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*Original Article*

# Genetic characterization of *Helicobacter pylori vac*A and *cag*A genes in Thai gastro-duodenal and hepatobiliary patients

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## Abstract

Introduction: *H. pylori* has been detected in patients with hepatobiliary diseases. It is currently unclear whether the *H. pylori* detected in hepatobiliary patients are genetically similar to those in gastro-duodenal patients. The aim of this study was to determine *H. pylori vac*A and *cag*A genotypes in Thai patients with gastro-duodenal and hepatobiliary diseases.

Methodology: *H. pylori* DNA was extracted from samples from gastric biopsies of gastro-duodenal patients (n=100) and from bile samples of hepatobiliary patients (n=80). The *vac*A and *cag*A genotypes were performed via polymerase chain reaction (PCR) followed by DNA sequencing.

Results: The *vac*A m1 was found in Thai hepatobiliary patients (90%) at a higher rate compared with gastro-duodenal patients (50%).The combined *vac*A s1a+c/m1 were mostly found in Thai gastro-duodenal and hepatobiliary patients. The *cag*A gene was detected in 94% of patients with gastro-duodenal diseases compared with 28.8% in those with hepatobiliary diseases ($p<0.05$). On the other hand, the Western type *cag*A was more prominent among hepatobiliary patients (100%) than gastro-duodenal patients (57.4%), and this type was grouped into same cluster with Thai gastro-duodenal patients via phylogenetic analysis.

Conclusions: Based on *vac*A and *cag*A analysis, we conclude that infection with *H. pylori* in gastro-duodenal and hepatobiliary patients may be caused by the different *H. pylori* strains.

**Key words:** *H. pylori*; *vac*A; *cag*A; gastro-duodenal diseases; hepatobiliary diseases.

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## Introduction

*Helicobacter pylori* (*H. pylori*) is a Gram-negative, non-spore-forming and microaerophilic bacteria. This bacterium is the primary agent in gastro-duodenal diseases, including chronic gastritis and peptic ulcers, which lead to gastric adenocarcinoma [1]. *H. pylori* was recently proposed to be involved in the diseases of the extra-gastroduodenal tract, particularly the hepatobiliary tract [2]. Cholangiocarcinoma (CCA) is a primary cancer of the biliary epithelium, and the incidence of this cancer is endemic to Northeast Thailand [3]. A report by Boonyanugomol *et al*. revealed that *H. pylori* was detected in specimens of hepatobiliary patients (especially in CCA patients) and that this bacteria may be a factor in CCA due to its acceleration of biliary inflammation and proliferation [4].

Vacuolating cytotoxin A (*vac*A) and cytotoxin-associated gene A (*cag*A) are the major virulence factors of *H. pylori* contributing to disease severity [5]. VacA is produced in all strains of *H. pylori*, which induces cytoplasmic vacuoles and increases membrane permeability and apoptotic cell death and leads to gastric epithelial cell damage [5]. Variations in *H. pylori vac*A at signal (s) and middle (m) regions have been studied. Allelic variations in the s-region and m-region have been classified into the s1 (s1a, s1b, and s1c) or s2 and m1 or m2 subtypes, respectively [6]. The *cag*A gene is an important virulence factor of *H. pylori* and is located at the end of the *cag* pathogenicity island

(*cag*PAI) [7]. Infections with *cag*A-positive strains are related to gastric ulcers, duodenal ulcers, and gastric cancer. However, the prevalence of *cag*A-positive strains depends on geographical distribution and the associated human population [8]. The 3' of the *cag*A geneis a variable region encoding the EPIYA motifs (Glu-Pro-Ile-Tyr-Ala). Due to differences in these motifs, the *cag*A gene can be classified into Western or East Asian types [9]. Several studies revealed that infections with East Asian *cag*A-positive strains are associated with severe inflammation and gastric cancer development compared with Western *cag*A-positive strains [9-11]. Therefore, infection with different *vac*A and *cag*A genotypes may lead to differing clinical outcomes.

