Helicobacter species ribosomal DNA in the pancreas, stomach and duodenum of pancreatic cancer patients

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Abstract

AIM: To determine whether gastric and enteric Helicobacter species are associated with pancreatic cancer.

METHODS: Patients with exocrine pancreatic cancer (n = 40), neuroendocrine cancer (n = 14), multiple endocrine neoplasia type 1 (n = 8), and chronic pancreatitis (n = 5) were studied. Other benign pancreatic diseases (n = 10) and specimens of normal pancreas (n = 7) were included as controls. Pancreatic tissue specimens were analyzed by Helicobacter-specific PCR-assay and products were characterized by denaturing gradient electrophoresis and DNA-sequencing. From a subset of the pancreatic cancer patients, gastric and/or duodenal tissue as well as gallbladder and ductus choledochus tissue were analyzed. Gallbladder and choledochus samples were included as controls. Stomach and duodenum samples were investigated to analyze whether a gastric Helicobacter might disseminate to the pancreas in pancreatic cancer patients. Pancreatic specimens were analyzed by Bacteroides-specific PCR for detecting the translocation of indigenous gut microbes to the diseased pancreas.

RESULTS: Helicobacter DNA was detected in pancreas (tumor and/or surrounding tissue) of 75% of patients with exocrine cancer, 57% of patients with neuroendocrine cancer, 38% of patients with multiple endocrine neoplasia, and 60% of patients with chronic pancreatitis. All samples from other benign pancreatic diseases and normal pancreas were negative. Thirty-three percent of the patients were Helicobacter-positive in gastroduodenal specimens. Surprisingly, H. bilis was identified in 60% of the positive gastroduodenal samples. All gallbladder and ductus choledochus specimens were negative for Helicobacter. Bacteroides PCR-assay was negative for all pancreatic samples.

CONCLUSION: Helicobacter DNA commonly detected in pancreatic cancer suggests a possible role of the emerging pathogens in the development of chronic pancreatitis and pancreatic cancer.

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Key words: Pancreatic cancer; Helicobacter species; Polymerase chain reaction; DNA-sequence analysis

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INTRODUCTION

Pancreatic carcinoma, an extremely aggressive cancer with very poor prognosis, is one of the leading causes of cancer-related death in the Western world[8]. Consistently reported risk factors are age and cigarette smoking[9], whereas approximately 5% of pancreatic cancers seem to be primarily related to genetic traits[9]. Some early case control and data register studies suggested that chronic pancreatitis is a risk factor for pancreatic cancer[3, 6], which has been confirmed recently in prospective studies of chronic pancreatitis[9, 5]. Helicobacter pylori (H. pylori), the prototype species of the genus Helicobacter, which colonizes the stomach mucosa and causes acute and chronic gastritis as well as peptic ulcer disease, is a major predisposing factor for gastric cancer in human beings[7]. Other Helicobacter and Campylobacter species, including bile tolerant enteric Helicobacter species, colonize the intestine and hepatobiliary tract of many mammals and birds. Helicobacter hepaticus is the type organism of the enteric Helicobacter species and induces chronic active hepatitis, liver fibrosis, hepatocellular carcinoma, as well as inflammatory bowel disease in susceptible inbred strains of mice[8, 9]. Enteric Helicobacter species such as H. pullorum, H. canis and H. suis, are associated with hepatitis in poultry, dogs, and macaques,
respectively, as well as gastroenteritis and bacteremia in human beings\(^9\). \textit{H. pylori} and the enteric species \textit{H. bilis}, as well as \textit{H. pylori}-like \textit{H.} species ‘liver’, are associated with biliary tract cancer, some chronic liver diseases as well as hepatocellular carcinoma in humans\(^10^{\text{-}14}\). Two serology-based case-control studies have shown an association between \textit{H. pylori} and pancreatic cancer\(^15^,16\), suggesting a possible relationship between helicobacter infections and pancreatic cancer development, which is supported by a recent study of \textit{Helicobacter} species in a small number of patients with pancreatic exocrine cancer\(^17\).

