HELICOBACTER PYLORI cagA, vacA and iceA Genotypes in Northern Thai Patients with Gastric Disease

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Abstract. Helicobacter pylori, a common infectious bacterium, has been linked to chronic gastritis, peptic ulcer and gastric cancer. Gastric biopsy specimens were obtained from 58 northern Thai patients with gastritis, 28 with gastric ulcer, 45 with duodenal ulcer and 4 with gastric cancer. cagA, vacA s1 and iceA gene was found in 88, 98, and 89% of the specimens, respectively. For vacA, the frequency of subtype s1a, s1c and combined s1a and s1c was 40, 16, and 41%, respectively. The frequency of subtype s1a/m1 and s1a/s1c/m1 was 27 and 20%, respectively. Fifty-three patients (39%) were infected with multiple vacA genotypes but there was no association with clinical outcome. cagA positive and mixed vacA s1a and s1c strains were found in significantly more cases of duodenal ulcer than gastritis (p<0.05). For iceA, subtype iceA1 reached a frequency of 60%, whereas subtype iceA2 was only 24%.

INTRODUCTION

Helicobacter pylori (H. pylori) has been recognized as the major risk factor for the development of gastric and duodenal ulcerations or gastric cancer (Marshall and Warren, 1984; Goodwin et al, 1997). Epidemiological studies demonstrated that about half of the world’s population are infected with this bacterium, ranging from 25% in developed to more than 80% in the less developed countries. However, only 20% of the infected patients develop serious disease and this might be related either to differences among the host or to differences in virulence of H. pylori strains (Taylor and Blaser, 1991).

Several H. pylori genes related to the risk of disease have been identified, such as cytotoxin associated geneA (cagA) that encodes a protein that enhances the virulence of the bacterium by increasing cytokine production of the host cell. The presence of cagA is associated with a more severe clinical outcome of gastro- duodenal diseases (Tummuru et al, 1995; Censini et al, 1996; Akopyants et al, 1998). Another virulence gene is the vacuolating cytotoxin gene (vacA) that induces vacuolation in epithelial cells leading to cell damage. Recently, Peek et al, (1998) identified the iceA gene, which exists as 2 subtypes, iceA1 and iceA2. The function of iceA1 is similar to that of type II restriction endonuclease.

Many studies have demonstrated the distribution and association between H. pylori virulence genes and the severity of gastro-duodenal diseases. However the results are inconsistent among different geographic re-
cagA, vacA and iceA H. pylori Genotypes in Thai Patients

In Thailand, H. pylori is found in 48.2% of dyspeptic patients (Atisook et al., 2003), but data on H. pylori virulence-related genes are scarce. Therefore, the purpose of this study was to determine the genotypes of H. pylori cagA, vacA and iceA and their relationship to clinical outcome in northern Thai patients with gastro-duodenal diseases.

MATERIALS AND METHODS

Gastric biopsy specimens

Gastric biopsy specimens were obtained from 2 sources: 91 tissue materials from patients with positive CLO test who underwent endoscopic examinations at the Gastroenterology Unit, Maharaj Nakhon Chiang Mai Hospital, Chiang Mai, Thailand and 94 specimens that were H. pylori positive by histology of paraffin-embedded tissues, obtained from the Department of Pathology, Faculty of Medicine, Chiang Mai University.

DNA extraction

After the CLO test were interpreted within 24 hours, the CLO positive samples were stored at 4ºC until DNA was extracted. Gastric biopsy samples were transferred to microcentrifuge tubes and washed twice with distilled water. DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions and was frozen at -20ºC until used in PCR.

Three to five 10-µm thick paraffin-embedded gastric tissue slices were deparaffinized in xylene by incubating at 55ºC for 15 minutes. The tissue samples were then washed with xylene : ethanol (1:1) and dried at room temperature. DNA was extracted as described above.

