## RESEARCH NOTE

# DETECTION OF VACA GENE SPECIFIC FOR *HELICOBACTOR PYLORI* IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA SPECIMENS OF THAI PATIENTS

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**Abstract.** In order to investigate and compare the presence of *Helicobacter pylori* VacA in primary liver cancer specimens (12 hepatocellular carcinoma and 6 cholangiocarcinoma) and control liver specimens (7 non-primary liver cancer) from Thai patients who underwent liver resection, *H. pylori* VacA gene was assayed in extracted DNA by real-time polymerase chain reaction. The selected amplicons revealed high homology compared with *H. pylori* VacA sequence. *H. pylori* VacA gene was detected in all primary liver cancer specimens and in 71% (5/7) of control liver specimens.

## INTRODUCTION

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) account for more than 80% of all primary cancers of the liver. These cancers rank third amongst organ-specific causes of cancer-related deaths in men worldwide and account for almost 4% of all human cancers (Parkin *et al*, 2000). The geographic areas most affected are located in Southeast Asia especially in Thailand. Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and aflatoxins are the main causes of HCC (Michielsen *et al*, 2005). The pathogenesis of cholangiocarcinoma remains unclear, although infection with *Opisthorchis viverrini* is an important risk factor (Srivatanakul *et al*, 2004). However, these liver cancers also occur in a considerable proportion of patients without recognized risk factors.

*Helicobacter pylori* is the most important cause of chronic gastritis and peptic ulcer disease. It induces a persistent infection and is thought to be a type I carcinogen because of its role in the development of gastric carcinoma and gastric mucosa associated lymphoid tissue lymphoma (Umehara *et al*, 2003). More recently, *Helicobacter* spp has been detected in bile and gallbladder tissue from patients with chronic cholecystitis (Silva *et al*, 2003; Apostolov *et al*, 2005) and in liver tissue from patient with HCC (Coppola *et al*, 2003; Xuan *et al*, 2006). It is possible that *H. pylori* may also be a risk factor for liver cancer.

In this study real-time PCR was used for identification of *H. pylori* VacA gene in liver tissue from hepatocellular carcinoma and cholangiocarcinoma patients. Sequence analysis and comparison showed that these DNA fragments were closely related to *H. pylori* VacA gene.

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## MATERIALS AND METHODS

### Patients and sample preparation

Twenty-five liver specimens were collected from patients who underwent surgery at the Rajavithi Hospital, Thailand, between October 2005 and May 2006. Informed consent for the study was obtained from all patients after approval of this study by the Rajavithi Hospital Ethics Committee on Research Involving Human Subject (85/2005). The final diagnosis of these patients was ascertained from histopathological reports by a pathologist of the Rajavithi Hospital. The diagnosis included HCC, CCA, intra-hepatic duct stones and colorectal cancer with liver metastasis.

### DNA extraction

DNA from liver samples was extracted using a commercially available kit (QIAmp® DNeasy Mini Kit, QIAGEN, USA) according to the manufacturer's protocols. DNA concentration was determined by UV spectrophotometer at 260 nm.

### Real-time PCR

Real-time PCR was performed with QuantiTect SYBR Green PCR (QIAGEN) in a standard PCR reaction mixture. The amplification primers were: *H. pylori*-specific VacA forward primer 5´ ATGGAAATACAACAAA CACAC 3´ and *H. pylori*-specific VacA reverse primer 5´ CTGCTTGAATGCGCCAAAC 3´. DNA from *H. pylori* colonies was extracted and used as positive control. Amplification and detection were performed in a Chromo 4™ System (Bio-Rad, USA). The conditions were as follows : 15 minutes, 95ºC; 35 cycling steps of 30 seconds at 94ºC, 30 seconds at 51ºC and 45 seconds at 72ºC Fluorescence measurement was taken at each extension step. The crossing points (Cp), the cycles when the fluorescence of a given sample significantly exceeded baseline signal, were recorded and expressed as a function of the cycle number. Melting curve analysis was also performed to assess the specificity of the amplicon.

