Microbiome insights into the perforated duodenal ulcer via metagenomic sequencing

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Xue Huang (Former Corresponding Author)  
Chongqing University

Shaying Ma  
Chongqing Emergency Medical Center

Jingjing Niu  
Southwest University

Jiang Li  
Southwest University

Xiaoyu Wang  
Southwest University at El Paso

Xiangke Duan  
Missouri State University

Shuangquan Yan  
southwest university

Jianping Xie (New Corresponding Author)  
georgex@swu.edu.cn

Corresponding Author
ORCiD: 0000-0002-8959-8146

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Abstract

Background: As a part of the digestive duct, duodenum, populates abundant commensal microbes, of which Helicobacter pylori was considered as the most widely and only recognized causative agent, especially in perforated duodenal ulcer patients. Although the most researches have been performed between duodenal ulcer diseases and the invader H. pylori, the relationship between perforated duodenal ulcer and the associated microbiome remained to be explored.

Results: Metagenomic sequencing were used to profile the microbes underlying duodenal ulcer. The metagenome results of 6 duodenal ulcer samples (3 ulcer foci and 3 sites around the ulcer foci) were obtained and compared. Ulcer foci and (healthy control (3 sites around the ulcer foci) were found to exist distinct microbial distributions. And interestingly, Streptococcus mitis was highly enriched in duodenal ulcer foci.

Conclusion: The dysbiosis of microbiomes might underlie duodenal ulcer diseases, while the increased abundance of S. mitis is a novel finding. Whether there is causal link between S. mitis and duodenal ulcer remains to be determined. This represents the first report on the gut microbiome of duodenum or duodenal ulcer, that revealed that gut microbiota dysbiosis, especially S. mitis, might play an unexpected role in the disease. The S. mitis and microbiome dysbiosis can be new direction in the duodenal ulcer study.

Background

Intestinal tract is one of the most important digestive apparatus closely relevant to human health [1, 2], of which digestive tract is populated by a large number of
microbes [2], with cell numbers ($10^{14}$) exceed that of body cells ($10^{13}$) by a factor of ten [3]. These “aboriginal inhabitants” protect the host through communication with immune system. Activation of Toll-like receptors (TLRs) by commensal microflora is critical for the protection against gut injury and associated mortality, for these bacteria are recognized by TLRs under physiological status [4]. Beyond that, gut microbiota monitor multiple aspects of host, for instance energy harvest from the diet and energy storage [5], intestinal angiogenesis [6] and host immunity [7].

The gut microbes contribute to resist numerous invading pathogens [8–10]. *Bacillus bifidus* can protect or resist the ulcerative inflammation in gut [11], while the *Escherichia coli* often causes diarrhea [12]. The immunologic response and endogenous or exogenous compounds metabolism in the liver are interweaved with the gut microbes [10]. *E. coli* evolution ability is associated with the gut microbiota homeostasis [13]. Inflammatory bowel diseases, Crohn’s disease and ulcerative colitis, are chronic idiopathic disorders causing inflammation of the gastro-intestinal tract [14]. It’s reported that more than 60 percent population was carrier of an opportunistic pathogen *H. pylori* [15, 16], the culprit of superficial gastritis. The duodenum, one of the most important digestive apparatus, is located between stomach and jejunum. Although *H. pylori* was widely considered as a culprit of these gastroduodenal disorders [17, 18], few direct evidences can be found for its role in duodenal ulcer, which was considered as the result of convulsion in stomach and duodenum. Thus, the further analysis between duodenal ulcer and its microbiome required to be performed.

In this study, metagenome sequencing was performed for 3 duodenal ulcer patients, 6 samples were collected including 3 ulcer foci and 3 sites around the ulcer foci.
Each patient contributed two samples, one from the ulcerate foci, the sites around the ulcer foci as control. Metagenomic data showed unexpected enrichment of *Streptococcus mitis* in the ulcer sites.

**Materials and methods**

**DNA Extraction and library construction**

Samples separated from 3 patients were harvested and sent to Shanghai Biotechnology Corporation for metagenomic sequencing. Briefly, DNA was extracted according to conventional protocol. For the quality monitoring, the degradation and potential contamination were determined on agarose gels. DNA concentration was measured. The contents above 1µg are used to construct library. DNA sample was fragmented by sonication to a size of 350bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Bioanalyzer and quantified using real-time PCR.

**Data Analysis**

The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated. The Clean Data is assembled and analyst [19] by SOAPdenovo software [20, 21]. All samples’ Clean Data are compared to each Scaffolds respectively by SoapAligner software (soap 2.21) to acquire the PE reads.