We have previously reported on the prevalence of *H. pylori vac*A and *cag*A genotypes in hepatobiliary patients [12]. However, data on the genetic association between *H. pylori* colonized in hepatobiliary and gastro-duodenal tracts remain lacking because those genes have not been completely compared between the two disease groups. The aims of this study were to characterize the genotypes of the *vac*A and *cag*A genes of *H. pylori* detected in Thai gastro-duodenal patients and to compare these genotypes with Thai hepatobiliary patients based on our previously published data [12]. The genotype patterns of *vac*A and *cag*A in these two groups were analyzed. Elucidating the genetic relationship between *H. pylori* genotypes detected in gastro-duodenal and hepatobiliary patients may contribute to a better understanding of the pathogenesis of *H. pylori* in hepatobiliary diseases.

## Methodology

*Specimens*

Gastric biopsies were performed on dyspeptic patients who underwent gastroendoscopy and examination at the endoscopic unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. Dyspeptic patients were characterized as having non-ulcer diseases (NUD), peptic ulcer diseases (PUD), or gastric cancer (GC). In this study, a total of 100 gastric samples were positive for the rapid urease test (RUT), taken to indicate *H. pylori* infection.

Based on previously published data, bile samples were taken from patients with different hepatobiliary disorders (CCA and cholelithiasis) at the surgical unit of the Srinagarind Hospital, Faculty of Medicine, Khon Kaen University [12]. A total of 80 bile samples were found to be positive for the *H. pylori ure*A gene via polymerase chain reaction (PCR). Patients with history of previous *H. pylori* treatment were excluded from this study.

Approval for this study was obtained from the human ethics committee of Khon Kaen University (HE581271), and informed consent was obtained from all patients.

*DNA extraction*

*H. pylori* genomic DNA was extracted from bile and gastric tissue samples. DNA extractions from bile samples were previously described by Boonyanugomol *et al.* [4]. The DNA samples from biopsies positive for RUT were extracted using a genomic DNA extraction

**Table 1.** Primer sequences and polymerase chain reaction (PCR) conditions for amplification of *H. pylori* virulence-associated genes.

| Genes | Primer sequences | PCR condition | PCR sizes | Ref. |
|---|---|---|---|---|
| *ure*A gene (nested PCR) | OF-GCTAATGGTAAATTAGTTCCTGG<br>ORCTCCTTAATTGTTTTTACATAGTTG<br>IF-AGTTCCTGGTGAGTTGTTCTTAA<br>IR-AACCACGCTCTTTAGCTCTGTC | 94°C 30 sec, 62°C 30 sec, 72°C 30 sec (40 cycles)<br>94°C 30 sec, 59°C 30 sec, 72°C 30 sec (40 cycles) | 411<br>350 | 13 |
| *vac*As1/s2 | F-5′–ATGGAAATACAACAAACACAC–3′<br>R-5′–CTGCTTGAATGCGCCAAAC–3′ | 94°C 1 min, 58°C 1 min, 72°C 1 min (35 cycles) | 259/286 | 14 |
| *vac*As1a | F-5′–GTCAGCATCACACCGCAAC–3′<br>R-5′–CTGCTTGAATGCGCCAAAC–3′ | 94°C 1 min, 60°C 1 min, 72°C; 1 min (35 cycles) | 190 | 14 |
| *vac*As1b | F-5′–AGCGCCATACCGCAAGAG–3′<br>R-5′–CTGCTTGAATGCGCCAAAC–3′ | 94°C 1 min ; 60°C 1 min, 72°C; 1 min (35 cycles) | 187 | 14 |
| *vac*As1c | F-5′–CTCTCGCTTTAGTGGGGYT–3′<br>R-5′–CTGCTTGAATGCGCCAAAC–3′ | 94°C 1 min, 60°C 1 min, 72°C 1 min (35 cycles) | 213 | 15 |
| *vac*Am1/m2 | F- 5′–CAATCTGTCCAATCAAGCGAG–3′<br>R- 5′–GCGTCAAAATAATTCCAAGG–3′ | 94°C 1 min, 55°C 1 min, 72°C 1 min (35 cycles) | 567/642 | 14 |
| *cag*A gene (nested PCR) | OF-5′–AGACAACTTGAGCGAGAAAG–3′<br>OR-5′–TATTGGGATTCTTGGAGGGG–3′<br>OF-5′–AGACAACTTGAGCGAGAAAG–3′<br>IR-5′–GGAGGCGTTGGTGTATTTGA–3′ | 94°C 30 sec, 55°C 30 sec, 72°C 1.5 min (35 cycles)<br>94°C 30 sec, 57°C 30 sec, 72°C 30 sec (35 cycles) | 320<br>307 | 16 |
| *cag*A sequencing | F-5′–GGAACCCTAGTCGGTAATG–3′<br>R-5′–ATCTTTGAGCTTGTCTATCG–3′ | 94°C 30 sec, 57°C 1 min, 72°C 30 sec (35 cycles) | 550-800 | 17 |