The purpose of the present investigation was to analyze the prevalence of gastric and enteric \textit{Helicobacter} species DNA in samples from pancreatic cancer and chronic pancreatitis. Specimens from some benign pancreatic diseases (cysts or adenoma) as well as normal pancreatic tissue from patients with colon or choledochus cancer were included as controls. Translocation of some enteric bacteria to a diseased pancreas was analyzed by \textit{Bacteroides} genus-specific PCR assay on pancreatic tissue.

**MATERIALS AND METHODS**

**Patients**

All patients were operated on at the Department of Surgery, Lund University Hospital. Formalin-fixed paraffin-embedded pancreatic tissue samples from 84 patients were obtained from the Department of Pathology at the same hospital. Prior to deembedding, one pathologist (U.S) reviewed all samples and approximately 100 mg of each tissue type was taken from the paraffin blocks with the tip of a scalpel. By carefully comparing the blocks with the slides, it was ascertained that a pure tissue type, i.e. tumor or normal etc., was obtained.

The tissue samples were from consecutive patients with primary exocrine cancer, predominantly of ductal type (PC) \(n = 40\), 20 females, mean age 59 years, range 44-77 years), neuroendocrine cancer (NE) \(n = 14\), 6 females, mean age 58 years, range 15-84 years), and multiple endocrine neoplasia type 1 (MEN) \(n = 8\), 1 female, mean age 52 years, range 42-69 years). In addition to a tumor specimen available from all 62 PC-, NE- and MEN-patients, a sample of adjacent normal tissue was obtained from 41 patients (66%). Atrophic pancreatic tissue was obtained from 13 of the PC patients. Thus, 116 pancreatic tissue samples (1.9 samples per patient in average) were available from 13 of the PC patients. Therefore, 116 pancreatic tissue samples from patients with cancer of the pancreas (PC) \(n = 40\), 20 females, mean age 59 years, range 44-77 years), neuroendocrine cancer (NE) \(n = 14\), 6 females, mean age 58 years, range 15-84 years), and multiple endocrine neoplasia type 1 (MEN) \(n = 8\), 1 female, mean age 52 years, range 42-69 years), were also examined.

Pancreatic tissue specimens from patients with benign (other than pancreatitis) pancreatic diseases \(n = 10\), 8 females, mean age 55 years, range 24-71 years) such as mucinous cystadenoma \(n = 5\), serous cystadenoma \(n = 3\), pancreatic cysts \(n = 2\), as well as histologically normal pancreatic tissue samples from patients with cancer of ductus choledochus \(n = 4\), 1 female, mean age 65 years, range 55-75 years), colon (male, mean age 60 years), duodenum (male, mean age 64 years), and retroperitoneal fibrosis (male, mean age 57 years), were included as controls (collectively denoted C). Gastric tissue samples of the antrum and/or fundus, as well as duodenal samples, were obtained from 23 PC patients and four NE- and MEN patients. An average of 2.5 stomach and/or duodenum specimens was tested per patient. Specimens of the gallbladder \(n = 18\) and ductus choledochus \(n = 8\) were also obtained from PC-, NE- and MEN patients. This study was approved by the Research Ethics Committee at Lund University (LU 726-02).

**Bacterial strains**

Bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Culture Collection of the University of Gothenburg (CCUG, Gothenburg, Sweden). Genomic DNA of \textit{Bacteroides fragilis} (ATCC 25285), \textit{H. pylori} (ATCC 17874), \textit{H. bilis} (CCUG 38995), \textit{H. pullorum} (CCUG 33840), \textit{H. sp} flexispira (CCUG 23435), \textit{H. hepaticus} (CCUG 33637), \textit{Escherichia coli} (ATCC 25922), \textit{Campylobacter jejuni} (ATCC 33560), \textit{Clostridium difficile} (ATCC 9689), \textit{Proteus mirabilis} (ATCC 43071), \textit{Klebsiella pneumoniae} (ATCC 10031), \textit{Enterobacter facalis} (ATCC 29212) and \textit{Enterobacter cloacae} (CCUG 6323) was extracted from 10\(^{7}\) bacterial cells by the QIAamp\textsuperscript{®} DNA Mini Kit (Qiagen) as described below.