Detection of H. pylori and genotyping of cagA, vacA and iceA genes by PCR

H. pylori was detected by PCR using primers specific to a 860 bp DNA fragment (Linpisarn et al., 2005). PCR was performed in a volume of 10 µl containing 1 µl of DNA extract, 20mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% bovine serum albumin and 0.05% Tween, 0.2 mM of each of the four deoxynucleoside triphosphates (New England Biolabs, USA), 0.25 U of Taq DNA polymerase (QIAGEN, Germany) and 0.25 µM of genotype-specific primers. The sequences of the primers are listed in Table 1. Amplifications for cagA were conducted as follows: 2.30 minutes denaturation at 95ºC, 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 1 minute, extension at 72ºC for 1 minute, and one final extension at 72ºC for 7 minutes. For vacA and iceA the conditions were similar to those for cagA but annealing was done at 52ºC for 1 minute. PCR products were checked on 2.0% agarose gels with 100 bp ladder size markers.

H. pylori strain J 99 (ATCC 700824) was used as positive control of cagA and vacA s1/m1 and s1b genes, and sterile distilled water was used as negative control.

Data analysis

A chi-square or Fisher's exact test was used to test whether differences between values were significant. A value of p<0.05 is considered to be significant.

RESULTS

Among the 135 PCR positive biopsy specimens, 85 were from positive CLO test and 50 from paraffin-embedded tissues identified H pylori by histological method. These samples were from 58 patients with gastritis, 28 patients with peptic ulcers, 45 patients with duodenal ulcer and 4 patients with gastric cancers.

cagA, vacA and iceA genotypes of H. pylori were analyzed by PCR and the genotyping results in association to clinical outcome are summarized in Table 2. cagA positive strains were detected in 119 of 135
Table 1

<table>
<thead>
<tr>
<th>Target gene</th>
<th>PCR product (bp)</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA</td>
<td>349</td>
<td>cagA-F: GAT AAC AGG CAA GCT TTT GAG G Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cagA-R: CTG CAA AAG ATT GTT TGG CAG A Rudi et al, 1998</td>
<td></td>
</tr>
<tr>
<td>vacA s1a</td>
<td>201</td>
<td>vacA-F: GAA ATA CAA CAA ACA CAC CGC Rudi et al, 1998</td>
<td></td>
</tr>
<tr>
<td>vacA s2b</td>
<td>228</td>
<td>vacA-R: GGC TTG TTT GAG CCC CCA G Atherton et al, 1995</td>
<td></td>
</tr>
<tr>
<td>vacA s1a</td>
<td>259</td>
<td>vacAs1-F: ATG GAA ATA CAA CAA ACA CAC Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacA s2b</td>
<td>286</td>
<td>vacAs1-R: GTG CTA GAA TGC GCC AAA C Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacA s1a</td>
<td>190</td>
<td>vacAs1a-F: ATG GAA ATA CAA CAA ACA CAC Atherton et al, 1995</td>
<td></td>
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<tr>
<td>vacA s2b</td>
<td></td>
<td>vacAs1a-R: GTG CTA GAA TGC GCC AAA C Atherton et al, 1995</td>
<td></td>
</tr>
<tr>
<td>vacA s1c</td>
<td>213</td>
<td>vacAs1c-F: CTY GCT TTA GTR GGG YTA Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacA s2</td>
<td>199</td>
<td>vacAs1c-R: CTY GCT TTA GTR GGG YTA Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacA m1</td>
<td>290</td>
<td>vacAm1-F: GGT CAA AAT GCG GTC ATG G Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacA m2</td>
<td>352</td>
<td>vacAm1-R: CCA TTG GTA CCT GTA GAA AC Atherton et al, 1995</td>
<td></td>
</tr>
<tr>
<td>vacA m2</td>
<td>352</td>
<td>vacAm2-F: GGT CAA AAT GCG GTC ATG G Atherton et al, 1995</td>
<td></td>
</tr>
<tr>
<td>vacAm</td>
<td>567</td>
<td>vacAm-R: CCA TCT GTG CAA CAA TCA AGC GAG Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>vacAm2</td>
<td>642</td>
<td>vacAm-R: CCA TCT GTG CAA CAA TCA AGC GAG Yamaoka et al, 1999</td>
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</tr>
<tr>
<td>iceA1</td>
<td>247</td>
<td>iceA1-F: GTG TTT TTA ACC AAA GTA TC Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>iceA1</td>
<td></td>
<td>iceA1-R: CTA TAG CCA STY TCT TTT CA S = C or G, Y = C or T Yamaoka et al, 1999</td>
<td></td>
</tr>
<tr>
<td>iceA2</td>
<td>229</td>
<td>iceA2-R: GTG TTT TTA ACC AAA GTA TC Yamaoka et al, 1999</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>iceA2-F: TTR CCC TAT TTT TCA GTA GGT R = A or G Yamaoka et al, 1999</td>
<td></td>
</tr>
</tbody>
</table>