and purification kit (Gentra System, Inc., Minneapolis, USA), according to the manufacturer's instructions.

*PCR assay*

PCR amplifications of *H. pylori* virulence genes were based on previously published protocols with slight modifications [13-17]. The PCR primer, annealing condition, and PCR product sizes are shown in Table 1.

The PCR was performed in a total volume of 50 µL containing 500 ng of genomic DNA from the sample, 1X PCR buffer (RBC, Bioscience, Taipei, Taiwan), 200 µM of dNTP (Gibco, Gaithersburg, USA), 0.2 µM of each primer (with the exception of 0.5 µM for *cag*A sequencing), and 1.5 U *Taq* polymerase (RBC, Bioscience, Taipei, Taiwan). PCR amplification was conducted using a GeneAmp PCR system 9600 (Perkin Elmer, Waltham, USA). The amplification was programmed for 35 cycles. Finally, PCR amplicons were examined via agarose gel electrophoresis and visualized under a UV illuminator after staining with ethidium bromide.

*DNA sequence analysis*

To confirm the subtypes of the *cag*A gene, amplicons were purified and sequenced using a DYEnamic ET Dye Terminator Cycle Sequencing Kit from the MegaBACE 1000 DNA Analysis System (GE Healthcare, Life Sciences, Little Chalfont, UK). The *cag*A types were investigated by translating the amino acid sequence usingthe BMC Search Launcher program (Baylor College of Medicine, Houston, Texas, USA).

*Phylogenetic analysis of cagA*

The genetic relationship was confirmed by conducting a phylogenetic analysis of a partial sequence of the Western-type *cag*A detected in patients with gastro-duodenaland hepatobiliary disease and comparing this type with other *cag*A DNA sequences of gastro-duodenal patients from various geographic locations. To clarify the relationship between *cag*A detected in gastro-duodenal and hepatobiliary patients in Thailand, a phylogenetic analysis was performed. The non-Thai *cag*A sequences of gastro-duodenal patients from various regions were also taken from GenBank. The accession numbers used in the tree construction came from the following regions: Japan (AB190943, AB190946, AB190954, AB190956, and AB190949), Australia (AF202973, AF083352, and AF282853), the United States (AB015416, DQ067454, and AB015414), and Europe (AY330639 and AY330644). The partial sequences of *cag*A were aligned using the CLUSTAL W program. The phylogenetic tree was constructed using a maximum parsimony protocol, and reliability was confirmed via reconstruction with 1,000 replicates (MEGA software version 4.0, Center for Evolutionary Medicine and Informatics, Tempe, USA).

*Statistical analysis*

Data were subjected to analysis via Fisher's exact test (two-tailed test). A p value of less than 0.05 was regarded as statistically significant.

## Results

Patients with gastro-duodenal and hepatobiliary diseases were evaluated for possible relationship to age, gender, and clinical manifestations (Table 2). Males and patients 41–60 years of age were found most frequently in the two patient groups.