Gene prediction and abundance analysis
The Scaftigs (≥ 500 bp) assembled from both single and mixed are all predicted for the ORF by MetaGeneMark (V2.10, http://topaz.gatech.edu/GeneMark/) software, and the length information shorter than 100nt were filtered. For ORF predicted, CD-HIT software is adopted to redundancy and obtain the unique initial gene catalogue. The Clean Data of each sample is mapped to initial gene catalogue using SoapAlignerand. Genes with reads ≤ 2 in each sample was filtered and the gene catalogue (Unigenes) was obtained for subsequent analysis. The number of mapped reads and length of gene determined the abundance of each gene per sample.

Taxonomy prediction

DIAMOND software is used to Blasting the Unigenes to the sequences of Bacteria, Fungi, Archaea and Viruses by DIAMOND software. The table containing the genes numbers and abundance per sample in each taxonomy hierarchy (kingdom, phylum, class, order, family, genus, species) is based on the LCA annotation result and the gene abundance table. Abundance of a specie per sample be equal with the sum of gene abundance annotated for the species; the gene number of a species in a sample equates to the number of genes with nonzero abundance. Kmoa analysis, the representation of relative abundance, the exhibition of abundance cluster heat map, PCA and NMDS decrease-dimension analysis were based on the abundance table of each taxonomic hierarchy.

Common functional database annotations

DIAMOND software (V0.7.9) was used to BLAST Unigenes to functional database. Functional database including KEGG database, eggNOG database, CAZy database. For the BLAST result of each sequence, the best Blast Hit is used for subsequent analysis. Statistic of the relative abundance of different functional hierarchy, the
relative abundance of each functional hierarchy equal to the sum of relative abundance annotated to that functional level. Based on the function annotation result and gene abundance table, the gene number table of each sample in each taxonomy hierarchy is obtained. The gene number of a function in a sample equal the gene number that annotated to this function and the abundance is nonzero. Based on the abundance table of each taxonomy hierarchy, the count of annotated gene numbers, general relative abundance, the abundance cluster heat map and the decrease-dimension analysis of PCA and NMDS are conducted, as well as the Anosim analysis of the difference between groups (inside) based on functional abundance. The metabolic pathways, Metatat and LEfSe analysis between groups are performed.

Resistance gene annotation

DIAMOND software (V0.7.9) was used to align the Unigenes to ARDB database (http://ardb.cbcb.umd.edu/) with the parameters: blastp, -e 1e-5. Filter the aligned result and choose the identity value bigger than the lowest identity value from the aligned result of each sequence, to make sure the resistance gene annotation is reliable. Based on the aligned result, count the relative abundance of each resistance gene. The bar charts and cluster heat map were used to visualize the abundance of resistance genes and associated species.

Results

The duodenal ulcer microbiota was significantly altered by metagenomic study.

To explore the metagenomic change of duodenal ulcer patients, 6 samples (3 ulcer foci and 3 healthy controls) were subjected to metagenomic sequencing. Consistent
with the role of gut microbiota change in metabolic disorders [8], multiple central metabolic pathways were underrepresented in duodenal ulcer patients, such as ABC transporters, amino acids biosynthesis, carbon metabolism and pyrimidine metabolism (Figure 1A). These underrepresented pathways were highlighted (Figure 1B). D-glutamine and D-glutamate metabolism, sphingolipid metabolism and nitrotoluene degradation were significantly decreased. Tetracycline biosynthesis pathway was enriched 3000 folds. Bacteria population density was auto-regulated [22]. The enrichment of tetracycline biosynthesis pathway and antibiotics resistance genes (with abundance over 0.03%) indicated the specifically proliferating of microbes bearing these genes (Figure 1C). The percentage of duodenum microbes is similar between ulcer foci and the healthy control (Figure 2A), except the virus. Bacteria are predominant (77%-92%) (Figure 2A). Among the top10 species which contribute 51%, Gemella haemolysans (10.6%), Gemella sanguinis (7.8%), Fusobacterium periodonticum (5.9%), Streptococcus mitis (7.3%), Prevotella sp. Oral taxon 473 (7.5%) are very prominent. Veillonella unclassified (6.6%), Neisseria unclassified (3.6%), Neisseria flavescens (3.4%), Porphyromonas sp. Oral texon 279 (3.7%) are predominant in the health control (Figure 2B). There is no overlap for the top 10 species between the ulcer foci and health control. This comparison indicated significant change of microbiota during duodenal ulcer.

