

Molecular identification of *Helicobacter* DNA in human gastric adenocarcinoma tissues using *Helicobacter* species-specific 16S rRNA PCR amplification and pyrosequencing analysis

HYE SEUNG HAN^{1,3}, KYUNG-YUNG LEE², SO DUG LIM¹, WAN SEOP KIM¹ and TAE SOOK HWANG¹

Departments of ¹Pathology, ²Surgery, and ³Research Institute of Medical Science, Konkuk University School of Medicine, Seoul 143-729, Korea

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Abstract. *Helicobacter pylori* (*H. pylori*) is a microaerophilic gram-negative bacterium known to be associated with chronic gastritis, peptic ulcer and gastric adenocarcinoma. In the present study, the presence of *Helicobacter* DNA was investigated using a *Helicobacter* species-specific 16S rRNA PCR amplification and pyrosequencing analysis in 51 resected gastric adenocarcinomas. DNA was extracted from paraffin-embedded tissues of resected gastric adenocarcinomas. PCR primers were designed to amplify the 133-bp PCR fragment in highly conserved regions of the 16S rRNA gene. The sequence of the PCR products was analyzed using a PSQ 96 system with SQA software. The pyrosequencing analysis of 16S rRNA showed that *H. pylori* was present in 47 (92.2%) of the 51 gastric adenocarcinomas. In the 4 *H. pylori*-negative cases, *Helicobacter cinaedi* (2 cases), *Helicobacter mustelae* (1 case) and *Campylobacter hyointestinalis* (1 case) were detected. Pyrosequencing technology was useful in the identification and differentiation of *H. pylori* from other species by analyzing the gene encoding 16S rRNA. Gastric adenocarcinoma tissues contain bacteria, and the majority are *H. pylori*. *Helicobacter cinaedi*, *Helicobacter mustelae* and *Campylobacter hyointestinalis* rarely occur. The roles of these organisms in the pathogenesis of gastric adenocarcinoma remain unclear.

Introduction

Helicobacter pylori (*H. pylori*) is a microaerophilic gram-negative bacterium known to be associated with chronic gastritis, peptic ulcer and gastric adenocarcinoma (1). It is of

great clinical importance to identify the organism in gastric specimens. Subsequently, several diagnostic assays exist. *H. pylori* infection in gastric specimens can be demonstrated through the use of culture, histological examination of biopsy specimens using different stains, assaying for urease activity and PCR assay with the aim of specifically detecting *H. pylori* DNA (2). Assays based on the use of PCR to detect the presence of *H. pylori* DNA using several different gene targets have been described (2-10). Moreover, it is well known that the PCR assay is highly reliable in the detection of *H. pylori*. The pyrosequencing analysis was employed to identify *H. pylori* by sequencing a part of the 16S rRNA gene covering the *H. pylori* signature sequence (6). The *H. pylori* signature sequence allows for the distinction of the organism from a set of other bacterial species (11). This study investigated the possibility of using pyrosequencing technology to verify the species identity of *H. pylori* from paraffin-embedded tissues of resected gastric adenocarcinomas by amplifying a part of the 16S rRNA gene using broadly reactive primers followed by the sequencing of a 20-bp sequence unique to the 16S rRNA gene of *H. pylori*.

Materials and methods

DNA extraction. DNA was extracted from paraffin sections of 51 resected gastric adenocarcinomas including 21 intestinal, 24 diffuse and 6 mixed types. Briefly, 50-100 μ l of DNA extraction buffer solution [50 mM Tris buffer (pH 8.3), 1 mM EDTA (pH 8.0), 5% Tween-20 and 100 μ g/ml proteinase K] with 10% resin was added to scraped tissue and incubated at 56°C for a minimum of 1 h. Following incubation, the tubes were heated at 100°C for 10 min. Tubes were centrifuged to pellet the debris, and 5 μ l of the supernatant was used in the PCR reaction.

PCR amplification for *Helicobacter pylori* identification. To identify *H. pylori* the primers used were: forward, 5'-biotin-AGGGGTAAAATCCGTAGAGAT-3' and reverse, 5'-CGTTTAGGGCGTGGACTA-3'. The latter primer amplifies a 133-bp DNA fragment from the '16S rRNA' region of *H. pylori*. Briefly, 5 μ l of DNA was added to reach 50 μ l of PCR solution mix, containing 0.2 mmol each of dNTP,

Correspondence to: Dr Tae Sook Hwang, Department of Pathology, Konkuk University Hospital, 4-12 Hwayang-dong, Gwangjin-gu, Seoul 143-729, Korea
E-mail: tshwang@kuh.ac.kr

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1.5 mmol/l MgCl₂, 1X PCR buffer, 1.5 units of Immolase DNA Taq polymerase (Bioline, London, UK) and 20 pmol of each primer. PCR was performed for 5 min at 95°C, 50 cycles (30 sec at 95°C, 30 sec at 52°C and 30 sec at 72°C) and 10 min at 72°C using a PTC-220 thermal cycler (Bio-Rad, USA). The PCR products were electrophoresed in an agarose gel to confirm successful amplification of the PCR product.

