Molecular identification of *Helicobacter* DNA present in human colorectal adenocarcinomas by 16S rDNA PCR amplification and pyrosequencing analysis

Niclas Grahn, Mounira Hmani-Aifa, Karin Fransén, Peter Söderkvist and Hans-Jürg Monstein

1 Molecular Biology Laboratory - LMC, University Hospital, S-581 85 Linköping, Sweden
2 Division of Cell Biology, Department of Biomedicine and Surgery, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden
3 Laboratory of Human Molecular Genetics, Faculty of Medicine of Sfax, Tunisia

Seroepidemiological studies have indicated that *Helicobacter pylori* infection might be a possible risk factor for colorectal adenocarcinoma (CRC) development. However, limited information is available as to whether or not *Helicobacter* species are present in CRC tissues. In this study the presence of *Helicobacter* DNA in 77 CRC biopsies was investigated by means of a *Helicobacter* species-specific 16S rDNA PCR assay and real-time DNA pyrosequencing of the 16S rDNA variable V3 region. Pyrosequencing revealed the presence of *Helicobacter* DNA sequences in 21 of 77 biopsy specimens (27%). 16S rDNA sequences corresponding to *H. pylori* 26695 and *H. pylori* J99 were most commonly found. Intriguingly, one sequence belonged to *Helicobacter mustelae*, previously identified in ferrets. No significant correlations were found in the prevalence of *Helicobacter* DNA between colon and rectum tumour biopsies ($P = 0.815$), nor between Dukes’ classes A/B and C/D ($P = 0.262$). 16S rDNA PCR amplification combined with pyrosequencing analysis of 16S rDNA variable V3 regions provides a powerful molecular tool to identify *Helicobacter* species in human biopsy specimens.

INTRODUCTION

*Helicobacter pylori* is a microaerophilic Gram-negative spiral-shaped bacterium (Marshall & Warren, 1983) associated with chronic gastritis, peptic ulcer and gastric adenocarcinoma development (Parsonnet *et al.*, 1991; Cover & Blaser, 1992; Logan, 1994). It has been shown that there is a significant geographical relationship between gastric cancer mortality rates and prevalence of *H. pylori* infection. Subtypes of *H. pylori* might differ in pathogenicity (Xiang *et al.*, 1995), and a number of studies have shown that the prevalence of *H. pylori* expressing the cytotoxin-associated gene A (*cagA*) in gastric cancer patients is much higher than in age- and gender-matched controls (Parsonnet *et al.*, 1997; Konturek *et al.*, 2003; Semino-Mora *et al.*, 2003). Recent reports imply that *H. pylori* may be an association factor involved in colorectal cancer (CRC) development in patients infected with *H. pylori* strains (Meucci *et al.*, 1997; Shmuely *et al.*, 2001). However, it is far from clear whether *H. pylori* is present in CRC tissues and whether or not *H. pylori* plays a similar role in colorectal carcinogenesis as has been proposed for gastric cancer development.

16S rDNA sequence analysis has demonstrated considerable genomic diversity among *H. pylori* clinical isolates, and numerous sequence-specific PCR assays, combined with 16S rDNA sequencing, have been developed to identify *Helicobacter* species (Thoreson *et al.*, 1995; Blom *et al.*, 2002). We recently described a method for the rapid simultaneous molecular identification and subtyping of *H. pylori* by pyrosequencing analysis of the 16S rDNA variable V1 and V3 regions (Monstein *et al.*, 2001).

In this report we describe the molecular identification of *Helicobacter* DNA in CRC biopsies by means of a 16S rDNA PCR amplification assay combined with pyrosequencing analysis.

METHODOLOGY

Tissue collection and DNA isolation. A total of 77 patients (45 women and 32 men, mean ± SD age, 71.9 ± 11.4 years) were included in the present study. Sampling and characterization of 42 colon and 35

Abbreviation: CRC, colorectal adenocarcinoma.
rectum cancer biopsy specimens have been described in a previous study (Fransen et al., 2004). Seventy-six of the 77 cancer patients were assessed according to the Dukes’ classification (Dukes, 1932; Cohen et al., 1993), 44 as Dukes’ class A/B and 32 as Dukes’ class C/D. Total DNA (cellular and bacterial DNA) from biopsy specimens was isolated using a Wizard Genomic DNA Purification Kit according to the supplier’s recommendations (Promega).

**Bioinformatics and primer design.** Sequence data for 16S rRNA genes from different Helicobacter species and Campylobacter species were obtained from GenBank (Benson et al., 2000) and aligned using CLUSTAL_W (Thompson et al., 1994). A complete list of accession numbers is available on request. Sequence alignment revealed some regions (in particular variable V2 and V3 regions) that were conserved among the different Helicobacter species but showed a substantial variation compared to Campylobacter species (data not shown). Based on this information, we designed primers that specifically targeted Helicobacter species over Campylobacter species and other bacterial species (Table 1, Fig. 1). However, some 16S rDNA sequence variation within the variable V2 and V3 regions of Helicobacter canis was found, and therefore the Helicobacter species-specific primers used would not be suitable to amplify H. canis 16S rDNA. Prior to PCR amplification, the primers were tested for their selectivity using H. pylori 26695 and Campylobacter jejuni DNA as described below. Furthermore, the H. pylori-specific primers were also tested with the BLAST tool (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI) for any sequence complementarities with other bacterial species.