### Sequencing and analysis

Selected amplified DNA fragments were purified and then sequenced using a Model 310 Genetic Analyzer (PE Biosystems) and a BigDye Terminator Cycle Sequencing Kit. The sequence homology search was conducted via World Wide Web at the location "http://ncbi.nlm.nih.gov/Recipon/bs-html (2006, March 12)" of the National Center of Biotechnology Information (NCBI), USA using the BLAST (Basic Local Alignment Search Tool) program.

## RESULTS

In the 25 patients who underwent surgery, diagnosis established by pathology 18 samples of primary liver cancer (12 HCC, 6 CCA) and 7 without primary liver carcinoma (3 intra-hepatic duct stones, 4 colorectal cancer with liver metastasis). Table 1 lists the main

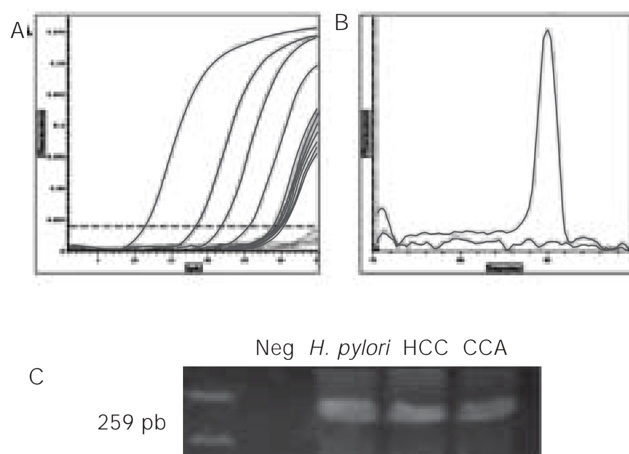


Fig 1–Real-time PCR analysis of VacA specific for *H. pylori*. (A) Continuous monitoring of fluorescent signals in parallel standard DNA from *H. pylori* colonies. (B) Melting profile of amplicon. (C) Electrophoretic analysis of 259 bp amplicon of *H. pylori* VacA generated from *H. pylori* and from hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) tissues.

characteristics of the study patients. The median age of three groups (HCC, CCA and controls without primary liver carcinoma) was 54 years (range of 26-79 years), 59 years (28-71 years) and 59 years (37-74 years), respectively.

Liver specimens were positive for *H. pylori* according to the detection of VacA DNA specific for *H. pylori* by PCR in 23 samples (92%) (Fig 1). All of HCC, CCA and intra-hepatic duct stones samples were positive for *H. pylori* detection (21/21) but only 2 samples of colorectal cancer with liver metastasis (2/4) were positive (Table 1).

The 259 bp amplicons (2 samples of primary liver cancers and 2 samples of controls) were sequenced and their homology were compared with the *H. pylori* VacA sequence (NCBI NC_000915; gi:15644634; gb:AY0 49007.1.) The DNA sequence of amplicons from tissues showed high homology (95-97%)

Table 1
Clinical data and detection of *H. pylori* VacA DNA.

| Case | Sex | Age(Year) | Serology test[a] | | *H. pylori* VacA DNA |
|---|---|---|---|---|---|
| | | | HBsAg | Anti-HCV | |
| HCC | | | | | |
| 1 | M | 63 | - | - | + |
| 2 | M | 55 | - | - | + |
| 3 | M | 62 | + | - | + (95%)[b] |
| 4 | M | 79 | - | - | + |
| 5 | M | 61 | - | - | + |
| 6 | M | 52 | - | - | + |
| 7 | M | 48 | + | - | + |
| 8 | F | 32 | + | - | + |
| 9 | F | 42 | + | - | + |
| 10 | F | 68 | - | - | + |
| 11 | F | 26 | + | - | + |
| 12 | F | 59 | + | - | + |
| CCA | | | | | |
| 1 | M | 53 | - | - | + |
| 2 | F | 67 | - | - | + (97%)[b] |
| 3 | F | 71 | - | + | + |
| 4 | F | 28 | - | + | + |
| 5 | M | 67 | - | - | + |
| 6 | M | 66 | - | - | + |
| Intrahepatic duct stones | | | | | |
| 1 | M | 37 | - | - | + |
| 2 | M | 74 | - | - | + |
| 3 | F | 56 | + | - | + |
| Liver metastasis | | | | | |
| 1 | M | 56 | - | - | + (97%)[b] |
| 2 | F | 62 | - | - | - |
| 3 | F | 43 | - | - | + (97%)[b] |
| 4 | M | 67 | - | - | - |