Note: a Nucleotide positions in the vacA gene of H. pylori 60190 (GenBank accession no. U05676).
b Nucleotide positions in the vacA gene of H. pylori Tx30a (GenBank accession no. U29401).

cases (88%). vacA s1 genotype was confirmed using two primer sets generating PCR products of 201 bp and 259 bp and the prevalence of these gene fragments was 98% and 89%, respectively.

Among the vacA type s1 allele, 56 specimens (41%) were found to have multiple subtype of s1a and s1c (p<0.0001 when compared with s1c) and 50 (40%) with s1a (p<0.0001 when compared with s1c). Neither s2 nor s1b allele was detected. The vacA m-region genotype m1 (54%) was detected in significantly more cases than type m2 (31%) vacA allele (p< 0.005). Both m1 and m2 were not associated with clinical outcome. The most prevalent of vacA subtypes was s1a/m1 (27%) followed by s1a/s1c/m1 (20%). Multiple vacA genotypes were found in 56 cases (39%) and
there was no association between multiple-strain infection and disease outcome (Table 3).

In the iceA subtypes, iceA1 was the most common (60%), followed by iceA2 (24%, p<0.0001). cagA positive and mixed vacA s1a and s1c strains were found in significantly more cases of duodenal ulcer than gastritis (p<0.05).

**DISCUSSION**

According to a recent nationwide survey in Thailand, H. pylori infection was high among dyspeptic patients (Atisook et al, 2003). Genotyping of reported virulence genes, such as cagA, vacA and iceA gene, should be useful for determining gastro-duodenal disease specificity. Although the aim of our study was to identify H. pylori virulence genes from paraffin-embedded tissue by PCR, however, only 53% of the extracted DNA successfully yielded PCR products of those virulence genes, which was lower than those from other studies (Köehler et al, 2002). Identification of cagA, vacA and iceA genotypes was highly successful by examining directly from gastric biopsy specimens obtained from the CLO test®. This method is simple, consumes less time and has been shown to be reliable when compared to the method using DNA from culture (Björkholm et al, 1998; van Doorn et al, 1998; Rudi et al, 1999; Wong et al, 2001).

Most of the H. pylori samples in our study carried the toxigenic vacA s1 alleles and cagA positive strains. Furthermore, the presence of cagA was associated with duodenal ulceration (p<0.05), similar to data from Brazil (Ashour et al, 2002). Several studies of H. pylori vacA subtypes showed that subtypes s1a and s1c were common in Asia but the prevalence of m1 and m2 subtypes were inconsistently found (Kim et al, 2001; Wong et al, 2001; Wang et al, 2003; Xue-jun et al, 2005). In ad-