**Table 2.** Distribution of 180 patients with different clinical characteristics based on age and gender.

| | **Hepatobiliary diseases n = 80** | | | **Gastro-duodenal diseases n = 100** | | | |
|---|---|---|---|---|---|---|---|
| | **CCA** | **Cholelithiasis** | **Total** | **Gastric cancer** | **Non-ulcer disease** | **Peptic ulcer diseases** | **Total** |
| | **n = 58 (%)** | **n = 22 (%)** | **n = 80 (%)** | **n = 12 (%)** | **n = 46 (%)** | **n = 42 (%)** | **n = 100 (%)** |
| **Age (years)** | | | | | | | |
| 15–40 | 0 (0) | 3 (13.7) | 3 (3.8) | 1 (8.0) | 14 (30.4) | 8 (19.0) | 23 (23.0) |
| 41–60 | 39 (67.2) | 7 (31.8) | 46 (57.4) | 4 (34.0) | 20 (43.5) | 18 (42.9) | 42 (42.0) |
| > 60 | 19 (32.8) | 12 (54.5) | 31 (38.8) | 6 (50.0) | 8 (17.4) | 9 (21.4) | 23 (23.0) |
| ND | - | - | - | 1 (8.0) | 4 (8.7) | 7 (16.7) | 12 (11.0) |
| **Gender** | | | | | | | |
| Male | 35 (60.3) | 6 (27.3) | 41 (51.3) | 5 (41.7) | 21 (45.7) | 25 (59.5) | 51 (51.0) |
| Female | 23 (39.7) | 16 (72.7) | 39 (48.7) | 7 (58.3) | 22 (47.8) | 11 (26.2) | 40 (40.0) |
| ND | - | - | - | - | 3 (6.5) | 6 (14.3) | 9 (9.0) |

ND: no determination due to no information.

*Prevalence of H. pylori vacA genotypes in gastro-duodenal and hepatobiliary diseases*

The genotype of the signal region (s) in *vac*A genes was type *vac*A s1 (100%) in both gastro-duodenal and hepatobiliary diseases. A detailed analysis of the *vac*A s1 type showed that the *vac*A s1a+c genotype was more predominant in these two groups. However, the *vac*A s2 and s1b types were absent. The variation in the middle region (m) of the *vac*A m1 and m2 alleles showed a nearly similar proportion of gastro-duodenal diseases (50% and 45%, respectively). In contrast, the *vac*A m1 allele (90%) was found more frequently than the m2 allele (10%) in hepatobiliary diseases. When comparing these two groups, the *vac*A m1 was significantly associated with hepatobiliary diseases, as shown in Table 3 ($p<0.05$).

Additionally, the gene combination of the *vac*A signal and the middle regions is shown in Table 4. The distribution of *vac*A s1a+c/m1 subtype was predominantly found compared to other subtypes in hepatobiliary diseases similar to gastro-duodenal disease.

*Prevalence of H. pylori cagA genotypes in gastro-duodenal and hepatobiliary diseases*

The *cag*A gene was found in 94% of gastro-duodenal patients, but in only 28.8% of hepatobiliary patients. Comparison among the two patient groups revealed that the *cag*A gene was significantly predominant in the gastro-duodenal patients.

According to PCR and DNA sequencing analysis, Western and East Asian *cag*A types were found in patients with gastro-duodenal diseases. The Western type (57.4%) was found significantly more frequently than the East Asian type (33%) ($p<0.05$).

In hepatobiliary patients, all *cag*A-positive samples were of the Western type (100%), especially in CCA patients. Our results revealed that the Western-type *cag*A gene was predominant in patients with hepatobiliary diseases.

*Phylogenetic analysis of H. pylori cagA Western type detected in gastro-duodenal and hepatobiliary diseases*

Due to the predominance of the *cag*A Western type in hepatobiliary diseases, DNA sequencing of the amplified product was performed. The DNA sequences of the *cag*A Western type in hepatobiliary diseases were studied for genetic relationships with random samples of the *cag*A Western type in Thai gastro-duodenal patients (n=20). In this study, the constructed tree showed two general clusters, cluster A (Thai cluster) and cluster B (mixed cluster) (Figure 1). Cluster A was the Thai cluster, which contained *cag*A sequences in Thai hepatobiliary and Thai gastro-duodenal patients. The interesting findings in cluster A included a genetic relationship between *H. pylori* detected in the gastro-

**Table 3.** Virulence-associated gene status of *H. pylori* with differing clinical outcomes in hepatobiliary and gastro-duodenal patients.