**Preparation of DNA**

Paraffin-embedded tissue samples were heated at 60 °C for 10 min to melt excess paraffin, aseptically transferred to new micro-centrifuge tubes, washed in xylene for 2 × 5 min, rehydrated through graded ethanol (990 mL/L and 950 mL/L for 2 × 5 min and 700 mL/L for 5 min), and finally washed for 5 min in double-distilled water. Subsequently, the samples were homogenized in 170 mmol/L phosphate-buffered saline pH 7.2 by using a plastic micro-centrifuge tube-adapter pestle. Pancreatic tissue samples were homogenized at 10 g/L and other gastrointestinal specimens at 40-50 g/L. DNA was extracted from 100 mL of each homogenate by the QIAamp\textsuperscript{®} DNA Mini Kit Tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All DNA samples were stored at -20°C.

**Helicobacter genus-specific PCR**

DNA-extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, US) by semi-nested PCR-assay for \textit{Helicobacter} species as previously described\(^18\) with primers constructed by Goto et al\(^19\). The forward primer 1F (5’-CTATGACGGTGATCCGGC-3’) and reverse primer 1R (5’-CTCAGGCGAGGTAGC-3’) were used in the first step. In the second step, primer 1F and reverse primer 2R (5’-TGGCGCTTCCGAATGATTTT-3’) were used. Precautions were taken to minimize the risk of PCR cross contamination as described recently\(^20\). Detection of PCR-products was done in agarose gels as described previously\(^21\).

**Bacteroides genus-specific PCR**

To rule out the non-specific translocation of a common gut microbe to the pancreas among the studied patients, a pancreatic tissue sample \(n = 84\) from each patient was amplified.
by nested PCR with primers for the glutamine synthase gene of *Bacteroides* species. Primers BFR-1 (5′-ACTCTTTGTATCCGACGGATT-3′) and BFR-2 (5′-GAGGTTGATGCGCTTGCATT-3′), described by Kane *et al.*[11], were used. For the second step, internal primers (forward primer BFR-3: 5′-GACAAAAACATCACCCGGGT-3′ and reverse primer BFR-4: 5′-GCCCCAGGCTTGACATCTCATTT-3′), based on the sequence of the BFR1-BFR2 PCR-product, were constructed using the Vector NTI Suite version 8.0 (Informax, Frederick, MD, US). The specificity of the *Bacteroides* species PCR-assay was evaluated using strains from the ATCC and CCUG. PCR-mixtures were prepared as described previously.[18] Amplification conditions for the first step were at 94 °C for 4 min; 30 cycles at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 45 s and finally at 72 °C for 5 min. Conditions for the second step were at 94 °C for 10 min; 35 cycles at 94 °C for 30 s, at 72 °C for 30 s, at 72 °C for 30 s and finally at 72 °C for 5 min. Genomic DNA (0.1 ng) of *B. fragilis* was used as a positive control.

**Denaturing gradient gel electrophoresis**
Denaturing gradient gel electrophoresis (DGGE) analysis of the V6-7 region of *Helicobacter* species 16S rDNA was performed as described previously.[18] Diluted (10×) first step PCR-products were amplified in the second step using forward primer GC-1F (5′-GCGGCCGCCCGTCCTTGCCGCGCAGGATCTGGTACGCCGGT-3′) and 2R as described above. Electrophoresis was carried out using a DCode™ electrophoresis unit (BioRad, Hercules, CA, US). A mixture of ampiclons of type strain DNA of *H. pylori* (CCUG 17874), *H. bilis* (CCUG 38995), *H. pullorum* (CCUG 33840), *H. sp. flexispira* (CCUG 23435) and *H. hepaticus* (CCUG 33637) was used as gel migration markers.

**DNA-sequence analysis**
Nucleic acid products of the *Helicobacter* genus-specific PCR-assay were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, US), or from DGGE-gels as described previously.[18] DNA-sequence reactions were performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems) with modifications. One microliter of a BigDye™ mix and 1.5 μL of sequencing buffer (10 μL of 10× PCR-buffer II, 6 μL 25 mmol/L MgCl₂, 4 μL double-distilled water) were prepared in a total volume of 10 μL with primers (1F or 2R) and template according to the manufacturer’s instructions. Products of the sequence reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis as described elsewhere.[17]