*Streptococcus mitis* was unexpectedly enriched in duodenal ulcer site samples

For the altered bacteria species, we corrected data about several pathogens in 353 patients. The directed enrich of *S. mitis* in patients was observed (Figure 3 and Table 1). The enhanced survival of *S. mitis* in old patients might hinted link between
the duodenal ulcer and *S. mitis*

Discussion

Gut microbiota is increasingly recognized as key player in host healthy [10, 20], largely due to close physical contact [23] and modulating the differentiation of immune cell subsets [24], the production of cytokines and chemokines to influence the T cell repertoire of the intestine and surrounding tissue, and the production of soluble immune mediator IgA [25, 26]. *Bifidobacterium* abundance was changed by gene-diet interaction [27]. However, to our knowledge, there is no reports on the gut microbiota of duodenum or duodenal ulcer.

In this study, metagenomic results showed that bacterium instead of virus or fungi dominated the duodenum (Figure 2A). Though both health control and ulcer sites are populated with bacteria, significant difference exists. *S. salivarius*, *S. parasanguinis*, *Megasphaera micronuciformis*, *Porphyromonas endodontails* are abundant in health control (Figure 2B), *G. sanguinis*, *Fusobacterium periodonticum*, *G. haemolysans*, *S. mitis* [28], bacteria frequently found in oral cavity, were found also dominant in ulcer foci (Figure 2B). In general, the top 10 species of the ulcer foci are more abundant than TOP 10 in health control (Figure 2B). The duodenal ulcer was accompanied by abundance change of dominant bacteria. Some hemolytic bacteria were found in duodenal ulcer, while absent in health control. *G. haemolysans*, *G. sanguinis*, *S. mitis* might underlie the duodenal ulcer. Gut type is a recently well recognized conception in gut microbiota field. *Prevotella*, *Bacteroides* and *Ruminococcaceae* were core for gut types identification [29]. In our results, *Prevotella* are predominant both in patients (6.9%) and health (6.9%), *Bacteroides* is presentin patients (0.2%) and absent in health. *Ruminococcaceae* (0. 0008%) was
found in patients only. The result suggested that ulcer patients and healthy control have different gut type.

*S. mitis* is one of mitis group *Streptococci*, which are abundant members of the microbiota on all surfaces in the oral cavity and pharynx birth and through lifespan [28]. As an oral parasite, *Streptococcus mitis* is opportunistic pathogens implicated in dental caries [30], subacute bacterial endocarditis [31], brain abscesses [32]. The role of *S. mitis* in duodenal ulcer warrants further study.

The copies of some bacterial genes involved in metabolic pathways are low in ulcer patients. The underrepresented KEGG [33] pathways are ABC transporters [34], amino acids biosynthesis [35] and carbon metabolism [36] (Figure 2A). The underlying mechanism of action remains elusive. The dysregulated metabolism of microbiota might underlie the duodenal ulcer.

The change of bacteria location was previously reported to be involved in diseases, such as cephalomeningitis, cirrhosis. Microbial metabolite imidazole propionate might contribute to the pathogenesis of type 2 diabetes by impairing insulin signal [37]. The presence of several conventional oral bacteria such as *S. mitis* in duodenal ulcer might be novel pathogen of duodenum ulcer, or underlying the dysbiosis of microbiota, resulted in significant abundance change (Figure 2B), culminated in ulcer. More studies into the role and mechanism of action of *S. mitis* might offer new insights into the duodenal ulcer.

Declarations

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Author Contributions

X. H., X. D., J.X, S. Y., and J. L. performed the experiments. X. H., and X. D., J. N. analyzed the data. S.M diagnosed the patients, collected samples, for all clinical related ethic approval and part of data analysis. X. H. and X. D., J. X. designed the study and wrote the paper. All authors have read and approved the manuscript.

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Ethics approval and consent to participate

Research conducted for this study was performed in accordance with approvals from the Institutional Review Board at the Hospital. All participants provided written informed consent prior to participation in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1. The age difference for the distribution of microbes among 353 patients

| Species                      | Counts | Ages   |
|------------------------------|--------|--------|
|                              |        | 0-20   | 20-39 | 40-60 | >60   |
| Streptococcus mitis          | 6      | 0      | 0     | 1     | 5     |
| Neisseria                    | 1      | 1      | 0     | 0     | 0     |
| Pseudomonas aeruginosa       | 2      | 0      | 0     | 0     | 2     |
| Klebsiella pneumoniae        | 10     | 0      | 1     | 0     | 9     |
| Escherichia coli             | 12     | 0      | 2     | 5     | 5     |
| Acinetobacter baumannii      | 1      | 0      | 0     | 1     | 0     |
| Enterobacter cloacae         | 9      | 0      | 3     | 1     | 5     |

Figures
**Figure 1**

Deregulated metabolism of the duodenal ulcer site. A. The deregulated metabolic

**Figure 2**

The microbiota difference between the duodenal ulcer foci and site around the ulcer.
Figure 3

Microbes ratio of 353 duodenal ulcer patients cultured on agar.