| Genes | Hepatobiliary diseases n = 80 | | | Gastro-duodenal diseases n = 100 | | | |
|---|---|---|---|---|---|---|---|
| | CCA | Cholelithiasis | Total | Gastric cancer | Non-ulcer diseases | Peptic ulcer disease | Total |
| | n = 58 (%) | n = 22 (%) | n = 80 (%) | n = 12 (%) | n = 46 (%) | n = 42 (%) | n = 100 (%) |
| ***vac*A s types** | | | | | | | |
| s1 | 58 (100) | 22 (100) | **80 (100)** | 11 (91.7) | 46 (100) | 41 (97.6) | **98 (98.0)** |
| s1a | 15 (25.9) | 7 (31.8) | **22 (27.5)** | 4 (36.4) | 21 (45.7) | 9 (22.0) | **34 (34.7)** |
| s1c | 10 (17.2) | 3 (13.6) | **13 (16.3)** | 4 (36.4) | 6 (13.0) | 9 (22.0) | **19 (19.4)** |
| s1a+c | 33 (56.9) | 12 (54.5) | **45 (56.2)** | 3 (27.2) | 19 (41.3) | 23 (56.0) | **45 (45.9)** |
| s2 | 0 (0) | 0 (0) | **0 (0)** | 0 (0) | 0 (0) | 0 (0) | **0 (0)** |
| ND | - | - | - | 1 (8.3) | - | 1 (2.4) | **2 (2.0)** |
| ***vac*A m types** | | | | | | | |
| m1 | 53 (91.4) | 19 (86.4) | **72 (90) *** | 7 (58.3) | 22 (47.8) | 21 (50.0) | **50 (50.0)** |
| m2 | 5 (8.6) | 3 (13.6) | **8 (10)** | 4 (33.3) | 22 (47.8) | 19 (45.2) | **45 (45.0)** |
| m1+m2 | 0 (0) | 0 (0) | **0 (0)** | 1 (8.4) | 1 (2.2) | 1 (2.4) | **3 (3.0)** |
| m negative | - | - | - | - | 1 (2.2) | 1 (2.4) | **2 (2.0)** |
| *cag*A$^+$ types | 21 (36.2) | 2 (9.1) | **23 (28.8)** | 11 (91.7) | 44 (95.7) | 39 (92.9) | **94 (94.0)**$^{\#}$ |
| Western | 21 (100) | 2 (100) | **23 (100)** | 6 (54.5) | 20 (45.5) | 28 (71.8) | **54 (57.4)** |
| East Asian | 0 (0) | 0 (0) | **0 (0)** | 5 (45.5) | 17 (38.6) | 9 (23.1) | **31 (33.0)** |
| Mixed | 0 (0) | 0 (0) | **0 (0)** | - | 7 (15.9) | 2 (5.1) | **9 (9.6)** |
| *cag*A$^-$ | 37 (63.8) | 20 (90.9) | **57 (71.2)** | 1 (8.3) | 2 (4.3) | 3 (7.1) | **6 (6.0)** |

* There was a statistically significant association between *vac*A m1 and *cag*A Western type in hepatobiliary diseases compared with gastro-duodenal diseases ($p<0.05$); $^{\#}$ There was a statistically significant association between *cag*A$^+$ and gastro-duodenal diseases compared with hepatobiliary diseases ($p<0.05$); ND: no determination due to no information.