**Table 2**

Distribution of cagA, vacA and iceA genotype of H. pylori in gastritis, peptic ulcer, duodenal ulcer and gastric cancer patients.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Gastritis (n = 58)</th>
<th>Peptic ulcer (n = 28)</th>
<th>Duodenal ulcer (n = 45)</th>
<th>Gastric cancer (n = 4)</th>
<th>Total (n = 135)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA* vacA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s1(201bp)</td>
<td>57 98</td>
<td>28 100</td>
<td>43 96</td>
<td>4 100</td>
<td>132 98</td>
</tr>
<tr>
<td>s1 (259bp)</td>
<td>49 84</td>
<td>26 93</td>
<td>41 91</td>
<td>4 100</td>
<td>120 89</td>
</tr>
<tr>
<td>s1a</td>
<td>28 48</td>
<td>9 32</td>
<td>15 33</td>
<td>20 50</td>
<td>54 40</td>
</tr>
<tr>
<td>s1c</td>
<td>10 17</td>
<td>3 11</td>
<td>7 16</td>
<td>1 25</td>
<td>21 16</td>
</tr>
<tr>
<td>s1a+s1c*</td>
<td>19 33</td>
<td>14 50</td>
<td>23 51</td>
<td>0 0</td>
<td>56 41</td>
</tr>
<tr>
<td>Not s1a and s1c</td>
<td>1 2</td>
<td>2 7</td>
<td>0 0</td>
<td>1 25</td>
<td>4 3</td>
</tr>
<tr>
<td>m1</td>
<td>29 50</td>
<td>14 50</td>
<td>27 60</td>
<td>3 75</td>
<td>73 54</td>
</tr>
<tr>
<td>m2</td>
<td>19 33</td>
<td>9 32</td>
<td>14 31</td>
<td>0 0</td>
<td>42 31</td>
</tr>
<tr>
<td>m1+m2</td>
<td>1 20</td>
<td>3 11</td>
<td>3 7</td>
<td>0 0</td>
<td>7 5</td>
</tr>
<tr>
<td>Not m1 and m2</td>
<td>9 15</td>
<td>2 7</td>
<td>1 2</td>
<td>1 25</td>
<td>13 10</td>
</tr>
<tr>
<td>iceA1</td>
<td>30 52</td>
<td>19 68</td>
<td>30 67</td>
<td>2 50</td>
<td>81 60</td>
</tr>
<tr>
<td>iceA2</td>
<td>17 29</td>
<td>6 21</td>
<td>9 20</td>
<td>1 25</td>
<td>33 24</td>
</tr>
<tr>
<td>iceA1+iceA2</td>
<td>3 5</td>
<td>0 0</td>
<td>3 7</td>
<td>0 0</td>
<td>6 4</td>
</tr>
<tr>
<td>No iceA1 and iceA2</td>
<td>8 14</td>
<td>3 11</td>
<td>3 7</td>
<td>1 25</td>
<td>15 11</td>
</tr>
</tbody>
</table>

*Compared between gastritis and duodenal ulcer group (p < 0.05).
dition, the presence of multiple vacA genotypes was low (Yamaoka et al, 1999; Kim et al, 2001; Wong et al, 2001; Wang et al, 2003; Xue-jun et al, 2005). In this study, the highest prevalence vacA subtype was mixed s1a and s1c genotype, which was significantly higher in duodenal ulcer than in gastritis group (p<0.05). One reason for this is that multiple strain infection increases the chance of infection with more pathogenic strains (Gonyalez-Valencia et al, 2000). For m-regions of H. pylori, m1 was significantly higher than m2 and this finding is consistent with reports from Korea, Malaysia and Srilanka (Kim et al, 2001; Fernando et al, 2002; Tan et al, 2005). s1a/m1 was the dominant vacA gene subtype, similar to that reported in Japan (Ito et al, 1997).

Multiple subtypes of vacA were more common in this study than reported in developed countries (Hirschl et al, 1994; van Doorn et al, 1998). However, the prevalence of multiple vacA gene was lower than those reported from a Chinese population (Gong et al, 2005). H. pylori infection with multiple genotypes was high in our studied population. These results may be related to the high prevalence of H. pylori and to a higher rate of childhood infection in Thailand (Perez-Perez et al, 1990; Boonyaritichaikij et al, 2001; Atisook et al, 2003).

In summary, the prevalence of cagA or mixed infection of vacA s1a and s1c genotypes were more prevalent in duodenal ulcer patients than in gastritis group. Multiple vacA genotypes were common in subjects in north Thailand.

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