**RESULTS**

**Helicobacter PCR of pancreas**
As a rule, two pancreatic specimens were obtained from each patient in the PC-, NE- and MEN-groups. If at least one specimen was positive, the patient was considered *Helicobacter*-positive. Hence, 75% (tumor and/or surrounding tissue) of the PC-, 57% of the NE-, and 38% of the MEN patients were positive for the genus *Helicobacter* (Table 1). Three

| Patient group | Tumor (n = 62) | Atrophic (n = 13) | Normal (n = 41) |
|---------------|---------------|------------------|-----------------|
| *Helicobacter* genus-specific 16S ribosomal DNA PCR-assay (positive/total) |
| **PC** | 30/40 | 7/23 | 0/12 | 0/6 |
| **NE** | 14/8 | 1/2 | 0/4 | 0/1 |
| **MEN** | 8 | 3/8 | 1/2 | 0/1 |
| **CP** | 5 | 3/5 | – | – |
| **C** | 17 | 0/17 | – | – |

PC: pancreatic exocrine cancer; NE: neuroendocrine cancer; MEN: multiple endocrine neoplasia type 1; CP: chronic pancreatitis; C: cysts, cystadenoma and remaining controls.

(= (n = 6) − 0 5)

PC: pancreatic exocrine cancer; NE: neuroendocrine cancer; MEN: multiple endocrine neoplasia type 1.

Stomach and/or duodenum samples demonstrated a *Helicobacter*-positive result in 33% of the patients (Table 1). Of the 40 PC patients, stomach and/or duodenum was obtained from 23. Among these 23 patients, 30% were positive in the stomach and/or duodenum whereas 74% were positive in the pancreas. Four PC patients and one
Table 3 Distribution of Helicobacter 16S ribosomal DNA sequences identified using DNA-sequence and/or DGGE-analysis of the V6-region

| Helicobacter species | Pancreatic tissue samples | Gastric or duodenal samples |
|----------------------|---------------------------|-----------------------------|
| H. pylori            | Normal¹                   | Tumor²                     |
|                      | Atrophic                  | CP³                         |
| H. bilis             | 7                         | 6                           | 2 |
| H. hepaticus         | 2                         | 6                           | 2 |
| H. sp. flexispira    | 7                         |                             | |
| H. cinaedi           | 2                         |                             | |

¹Tissue adjacent to tumor; ²Atrophic tissue (PC only); ³Tumor from PC, NE- and MEN patients; ⁴Chronic pancreatitis.

DISCUSSION

We analyzed the prevalence of Helicobacter species ribosomal DNA by PCR in paraffin-embedded pancreatic and gastroduodenal samples from patients with pancreatic cancer and tissues of benign pancreatic diseases as well as controls of normal pancreas (from choledocholes and colon cancer patients). Helicobacter species DNA was identified in the pancreas of 75% of the PC patients, 57% of the NE patients, 38% of the MEN patients, and 60% of the patients with chronic pancreatitis. Other benign pancreatic diseases and the normal pancreas controls were all helicobacter-negative (Table 1). Detection of Helicobacter species in pancreas is thus related to patients suffering from pancreatic cancer and chronic pancreatitis. Serological studies have previously demonstrated an association between serum antibodies to H. pylori and pancreatic cancer (15, 16). Moreover, H. pylori increases the severity of tissue inflammation and production of proinflammatory cytokines in a rat model of ischemia/reperfusion-induced pancreatitis (17).

The distribution of Helicobacter ribosomal DNA in tumor and normal tissue of pancreatic cancer patients was also studied. Helicobacter species was commonly detected in tumors (48% of PC, 57% of NE, and 38% of MEN). The prevalence of helicobacter was much lower in the normal pancreas surrounding NE and MEN (5%) than surrounding PC (36%) (Table 2). There are at least two possible explanations. One is that helicobacter bacteria and/or helicobacter DNA may be taken up and retained by the diseased tissues such as tumors, the other is that helicobacter cells and/or cell debris in pancreas may be implicated in the genesis of PC and are therefore also found in the non-tumor pancreas, while in NE and MEN helicobacters may be an epiphenomenon and are thus uncommon in the non-tumor pancreas. Similar to the findings in normal tissues surrounding tumors in PC patients compared with NE and MEN, we have previously detected Helicobacter species in liver tissue.
surrounding primary liver carcinoma but not colorectal liver metastases. Hence, a possible participation of Helicobacter species in the genesis of exocrine pancreatic and liver carcinoma has to be further explored. Moreover, PCR detection of H. pylori in the liver is associated with cirrhosis in hepatitis C patients with or without hepatocellular carcinoma, in analogy to H. pylori-associated tissue inflammation in chronic atrophic gastritis progressing to gastric cancer.