**Helicobacter species-specific PCR and pyrosequencing analysis.** PCR amplifications (first round and semi-nested) were carried out using selective primers HP-NG.SE and HplBS.V3.SE, targeting Helicobacter species 16S rDNA variable V2 and V3 regions, respectively, and a 16S rDNA broad-range primer p13B.AS (for details see Table 1, Fig. 1). In brief, approximately 50 ng total DNA was used as template in a first round PCR amplification with primers HP-NG.SE (specific) and p13B.AS (broad-range). PCR amplicons were diluted 1:100 and 1 μl was used as template DNA in a semi-nested PCR amplification step using primers HplBS.V3.SE (specific) and p13B.AS (broad-range). Subsequently, 1 μl (diluted 1:100) of the semi-nested PCR amplicons and primers BIO-HplBS.V3.SE and B-V3.AS were used to generate Helicobacter-specific amplicons suitable for pyrosequencing analysis (see below). First and semi-nested PCR amplifications were carried out using puRe Taq Ready to Go PCR beads (Amersham Biosciences) in a final 25 μl reaction volume. First-round PCR amplification was performed with an initial denaturing step at 95 °C for 10 min followed by 30 cycles of denaturing at 95 °C for 40 s, annealing at 55 °C for 40 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Semi-nested PCR amplification was performed using an initial denaturing step at 95 °C for 5 min, followed by 25 cycles under the conditions described above. Third-round PCR amplification (pyrosequencing template generation) was performed using a HotStar Taq Master-mix kit (Qiagen) in a final 25 μl reaction volume. An initial DNA denaturation and Taq DNA polymerase activation was performed at 95 °C for 15 min, followed by 25 cycles of denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. All PCR reactions were performed on a Mastercycler gradient (Eppendorf). Pyrosequencing analysis of the PCR amplicons derived from the 16S rDNA variable V3 region was performed as described previously (Monstein et al., 2001; Grahn et al., 2003) using a PSQ 96MA (Biotage AB). Helicobacter species sequences were identified using catalogued sequences in GenBank with the BLAST tool at NCBI.

**Statistical analyses.** Chi square and Fisher’s exact test analyses were performed with SPSS 11.0.0 statistical software package. The results were considered to be significant at the level $P < 0.05$.

**Fig. 1.** Schematic illustration (not to scale) of the three-round PCR assay used in this study. (a) 16S rDNA variable regions are indicated (boxes) and numbered according to Gray et al. (1984). Arrows indicate primer positions. (b) PCR amplicons generated using primers and DNA templates as described in Methods are indicated (first-round, semi-nested and pyrosequencing). Primer sequences and PCR amplicon lengths are given in Table 1.

**Table 1.** Primers used in PCR amplification and pyrosequencing analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Amplified region</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP-NG.SE</td>
<td>TAGCCACTGAAAAACGGGTGAT</td>
<td>16S rDNA</td>
<td>~1200 bp</td>
</tr>
<tr>
<td>p13B.AS*</td>
<td>GTGTACTAGGCCCAGGAGTATTTC</td>
<td>V3, V4, V9 regions</td>
<td>~430 bp</td>
</tr>
<tr>
<td>HplBS.V3.SE</td>
<td>CCTTACCTAGGCTGTACATTG</td>
<td>V3 region</td>
<td>~85 bp§</td>
</tr>
<tr>
<td>p13B.AS</td>
<td>GTGTACTAGGCCCACGAGTATTTC</td>
<td>V3 region</td>
<td>~85 bp§</td>
</tr>
<tr>
<td>BIO-HplBS.V3.SE</td>
<td>As above, 5’-biotinylated</td>
<td>V3 region</td>
<td>~85 bp§</td>
</tr>
<tr>
<td>B-V3.AS†</td>
<td>ACGACAGCCATGCAGCACCT</td>
<td>V3 region</td>
<td>~85 bp§</td>
</tr>
</tbody>
</table>

* Primer from Tiveljung et al. (1995).
† Primer from Jonasson et al. (2002).
§ PCR amplicons were used for pyrosequencing analysis.
RESULTS

Helicobacter-specific PCR amplification and pyrosequencing analysis

PCR amplification and subsequent pyrosequencing analysis revealed the presence of Helicobacter-specific 16S rDNA variable V3 region sequences in 21 out of 77 (27%) biopsy specimens. More specifically, Helicobacter DNA was identified in 11 out of 42 (26%) colon cancer and 10 out of 35 (29%) rectum cancer biopsies. The difference in Helicobacter DNA prevalence between colon and rectum tumour biopsies was not significant (P = 0.815; χ² test). There was also no significant difference found in Helicobacter DNA prevalence between Dukes’ classes A/B and C/D, where 10 out of 44 and 11 out of 32 biopsies, respectively, were Helicobacter positive (P = 0.262, χ² test). Moreover, no significant difference in Helicobacter DNA prevalence between men (11/32) and women (10/45) was observed (P = 0.238; χ² test).