**Table 4.** Gene combination of *H. pylori vac*A s- and m-regions in hepatobiliary and gastro-duodenal patients.

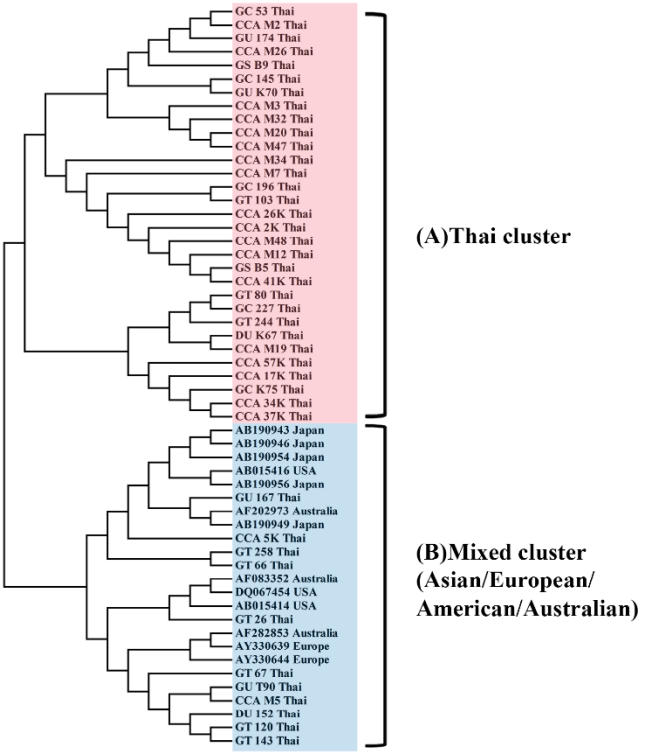
| Combination of *vac*A gene | Hepatobiliary diseases (%) | Gastro-duodenal diseases (%) |
|---|---|---|
| *vac*A s1 a / m1 | 21 (26.3) | 18 (18.0) |
| *vac*A s1 c / m1 | 11 (13.8) | 9 (9.0) |
| *vac*A s1 a+c / m1 | 40 (50.0) | 23 (23.0) |
| *vac*A s1 a / m2 | 1 (1.2) | 13 (13.0) |
| *vac*A s1 c / m2 | 2 (2.5) | 10 (10.0) |
| *vac*A s1 a+c / m2 | 5 (6.2) | 22 (22.0) |
| *vac*A s1 a / m1+ m2 | 0 (0) | 3 (3.0) |
| UD, undetectable | 0 (0) | 2 (2.0) |

duodenal and hepatobiliary tract with a unique genetic presence in the Thai population. However, cluster B was a mixed group that included Asian/European/American/Australian sequences. Findings in cluster B showed that some *cag*A sequences of Thai patients (gastro-duodenal and hepatobiliary diseases) had genetic similarity with *cag*A sequences from Japan and the Western geographic region, which diverged from the Thai cluster.

## Discussion

*Helicobacter pylori* is widely known to be the primary agent of gastro-duodenal diseases, including gastric cancer. The gastro-intestinal tract connects with the hepatobiliary tract via the common bile duct at the duodenal papilla; thus, the bacterium in the stomach could pass to the duodenum through the biliary system. *Helicobacter* species can colonize diverse regions of the digestive system, including the gallbladder, intrahepatic bile duct, and liver [18,19]. This knowledge led to investigations into a potential relationship between *Helicobacter* species and hepatobiliary diseases. The relationship between *H. pylori* and liver diseases has been assessed in humans, and several reports have demonstrated the prevalence of *H. pylori* in hepatobiliary diseases [20-22]. Previous study reported that *H. pylori* or related similar *H. pylori* could be detected in liver tissues from patients with cholestatic liver diseases [21]. In agreement with our previous report, we demonstrated that *H. pylori* was found significantly in patients with CCA compared to a cholelithiasis and control groups [4]. Additionally, our previous study found that the presence of *H. pylori* in CCA patients was associated with biliary cell inflammation and proliferation, as determined via inflammatory grading and Ki67-labelling immunohistochemistry techniques, respectively [4]. Together, our previous results indicated that *H. pylori* was strongly associated with CCA through the acceleration of biliary cell inflammation and the disturbance of biliary cell kinetics. We subsequently reported on the genotypes of *vac*A and *cag*A genes in hepatobiliary patients [12]. However, it remains unclear whether the gastro-duodenal and hepatobiliary patients suffering from *H. pylori* infection had the same strain. In the present study, molecular characterization was carried out to determine the genetic characteristics of *H. pylori* detected in gastro-duodenal diseases and compared with our previous data of hepatobiliary diseases [12].

**Figure 1.** Phylogenetic analysis of partial *cag*A gene (Western type) detected in hepatobiliary and gastro-duodenal (dyspeptic patients) diseases in a Thai population. The *cag*A Western type DNA sequences from different geographic locations were also analyzed: Japan (AB190943, AB190946, AB190954, AB190956, and AB190949), USA (AB015416, DQ067454, and AB015414), Europe (AY330639 and AY330644), Australia (AF202973, AF083352, and AF282853).