A majority of the PCR-products from pancreatic cancer samples are related to 16S ribosomal DNA sequences of H. pylori previously identified in gallbladder tissue samples, and to H. sp. ‘liver’, a putative subspecies of H. pylori with liver tropism, detected in hepatocellular carcinoma, supporting the hypothesis of a H. pylori subpopulation with hepatobiliary tropism. We also identified H. cinaedi and H. sp. flexispira taxon 8 in some exocrine pancreatic cancer samples (Table 3). These species with a broad mammal host-range have been isolated from patients with bacteremia and gastroenteritis.

A low prevalence of H. pylori DNA was found in gastric and duodenal samples from the patients in this study (Table 3). At least half of the adult human population is infected with H. pylori, but only 14% of the PCR-products from the stomach of patients with exocrine pancreatic cancer could be identified as H. pylori. However, H. bilis has been identified in 60% of helicobacter-positive gastroduodenal specimens. H. bilis is associated with chronic hepatitis and chronic enteric inflammation in susceptible laboratory mouse strains and has been recently identified in both diseased human gallbladder and bile by PCR-based methods. To our knowledge, H. bilis has not previously been detected in tissue samples of the human stomach and small intestine. The reason why the detection rate of H. pylori is low in the stomach and duodenum of PC patients remains obscure. This may partly be explained by the fact that most or all patients were given metronidazole preoperatively.

We analyzed stomach and duodenum tissue specimens from pancreatic cancer patients to detect whether gastric Helicobacter species, such as H. pylori, may disseminate to the pancreas in pancreatic cancer patients. However, DNA of different Helicobacter species in the pancreas compared with gastroduodenal tissue was identified in patients who were Helicobacter-positive both in the stomach and pancreas (Figure 1). Moreover, many pancreas-positive PC patients were negative in stomach samples and vice versa, not supporting migration of helicobacter microorganisms colonizing the stomach to the pancreas in the studied PC patients.

B. fragilis constitutes a part of the indigenous microflora of the human gut, predominantly of the colon. A nested PCR-assay for the genus Bacteroides was designed to study whether a major constituent of the normal microflora of the lower bowel could translocate to a diseased pancreas. None of the pancreatic tissue specimens was positive for Bacteroides spp., suggesting that bacterial translocation from the bowel to pancreas does not frequently occur in patients with cancer or a benign pancreas disease. However, the indigenous microflora may more easily be washed off tissues during sample preparations such as paraffin deembedding. Putative pathogens, such as some Helicobacter species, might be more closely associated with the gut mucosa.

Chronic inflammation is a characteristic feature of gastric-, colon- and hepatobiliary tract cancers. Bacterial cell-wall peptidoglycan and lipopolysaccharide can stimulate the human innate immunity and induce inflammation. Bacterial DNA, so called CpG motifs, has been shown to activate macrophages, neutrophils as well as cell migration and to induce B cell activation and hyper-IgM production in patients with primary biliary cirrhosis. Proinflammatory cytokines, reactive oxygen species and other inflammatory mediators are associated with a chronic Helicobacter-induced tissue inflammation. Such factors probably increase genomic DNA damage and cell proliferation as well as inactivate tumor-suppressor genes, events also associated with malignant transformation of pancreatic cells. Tumor-associated chronic inflammation may be induced and maintained by bacteria or bacterial cell debris, originating from helicobacter and/or other microbial species.

In conclusion, 16S ribosomal DNA of gastric H. pylori and some enteric Helicobacter species is commonly detectable in tissue samples from patients with pancreatic cancer but not from controls. To further explore a possible role of gastric and enterohepatic Helicobacter species in pancreatic malignancy, more studies of pancreatic cancer and the emerging Helicobacter genus and related organisms are needed.

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