Partial sequences within the 16S rDNA variable V3 region were obtained from 21 PCR amplicons and two reference strains: H. pylori 26695 (Tomb et al., 1997) and J99 (Alm et al., 1999). Based on 16S rDNA variable V3 region nucleotide sequences, the 21 Helicobacter-specific PCR amplicons (isolates) were divided into six different clusters (Fig. 2). Cluster I, comprising 14 isolates, had an identical sequence to that of H. pylori 26695. Double nucleotide mutations were observed in cluster II (three isolates), which demonstrated DNA sequence identity with the corresponding region of reference strain H. pylori J99 (Fig. 2). Single nucleotide mutations were observed in clusters III and IV (one isolate in each group). Interestingly, multiple nucleotide sequence mutations were present in cluster V (one isolate), which differs significantly from the other isolates (Fig. 2a). A GenBank database search revealed DNA sequence identity with the corresponding region of Helicobacter mustelae, which has so far only been identified in ferrets (Fox et al., 1990). One isolate was assigned to cluster VI. However, only limited DNA sequence information could be obtained from the pyrogram, indicating that either two Helicobacter strains were present in the biopsy specimen and/or the 16S rDNA gene sequences (variable V3 region) were heterogeneous (Fig. 2a). Therefore, this particular PCR amplicon was cloned (Grahn et al., 2003) and, subsequently, DNA sequence analysis (pyrosequencing) revealed the presence of three different H. pylori-like 16S rDNA variable V3 region signatures (Fig. 2b).

DISCUSSION

Seroepidemiological studies have indicated that infection with H. pylori is a risk factor for gastric cancer and that antibodies against H. pylori are present in patients with colorectal polyps and adenocarcinomas (Cover & Blaser, 1992; Siddheshwar et al., 2001). However, even though measurement of circulating H. pylori antibodies is commonly used and highly specific it may not always reflect a real-time H. pylori infection (Moss et al., 1995). It is still a matter of debate as to whether or not H. pylori infection is an association factor in colorectal cancer development (Meucci et al., 1997; Shmuely et al., 2001).

By means of 16S-rDNA-based molecular methods (Helicobacter-specific semi-nested PCR amplification combined with pyrosequencing analysis of the PCR amplicons) we found that Helicobacter DNA was present in 27% (21/77) of the investigated colorectal adenocarcinomas. A drawback of this study is that we had no access to control biopsy specimens, and therefore we cannot conclude as to whether or not the presence of H. pylori is statistically significant. Nevertheless, colon and rectum tumour biopsies were selected, assessed according to Dukes’ classification (Dukes, 1932; Cohen et al., 1993) and analysed separately, and the results were compared. No statistically significant differences in the presence of H. pylori DNA in colon and rectum tumour biopsies or in Dukes’ classes A/B and C/D were found. Apparently, the prevalence of H. pylori DNA in colorectal cancer biopsies, using molecular methods, is not in good agreement with the frequency found using seroepidemiological methods. A comparison revealed that the prevalence of H. pylori antibodies among patients with CRC was approximately two to three times higher compared to the prevalence of H. pylori DNA detected using molecular methods (Meucci et al., 1997; Shmuely et al., 2001).

In this study, we used Helicobacter-specific primers designed to amplify 16S rDNA sequences common to Helicobacter species as identified by BLAST searches. To assess the nature of the Helicobacter species present in colon and rectum aden-
carcinomas, real-time DNA pyrosequencing was performed. Sequence analysis revealed that 16S rDNA variable V3 region sequences corresponding to *H. pylori* 26695 and J99 were most commonly found. Unexpectedly, one biopsy specimen revealed the presence of 16S rDNA variable V3 region sequences corresponding to *H. mustelae*, initially isolated from ferrets (Fox et al., 1990) and found to be a risk factor in gastric adenocarcinoma development in ferrets (Fox et al., 1997).

It is believed that *H. pylori* activate the synthesis and release of gastrin due to excessive production of cytokines and growth factors such as TNFa and EGF (Konturek et al., 2003). In a recent clinical study, we were able to show statistically significant gastrin mRNA expression in CRC tissues (Monstein et al., 2004). Although we have no statistically significant evidence for *Helicobacter* species infection, it is tempting to speculate that the presence of *Helicobacter* species in colorectal tumours may have an impact on changes in the cellular gene expression profiles, similar to those observed in gastric adenocarcinomas (Konturek et al., 2003).

In conclusion, the present study confirms the potential of the *Helicobacter*-specific PCR amplification assay used combined with real-time pyrosequencing to detect and subtype *Helicobacter* DNA in biopsy specimens. The results indicate that molecular methods might be an alternative to serological methods although we are aware that the method involving a three-round PCR is not particularly suitable in a clinical routine setting. Moreover, cDNA microarray expression profiling and quantitative real-time RT-PCR analyses should provide powerful means by which to study changes in cellular and *Helicobacter* gene expression in colorectal tumour and non-tumour tissue.

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REFERENCES