CCA: Thai cholangiocarcinoma patients; GS: Thai cholelithiasis patients; GT: Thai gastritis patients; GU: Thai gastric ulcer patients; DU: Thai duodenal ulcer patients; GC: Thai gastric cancer patients.

VacA is the major protein toxin secreted by *H. pylori*, and this toxin is present in all *H. pylori* strains [5]. Multiple effects of VacA on infected cells have been investigated, including the induction of cell vacuolation, inflammatory responses, apoptosis, and cell damage [8]. In the present study, we detected the *vac*A s1 type in both gastro-duodenal and hepatobiliary patients; however, *vac*A s2 was not found. Within the *vac*A s1 subtype, the *vac*A s1a+c subtype appeared to be present at a high proportion in hepatobiliary and gastro-duodenal patients (although with no significant differences in gastro-duodenal patients). Previous studies showed that *vac*A s1a and s1c, but not s1b and s2 strains, were found in a Southeast Asian population [23]. Conversely, *vac*A s1b and s2 appeared in East Asian and African Arab populations [15,24,25].Our findings are in agreement with a previous report that showed that mixed *vac*A s1a+c was more frequently found in patients with various gastric diseases in North Thailand [26].We assumed that the prevalence of *vac*A subtypes varies among different ethnic and geographic populations. The presence of mixed *H. pylori vac*A s1a and s1c (*vac*A s1a+c) may result from genetic recombination, leading to mutation inside a strain [27]. We suggested that mixed subtypes of *vac*A (*vac*A s1a+c) increase the chance of infection with more *H. pylori* pathogenic strains [26].

The *vac*A m-region is involved in cell binding, and *vac*A m1 and m2 genotypes ultimately affect the specificity to various cellular receptors [28]. The glycoprotein receptor protein tyrosine phosphatase (RPTPs) is a receptor for the VacA toxin [29]. Our findings showed that *H. pylori vac*A m1 strains were more frequent in hepatobiliary diseases ($p<0.05$), whereas *vac*A m1 and m2 strains were more frequent in gastro-duodenal patients in the same proportion. The difference in *vac*A m genotypes in hepatobiliary and gastro-duodenal patients may depend on different organs, specific binding to individual cell types, and differing post-translational modifications of RPTPs in each cell type [29]. However, gene combination of *vac*A s- and m-region revealed that *vac*A s1a+c/m1 was mostly found in both gastro-duodenal and hepatobiliary diseases. The activity of *vac*A s1/m1 causes more severe inflammation and gastric cell damage than the other subtypes, and an especially low and absent activity has been observed in the s2/m2 subtype [6]. We concluded that *vac*As1/m1 might be involved with severity of hepatobiliary diseases, especially in CCA.

*H. pylori cag*A plays an important role in gastric mucosal inflammation. The tyrosine phosphorylation of CagA is widely known to occur at the EPIYA motif [7]. The genetic variation of the EPIYA motif has been investigated, and four distinct segments were determined: EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D. The East Asian and Western CagA are characterized by the presence of EPIYA-ABD and EPIYA-ABC, respectively. The co-culture of AGS cells with East Asian and Western CagA strains showed that East Asian CagA strains induced significantly more IL-8 production than Western CagA strains [9]. However, *H. pylori* expressing the CagA Western type were also related to the severity of inflammation and gastric cancer development [9]. Most *H. pylori* samples in gastro-duodenal patients of our study carried the *cag*A gene (94%), whereas the *cag*A gene was found in 28.8% of hepatobiliary patients ($p< 0.05$). The difference in *cag*A prevalence between the two patient groups may be due to other virulence-associated genes that are associated with the presence of the *cag*A gene. One suggestion by a previous report stated that bacterial binding to the fucosylated blood group antigen Lewis b ($Le^b$) is coded by the *bab*$A_2$ gene and is associated with the presence of the *cag*A gene [30]. Thus, differences in the prevalence of *cag*A between the two patient groups may depend on other factors such as the level of $Le^b$ expression in biliary and gastric cells. In addition to the *cag*A gene, another *H. pylori* virulence factor may play a role in hepatobiliary diseases and should be further studied, such as our previous report investigating the effects of *H. pylori* γ-glutamyltranspeptidase (GGT) in biliary cells [31].

