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Characterization of the duodenal bacterial microbiota in patients with pancreatic head cancer vs. healthy controls

Qi-Xiang Mei [a], Chun-Lan Huang [a], Sheng-Zheng Luo [a], Xue-Mei Zhang [b], Yue Zeng [a, *], Ying-Ying Lu [a, **]

[a] Shanghai Key Laboratory of Pancreatic Diseases & Department of Gastroenterology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 200080 Shanghai, PR China
[b] Department of Pathology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 200080 Shanghai, PR China

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ABSTRACT

An increasing number of reports have demonstrated that there is an association between the presence of pathogenic microorganisms and pancreatic cancer. However, the role of the duodenal microbiota in pancreatic carcinogenesis remains unknown. In this study, duodenal mucosal microbiota was analyzed in 14 patients with pancreatic head cancer and 14 healthy controls using 16S rRNA gene pyrosequencing methods. Plasma endotoxin activity and the concentrations of the proinflammatory cytokine IL-6 and C-reactive protein (CRP) were measured in blood samples. The urea breath test was used to detect *Helicobacter pylori* infections. Endoscopic duodenal mucosal biopsies were evaluated by histological examinations. Statistical comparisons of inflammatory factors revealed significantly higher levels of CRP and IL-6 in the pancreatic cancer group as compared to healthy controls. Patients with pancreatic cancer also had a higher incidence of *H. pylori* infections and showed mucosal changes, including villous abnormalities and diffuse inflammatory cell infiltration in the lamina propria. The sequences analysis showed that based on linear discriminant analysis effect size (LEfSe) analysis at the genus level, *Acinetobacter, Aquabacterium, Oceanobacillus, Rahnella, Massilia, Delftia, Deinococcus,* and *Sphingobium* were more abundant in the duodenal mucosa of pancreatic cancer patients, whereas the duodenal microbiotas of healthy controls were enriched with *Porphyromonas, Paenibacillus, Enhydrobacter, Escherichia, Shigella, and Pseudomonas.* These results reveal a picture of duodenal microbiota in pancreatic head cancer patients that could be useful in future trials investigating the role of gut microbiota in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDCA) is the fourth leading cause of cancer-related death in the United States [1]. However, the pathogenesis of pancreatic cancer has not been completely elucidated. Inflammation is clearly a critical factor in pancreatic carcinogenesis [2,3], and the microbiome has been associated with inflammation-initiated cancers [4].

The gastrointestinal tract is the largest microbial compartment in the body, and the gut microbiome has emerged as an important factor in human physiology and pathophysiology [5]. Several studies have presented tangential evidence suggesting a possible role of microbes in the pathogenesis of pancreatic cancer [6]. A recent study showed that variations in oral bacteria measured in saliva were associated with pancreatic cancer [7]. Additional data also supported using *H. pylori* colonization as a risk factor for pancreatic cancer [8], as these microorganisms usually infect the pancreas via translocation from the gut [9]. Mitsuhashi et al. reported that bacteria can reach the pancreas through the circulation, and it has been suggested that this also occurs through the biliary tract (transductal transmission), acting synergistically with other risk factors [10]. The incidence rate for pancreatic head cancer has remained at 5.6% per 100,000 [11], approximately 65% of pancreatic cancers occur in the head of the pancreas, with 15% occurring in the body and tail [12]. Since the duodenum is the first site of contact with nutrients in the intestine, orally administrated drugs, and potential pathogens, and the head of the pancreas lies in the C-shaped duodenal curve, we propose that inflammation in the

* Corresponding author.
** Corresponding author.
E-mail addresses: zengyue1592@yahoo.com (Y. Zeng), le_voyageur@qq.com (Y.-Y. Lu).

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mucosa of the duodenal descending region, dysbiosis of duodenal microbiota, and bacterial dissemination to the pancreatic head may be linked to tumor progression. This microenvironment could cause the higher incidence of pancreatic head cancer than body/tail pancreatic cancer, in addition to other factors like genetic differences, molecular diversity between the two subtypes and the different head and body/tail volumes of the pancreas.

To date, no studies have reported an association between the intestinal bacterial microbiota composition and pancreatic head cancer. In this study, our aim was to characterize the specific composition of the duodenal microbiota in pancreatic cancer patients using 16S ribosomal RNA (rRNA) pyrosequencing methods.