[a]Serology test for HBsAg and HCV antibody were assayed from serum.
[b]Percent identity of the amplicons compared with the *H. pylori* VacA sequence

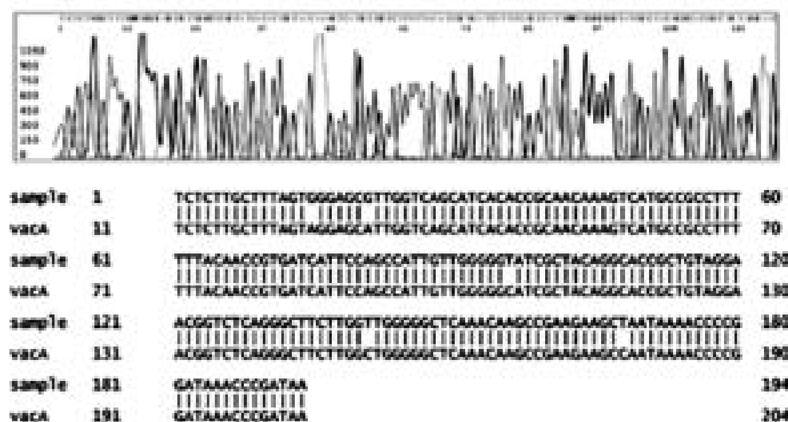

Fig 2–Comparison of nucleotide sequence of *H. pylori* VacA from primary liver cancer with that at NCBI GenBank (gi15644634).

with VacA gene specific for *H. pylori* (see Fig 2 for a typical result).

## DISCUSSION

*H. pylori* infection is chronic. In most instances, it is acquired during childhood, and is often associated with low socio-economic class (Mendall *et al*, 1992; Deankanob *et al*, 2006). The presence of this bacterium has been established as the main cause of several gastroduodenal diseases, including peptic ulcer disease (Hopkins *et al*, 1996), gastric carcinoma (Pritchar and Crabtree, 2006), and gastric MALT lymphoma (Wotherspoon *et al*, 2002). Gastric mucosal damage by *H. pylori* is caused by the chronic release of various bacterial and host-dependent cytotoxic substances. The role of *H. pylori* in the pathogenesis of extra-gastroduodenal manifestations is still under investigation. Previous studies found that *H. pylori* could damage hepatocytes by a cytopathic effect and induce hepatitis (Francavilla *et al*, 1999). Vacuolating cytotoxin of *H. pylori* could reach and damage the hepatocytes of patients with both *H. pylori* infection and isolated hyper-transaminasemia without signs of known causes of liver diseases.

We detected *H. pylori* DNA by PCR in liver specimens from all 18 patients with primary liver cancer (hepatocellular carcinoma and cholangiocarcinoma) and in 5 of 7 (71%) samples from non-primary liver cancer disease. These results are in contrast with a previous study demonstrating *Helicobacter* spp DNA in 40% samples of primary liver carcinoma, whereas none of the controls harbored this DNA (Huang *et al*, 2004). Our findings may be the result of the high incidence of *H. pylori* infection in Thai population (Vilaichon *et al*, 2004). Similar findings have been reported by Avenaud *et al*, 2000 who showed the presence of *H. pylori* in 100% (7/7) of primary liver cancer. In another study, 75% of liver samples from patients with CCA or HCC were PCR positive for *Helicobacter* spp as determined by using genus-specific primers (Nilsson *et al*, 2001). Fox *et al* (1998) demonstrated that several *Helicobacter* spp could survive in the human biliary tract, some of which are able to produce toxins capable to cause liver cell damage, inflammation and hepatitis.

The presence of *Helicobacter* species in the liver of our patients may have been the consequence of the tumor process or innocent bystander to the development of primary liver cancer. Prospective studies are necessary to assess the potential role of *Helicobacter* species in the development of primary liver cancer in humans.

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