Thailand is located in Southeast Asia, an area that is at the cultural crossroads of East and South Asia and is host to the migration of several geographic populations. The distribution of East Asian CagA strains is predominant in East Asian countries including Japan, China, and Korea. In contrast, Western CagA strains are widely distributed among European, South and Central Asian, North and South American, and African populations [32]. In the present study, the Western CagA type was more common than the East Asian CagA type in Thai gastro-duodenal patients. We showed that the Western CagA type was predominant in hepatobiliary diseases and gastro-duodenal diseases. One possible explanation of the Western *cag*A type predominant in the Thai population might be dependent on geography and ethnic groups. We found that gastro-duodenal patients in the Isan ethnic group (who are considered to live in Northeast Thailand) were significantly associated with the Western CagA type (data not shown), which was consistent with a previous epidemiological report that revealed that the Isan ethnic group in Northeast Thailand had a high percentage of

CCA [33]. Additionally, relationships between host and pathogen genetic variations and other environmental factors play important roles in the variability of infection outcomes [34]. Therefore, the host genetics of local ethnic populations in Northeast Thailand may also select for *H. pylori* Western *cag*A strain infection.

To clarify the genetic relationships between the *H. pylori* in the two patient groups, Western *cag*A sequences in hepatobiliary diseases were analyzed and compared with randomized Western *cag*A sequences in gastro-duodenal diseases (via phylogenetic analysis). Our constructed phylogenetic tree included two groups. Most of the Western *cag*A sequences (74%) in Thai gastro-duodenal and Thai hepatobiliary patients were grouped in same cluster, designated the Thai cluster, which diverged from the mixed cluster, which contained Asian/European/American/Australian sequences. However, 26% of the Western *cag*A sequences of Thai gastro-duodenal patients and Thai hepatobiliary patients were classified as being in the mixed cluster. Several geographic populations live together in Southeast Asia; thus, migration from various regions may be linked to diverse strains of *H. pylori*, as previously described by Breurec*et al*. in 2011 [35]. These two patient groups were classified into the same group (the Thai population cluster) because they both exhibited the similarity of Western-type *cag*A sequences. However, we suggest that other *H. pylori* genes should be used in classifying these two patient groups by phylogenetic analysis.

The high percentage of CCA in Northeast Thailand has been clearly demonstrated to be associated with liver fluke (*Opisthorchis viverrini*) infection. However, other factors in bile duct carcinogenesis should not be neglected, such as bacterial infection. Our previously published data showed that *cag*PAI especially *cag*A of *H. pylori* can induce inflammation and disturbance of cell kinetic on hepatobiliary cell lines [31,36,37]. Therefore, we suggest that, in addition to *O. viverrini* infection, *H. pylori* may be a co-factor of the *O. viverrini* pathogenesis in Thai CCA patients as previously described by our colleagues, showing the significant relationship between *O. viverrini* and *H. pylori* [38]. However, the association and role of *H. pylori* in CCA should be further investigated.

Our findings showed that the *cag*A gene was significantly more predominant in gastro-duodenal patients (94%) than in hepatobiliary patients (28.8%) ($p<0.05$). Moreover, high prevalence of *vac*A m1 (90%) and Western-type *cag*A (100%) were detected in hepatobiliary patients, and low prevalence was detectedin gastro-duodenal patients. Therefore, these findings indicate that *H. pylori* infection in gastro-duodenal and hepatobiliary patients may be caused by the different *H. pylori* strains.

## Conclusions

Our study is the first to show the genetic characterization between *H. pylori* in diseases of the gastro-duodenal and hepatobiliary tracts. Infection with *H. pylori* in gastro-duodenal patients and hepatobiliary patients may be caused by different *H. pylori* strains. However, the pathogenesis of this bacteria in the hepatobiliary tract should be further elucidated.

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