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 *iceA*1 reached a frequency of 60%, whereas subtype *iceA*2 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).

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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, *iceA*1 and *iceA*2. The function of *iceA*1 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 gastroduodenal diseases. However the results are inconsistent among different geographic re-

gions (van Doorn *et al*, 1999; Yamaoka *et al,* 1999; Azuma *et al*, 2004; Xue-jun *et al*, 2005). 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 $MgCl_2$, 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* was 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
Primer specific to *cagA*, *vacA* and *iceA* subtypes of *H. pylori.*

Target gene	PCR product (bp)		Primer sequence	Reference
cagA	349	cagA-F	GAT AAC AGG CAA GCT TTT GAG G	Yamaoka *et al*, 1999
		cagA-R	CTG CAA AAG ATT GTT TGG CAG A	
vacA s1[a]	201	vacA-F	GAA ATA CAA CAA ACA CAC CGC	Rudi *et al*, 1998
s2[b]	228	vacA-R	GGC TTG TTT GAG CCC CCA G	
vacA s1[a]	259	vacAs1-F	ATG GAA ATA CAA CAA ACA CAC	Yamaoka *et al*, 1999
s2[b]	286	vacAs1-R	CTG CTT GAA TGC GCC AAA C	
vacA s1a	190	vacAs1a-F	GTC AGC ATC ACA CCG CAA C	Atherton *et al*, 1995
		vacAs1a-R	CTG CTT GAA TGC GCC AAA C	
vacA s1b	187	vacAs1b-F	AGC GCC ATA CCG CAA GAG	Yamaoka *et al*, 1999
		vacAs1b-R	CTG CTT GAA TGC GCC AAA C	
vacA s1c	213	vacAs1c-F	CTY GCT TTA GTR GGG YTA Y = C or T, R = A or G	Yamaoka *et al*, 1999
		vacAs1c-R	CTG CTT GAA TGC GCC AAA C	
vacA s2	199	vacAs2-Fa	GCT AAC ACG CCA AAT GAT CC	Sillakivi *et al*, 2001
		vacAs2-R	CTG CTT GAA TGC GCC AAA C	
vacA m1	290	vacAm1-F	GGT CAA AAT GCG GTC ATG G	Atherton *et al*, 1995
		vacAm1-R	CCA TTG GTA CCT GTA GAA AC	
vacA m2	352	vacAm2-F	GGA GCC CCA GGA AAC ATT G	Atherton *et al*, 1995
		vacAm2-R	CAT AAC TAG CGC CTT GCA C	
*vacA*m	vacAm1	vacAm-F	CAA TCT GTC CAA TCA AGC GAG	Yamaoka *et al*, 1999
	567	vacAm-R	GCG TCA AAA TAA TTC CAA GG	
	vacAm2			
	642	vacAm-F	CAA TCT GTC CAA TCA AGC GAG	
		vacAm-R	GCG TCA AAA TAA TTC CAA GG	
*iceA*1	247	iceA1-F	GTG TTT TTA ACC AAA GTA TC	Yamaoka *et al*, 1999
		iceA1-R	CTA TAG CCA STY TCT TTG CA S = C or G, Y = C or T	
*iceA*2	229	iceA2-F	GTT GGG TAT ATC ACA ATT TAT	Yamaoka *et al*, 1999
		iceA2-F	TTR CCC TAT TTT CTA GTA GGT R = A or G	

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 prevalen of *vacA* subtypes was s1a/m1 (27%) followed by s1a/s1c/m1 (20%). Multiple *vacA* genotypes were found in 56 cases (39%) and

Table 2
Distribution of *cagA*, *vacA* and *iceA* genotype of *H. pylori* in gastritis, peptic ulcer, duodenal ulcer and gastric cancer patients.

Target gene	Gastritis		Peptic ulcer		Duodenal ulcer		Gastric cancer		Total	
	n = 58	%	n = 28	%	n = 45	%	n = 4	%	n = 135	%
*cagA**	48	83	25	89	43	96	3	75	119	88
vacA										
s1(201bp)	57	98	28	100	43	96	4	100	132	98
s1 (259bp)	49	84	26	93	41	91	4	100	120	89
s1a	28	48	9	32	15	33	2	50	54	40
s1c	10	17	3	11	7	16	1	25	21	16
s1a+s1c*	19	33	14	50	23	51	0	0	56	41
Not s1a and s1c	1	2	2	7	0	0	1	25	4	3
m1	29	50	14	50	27	60	3	75	73	54
m2	19	33	9	32	14	31	0	0	42	31
m1+m2	1	2	3	11	3	7	0	0	7	5
Not m1 and m2	9	15	2	7	1	2	1	25	13	10
*iceA*1	30	52	19	68	30	67	2	50	81	60
*iceA*2	17	29	6	21	9	20	1	25	33	24
*iceA*1+*iceA*2	3	5	0	0	3	7	0	0	6	4
No *iceA*1 and *iceA*2	8	14	3	11	3	7	1	25	15	11

*Compared between gastritis and duodenal ulcer group ($p < 0.05$).

there was no association between multiple-strain infection and disease outcome (Table 3).

In the *iceA* subtypes, *iceA*1 was the most common (60%), followed by *iceA*2 (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 realiable 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 3
vacA subtypes of s-and m-region and multiple subtypes from single biopsy specimens.

vacA genotype	Prevalence (%)									
	Gastritis		Peptic ulcer		Duodenal ulcer		Gastric cancer		Total	
	n = 58	%	n = 28	%	n = 45	%	n = 4	%	n = 135	%
s1a/m1	17	29	6	21	12	27	2	50	37	27
s1a/m2	8	14	3	11	3	7	0	0	14	10
s1c/m1	4	7	1	4	3	7	1	25	9	7
s1c/m2	4	7	1	4	3	7	0	0	8	6
s1c/m1m2	0	0	1	4	1	2	0	0	2	1
s1a/s1c/m1	8	14	7	25	12	27	0	0	27	20
s1a/s1c/m2	7	12	4	14	8	18	0	0	19	14
s1a/s1c/m1m2	1	2	2	7	2	4	0	0	5	4

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). *iceA*1 gene was the predominant subtype and showed no difference in patients with ulcer and non ulceration, a finding consistent with reports from East Asia (Yamaoka *et al*,1999; Kim *et al,* 1999; Wong *et al,* 2001). In the United State *iceA*2 is predominant (Yamaoka *et al,* 1999). Mixed infection of *iceA*1 and *iceA*2 was much lower than in other Asian countries such as China, Korea and Japan (Yamaoka *et al*, 1999; Li *et al*, 2002; 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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