metabolism were also performed via the metabolite profiling.

2. Materials and methods

2.1. Helicobacter pylori culture

The *H. pylori* SS1 strain, which was originally collected from the patient with peptic ulcer disease and is available for infecting mice, was used in this study. *H. pylori* SS1 was cultured on Columbia agar plates (Becton, Dickinson and Company, Tokyo, Japan) under microaerobic conditions (5% O_2, 5% CO_2, and 90% N_2) at 37 °C. One colony was picked from each culture plate, inoculated on a new agar plate, and cultured under the same conditions. Before the animal experiments, *H. pylori* SS1 were grown in Brucella broth supplemented with 10% fetal bovine serum overnight.

2.2. Animal experiments

All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). All mice were housed and bred at the animal unit of the Kobe University School of Medicine in a specific pathogen-free facility under an approved experimental protocol. C57BL/6J mice (female, 5-weeks-old, N = 12) were orally infected with *H. pylori* SS1 (2×10^8 CFU per an injection). As a negative control, C57BL/6J mice (female, 5-weeks-old, N = 12) were orally injected with PBS alone. In this study, the *H. pylori* SS1 infection was performed by its single injection. One, 3, and 6 months after the infection procedure, the mice were sacrificed, and then their stomach tissue was collected. All mice for 1, 3 and 6 month *H. pylori* SS1 infection were subjected to its orally single injection at the same time. In this study, 12 of C57BL/6J mice orally infected with *H. pylori* SS1, and then each the 4 mice infected with *H. pylori* SS1 were sacrificed at each the period of 1, 3, or 6 months. In addition, as a negative control, 12 of C57BL/6J mice were orally injected with PBS alone, and each the 4 mice were sacrificed as the corresponding control mice for each the infection period. Therefore, the number of infection groups and the corresponding non-infection groups was N = 4 each. The existence of *H. pylori* SS1 in the stomach tissue was confirmed by PCR. The collected stomach tissue samples were subjected to hematoxylin and eosin (HE) staining for pathological evaluation, and LC/MS and GC/MS analyses to obtain metabolite measurements.

2.3. HE staining

The stomach tissue samples collected from the mice were dissected and fixed with 10% formalin, and then the paraffin-embedded tissue was sliced at 5 µm and stained with HE in a blinded manner. The resultant sections were examined using a microscope (BX51; OLYMPUS, Tokyo, Japan).

2.4. LC/MS analysis

During the LC/MS-based measurement of anionic and cationic metabolites, metabolites were extracted from the stomach tissue samples according to the methods described in our previous report [8]. The resultant solution containing the extracted metabolites was subjected to LC/MS analysis. The LC/MS measurements were carried out using a Nexera LC system (Shimadzu Corp., Kyoto, Japan) equipped with two LC-30AD pumps, a DGU-20A5 degasser, an SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A control module, coupled to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp.). The data analysis for the semi-quantitative evaluation was performed in accordance with the previously described method [8,9].

2.5. GC/MS analysis

During the GC/MS analysis, metabolites were extracted from the stomach tissue samples in accordance with the methods described in our previous report [10]. The GC/MS measurements were carried out using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; inner diameter: 30 m × 0.25 mm, film thickness: 0.25 µm; Agilent Co., Palo Alto, CA). The data analysis for the semi-quantitative evaluation was performed in accordance with the method described in our previous report [10].

2.6. Statistical analysis

The F-test was used to compare the variances of each group, and then the Student's t-test was employed to evaluate the significance of the differences between each group. The P-values of less than 0.05 were judged to indicate a significant difference. Principal component analysis was performed using the JMP9 software (SAS Institute Inc., Cary, NC), and the score plots for the first three components were evaluated.

3. Results

In this study, C57BL/6J mice were infected with *H. pylori* SS1, which is a mouse-adapted *H. pylori* strain. *H. pylori* SS1 is a cagA-positive and vacA s2/m2-positive strain and is used to produce mouse infection models. First, the pathological appearance of the stomach tissues of the mice was confirmed at 1, 3, and 6 months after the *H. pylori* SS1 infection (Fig. 1). As a result, it was found that *H. pylori* SS1 infection causes the development of abnormal gastric mucosa architecture. Inflammatory cell invasion was also detected throughout the observation period. Six months after the infection, foveolar hyperplasia was observed, and the gastric pits and glands were longer than those of uninfected age-matched mice.

Next, using LC/MS we analyzed the metabolites in the stomach tissue samples collected from the mice at 1, 3, and 6 months after the *H. pylori* SS1 infection and the corresponding control mice. Then, evaluations based on particular metabolic pathways, including the glycolytic pathway, TCA cycle, choline pathway, urea cycle, glutathione cycle, purine pathway, pyrimidine pathway, and amino acid metabolism, were carried out (Table 1, Supplemental Fig. 1). GC/MS analysis was used to detect metabolites related to these pathways that were not detected by LC/MS, and the results of this analysis were added into the pathway-based evaluations (Table 1, Supplemental Fig. 1). The levels of metabolites existed in the glycolytic pathway tended to be upregulated in the mice that had been infected with *H. pylori* SS1 for 1 month, but no such tendency was observed at 3 or 6 months after the *H. pylori* SS1 infection. The levels of metabolites linked to the TCA cycle tended

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