Pyrosequencing analysis for Helicobacter pylori identification. Biotinylated PCR products were immobilized to streptavidin-coated beads (Amersham Pharmacia Biotech AB, Sweden) using solution from the PSQ™ 96 Sample Preparation kit (Pyrosequencing AB, UK), following a standard protocol. Beads (10 µl) were diluted in binding buffer with biotinylated PCR products and incubated for 10 min at room temperature. The beads were transferred to a filter probe, and liquid was removed by vacuum filtration. DNA was separated in denaturation solution for 2 min. The templates were washed with washing buffer, transferred to a PSQ 96 SQA plate and annealed with the sequencing primer, reverse, 5'-CTCCCA CGCTTT-3' in annealing buffer at room temperature. Samples were analyzed using the PyroMark ID system (Biotage, UK) with SQA software and the SQA reagent kit (Biotage) for sequence analysis.

Results

DNA was extracted from the paraffin-embedded tissues of 51 resected gastric adenocarcinomas. PCR primers were designed to amplify the 133-bp PCR fragment in highly conserved regions of the 16S rRNA gene. The sequence of the PCR products was analyzed using the PyroMark ID system with SQA software and the SQA reagent kit. Sequence analysis for the identification of *H. pylori* by sequencing a section of the 16S rRNA gene covering the *H. pylori* signature sequence was carried out. Fig. 1 shows the representative results from the analysis of the paraffin-embedded tissues of 51 resected gastric adenocarcinomas. Pyrosequencing analysis of 16S rRNA showed that *H. pylori* was present in 47 (92.2%) of the 51 gastric adenocarcinomas: 18 of the 21 intestinal-, 23 of the 24 diffuse- and all of the 6 mixed-type. In the 4 *H. pylori*-negative cases, *Helicobacter cinaedi* (*H. cinaedi*) (2 cases), *Helicobacter mustelae* (*H. mustelae*) (1 case) and *Campylobacter hyointestinalis* (*C. hyointestinalis*) (1 case) were detected. Two *H. cinaedi*- and 1 *C. hyointestinalis*-positive cases were detected in the intestinal-type, and 1 *H. mustelae* case was detected in the diffuse-type adenocarcinomas.

Discussion

A variety of diagnostic procedures are used to identify *H. pylori* in clinical samples. No single test is optimal due to the length of time required to perform the test, lack of sensitivity or irreproducibility (5). Culture and histological examination of biopsy specimens using different stains and assaying for urease activity have the disadvantages of lack of sensitivity and long incubation periods. Assays based on the use of PCR to detect the presence of *H. pylori* DNA using several different gene targets showed that PCR is feasible

for the rapid, sensitive and specific detection of *H. pylori* (2-5,7-10,12). Using the PyroMark ID system with SQA software and the SQA reagent kit to amplify a section of the 16S rRNA gene it was possible to analyze bacterial genetic targets in DNA extracted directly from human gastric tissues without the prolonged culturing of bacteria. Subsequently, the organism was differentiated from a set of other bacterial species (6).

H. pylori has been classified as a Group I carcinogen. Previous epidemiologic studies established a strong causal relationship between *H. pylori* infection and gastric cancer (13-16). A nationwide survey conducted in South Korea in 1998 on the serologic prevalence of *H. pylori* infection concluded that the prevalence of *H. pylori* was 66.9% among adults (≥16 years), a percentage that decreased to 59.6% in 2005 (17). In this study, *H. pylori* was present in 47 (92.2%) of 51 gastric adenocarcinoma tissues from Korean patients. Persistent infection of the gastric mucosa by *H. pylori* initiates an inflammatory cascade that progresses into atrophic gastritis, a condition associated with a reduced capacity for the secretion of gastric acid and an increased risk of developing gastric cancer (18).

Two cases of *H. cinaedi* infection, 1 case of *C. hyointestinalis* infection and 1 case of *H. mustelae* infection in the 4 *H. pylori*-negative cases were noted in the present study. Two *H. cinaedi*- and 1 *C. hyointestinalis*-positive cases were detected in the intestinal-type and 1 *H. mustelae* in the diffuse-type adenocarcinomas. Molecular evidence of *H. cinaedi* organisms in 2 of 126 urease-negative human gastric biopsy specimens was previously reported (19). *H. cinaedi* was found to cause gastroenteritis (20) and extraintestinal infection, particularly in immunocompromised patients (21). *C. hyointestinalis* was initially described by Gebhart *et al* as a possible cause of porcine proliferative enteritis (22). The organism has subsequently been isolated from the feces of humans with gastroenteritis and, in a few cases, from the blood of patients with bacteremia (23). Although *H. cinaedi* and *C. hyointestinalis* were previously associated with gastroenteritis, the incidence and roles of these organisms in gastric carcinogenesis remain unclear. *H. mustelae* is a gastric pathogen that has many biochemical, molecular and phenotypic characteristics similar to those of *H. pylori* (24). *H. mustelae* infection was found to increase gastric epithelial proliferation, as noted in *H. pylori*-infected humans, presumably due to a chronic inflammatory response (25). A previous study suggested that the high tumor incidence reported in MNNG-treated ferrets reflected the involvement of *H. mustelae* infection in the carcinogenic process in these animals (26). A previously reported case linking *H. mustelae* and gastric adenocarcinoma supports the hypothesis that *H. mustelae*, similar to *H. pylori* in humans, may be a gastric co-carcinogen in ferrets (27). However, this hypothesis has yet to be confirmed in humans.

Pyrosequencing technology is useful in the identification and differentiation of *H. pylori* from other species by analyzing the gene encoding 16S rRNA. Gastric adenocarcinoma tissues contain bacteria and the majority are *H. pylori*. *H. cinaedi*, *H. mustelae* and *C. hyointestinalis* rarely occur. The roles of these organisms in the pathogenesis of gastric adenocarcinoma remain unclear.

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