Methods

Patients and sampling

A total of 14 patients with pancreatic head carcinoma and 14 healthy subjects were recruited at Shanghai General Hospital. Pancreatic cancer was diagnosed on the basis of an imaging method such as computerized tomography (CT) or magnetic resonance cholangiopancreatography (MRCP), and further confirmed by pathological evaluation using endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) and diagnosed as pancreatic ductal adenocarcinoma. Most patients with pancreatic cancer had symptoms such as jaundice, abdominal pain, nausea without obvious weight loss. Pancreatic cancer was staged according to the Tumor Node Metastasis staging system from the American Joint Committee on Cancer. Four patients were classified as stage II. Nine patients were diagnosed with locally advanced, unresectable pancreatic cancer and one patient had stage IV pancreatic cancer with metastasis to the liver. The duodenum was not found to be infiltrated by cancer during EUS and the duodenal samples were collected before treatments. Health controls were recruited from our Health Physical Examination Center with normal test results after a routine physical examination who were age and sex matched to the patient group. Subjects were excluded if they were currently taking or had received antibiotics in the last two months. All samples were collected in accordance with the relevant guidelines and regulations, and the study was approved by the Research Ethics Boards of Shanghai General Hospital. Informed consent was provided by all individuals. All of the biochemical analyses were performed in the Shanghai General Hospital Laboratory. Blood samples were drawn after an overnight fast on the second day of admission. Plasma endotoxin activity and C-reactive protein (CRP) were measured with an immunoturbidimetric assay and serum IL-6 level was determined by electro-chemiluminescence immunoassay. The normal reference value of IL-6 and CRP ranges from 0 to 7 pg/mL and 0–10 mg/L,respectively, while the normal range of endotoxin level is below 0.053Eu/mL. The urea breath test was used to detect *H. pylori* infection. All patients received standard instructions to prepare for the gastroscopy. Two biopsy samples were taken from the duodenum (5 cm regions in diameter from the location of the major duodenal papilla) of each individual during the upper endoscopy. One tissue sample was frozen immediately after sampling and stored at −80 °C, and the other was immediately fixed in 10% formaldehyde in phosphate-buffered saline (PBS; pH 7.4) and embedded in paraffin.

Histological examinations

Samples of the duodenal biopsy tissues were fixed in a 10% buffered formalin solution, embedded in paraffin using standard methods, cut into 5-μm sections, stained with hematoxylin and eosin (H&E), and evaluated using light microscopy by two pathologists blinded to experimental groups, who scored the histological alterations according to a modified Chiu's score: grade 0, normal mucosal villi, absence of inflammatory reactions, ulceration, or tissue destruction; Grade 1, development of subepithelial Gruenhagen space, usually at the apex of the villus, often with capillary congestion, localized inflammatory cell infiltration in the lamina propria; Grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria, diffuse inflammatory cell infiltration in the lamina propria; Grade 3, massive epithelial lifting down the sides of the villi, localized inflammatory cell infiltration in the subendothelium; Grade 4, denuded villi with lamina propria and dilated capillaries exposed, diffuse inflammatory cell infiltration in the subendothelium; and Grade 5, digestion and disintegration of lamina propria, hemorrhage and ulceration with intense inflammatory reactions [13].

DNA extraction and PCR amplification

Microbial genomic DNA was extracted from the duodenal biopsies using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA.) according to manufacturer's protocols. Samples were placed into bead tubes with 500 mg of glass beads. One milliliter of SLX Mlus Buffer was added to the samples, which were then incubated at 70 °C for 10 min. The samples were then subjected to bead beating at 6.5 m/s for 5 min using a FastPrep-24 bead beater (MP Biomedicals, USA). Total DNA was eluted in 60 μL of Elution buffer. The amount of DNA was determined using NanoDrop2000 (Thermo Scientific). The integrity and size of the DNA were checked by 1% (wt/vol) agarose gel electrophoresis in 0.5 mg/ml ethidium bromide. All DNA samples were stored at −20 °C until further processing. The V3-V4 region of the bacterial 16S rRNA gene from each sample was amplified using the bacterial universal primers 338F 5′-barcode-ACTCCTACGGGAGGCAGCA-3′ and 806R 5′-barcode-GGACTACHVGGGTWTCTAAT-3′, where the barcode is a six to eight base sequence unique to each sample. PCR reactions were performed in triplicate in 20 μL reaction mixtures containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM) (Sangon Biotech, Shanghai, China), 0.4 μL of FastPfu Polymerase (TransStart® FastPfu DNA Polymerase, Cat. no. AP221-01; TransGen BioTech, Beijing, China), and 10 ng of template DNA. PCR was performed on a Thermal Cycler (Bio-Rad, USA) with the following procedure: 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. 3 μL of PCR products were evaluated using 2% (wt/vol) agarose gel electrophoresis.

Pyrosequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega, USA). Purified amplicons were pooled using equimolar concentrations. Index PCR and sequencing was performed according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library preparation.html). Briefly, samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., USA) according to the manufacturer's instructions. The final library was paired-end sequenced at 2 × 250 bp using a MiSeq Reagent Kit v2 on the Illumina MiSeq platform.

Raw fastq files were demultiplexed and quality filtered using Trimmomatic [14] and FLASH [15] software with the following criteria: (I) the 300-bp reads were truncated at any site receiving an average quality score of <20 over a 50-bp sliding window,

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