PREVALENCE OF *CAG*A EPIYA MOTIFS IN *HELICOBACTER PYLORI* AMONG DYSPEPTIC PATIENTS IN NORTHEAST THAILAND

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Abstract. The aims of this study were to determine the prevalence of *cag*A type in *Helicobacter pylori* isolated from dyspeptic patients in northeastern Thailand and to determine whether the pattern of *cag*A EPIYA motifs were associated with clinical outcomes. One hundred and forty-seven *H. pylori*-infected dyspeptic patients were enrolled, of whom 68 had non-ulcer dyspepsia (NUD), 57 peptic ulcer disease (PUD), 18 gastric cancer (GCA), and 4 other gastroduodenal diseases. PCR and DNA sequence analysis were used to determine the *cag*A genotype and the pattern of EPIYA motifs. *cagA*-positive *H. pylori* were identified in 138 (94%) of *H. pylori*-infected dyspeptic patients of whom 75 (54%) were of the Western-type, 44 (32%) the East Asian type and 19 (14%) of the other types. The Western type is significantly found in PUD patients ($p = 0.0175$). The majority of *cag*A EPIYA was EPIYA-ABC (43%) and EPIYA-ABD (28%). There is no significant correlation between the increase in number of EPIYA-C motifs and clinical outcomes. Thus, the most frequent *cag*A type found among northeastern Thai dyspeptic patients was the Western *cag*A type, which is significantly associated with PUD indicating a possible predictive parameter for clinical outcome.

Keywords: *Helicobacter pylori*, *cag*A genotype, dyspeptic patients

INTRODUCTION

Helicobacter pylori infection is associated with the development of gastroduodenal diseases, such as chronic gastritis, peptic ulcer and gastric cancer (Atherton, 2006). The cytotoxin-associated gene A (*cag*A) is one of the virulence genes related to pathogenicity (Atherton, 2000). Epidemiological studies have suggested that the presence of *cag*A is associated with the development of peptic ulcer and gastric cancer (Blaser *et al*, 1995; Nomura *et al*, 2002; Salih *et al*, 2010), increased mucosal inflammation (Peek *et al*, 1995) and cellular proliferation (Peek *et al*, 1997). *H. pylori cag*A-positive strains were found in 50-70% of *H. pylori* isolated from Western populations (Rudi *et al*, 1998; Podzorski *et al*, 2003; Ribeiro *et al*, 2003; Erzin *et al*, 2006), whereas 80-100% of strains found in the Asian populations were *cag*A-positive strains (Maeda *et al*, 1998; Kim *et al*, 2001).

*Cag*A is located on the *cag* pathogenicity island (*cag*PAI) (Censini *et al*, 1996), which encodes a bacterial type IV secre-

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tion system (Tummuru *et al*, 1993; Naito *et al*, 2006). Phosphorylation of CagA occurs at the tyrosine phosphorylation motifs (TPMs), comprising of repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence in the C-terminal variable region, catalyzed by members of the cellular Src kinase family (Segal *et al*, 1999; Asahi *et al*, 2000; Higashi *et al*, 2002; Stein *et al*, 2002; Sicinschi *et al*, 2009).

CagA protein can be classified into two major types, Western and East Asian type, on the basis of the amino acid sequence surrounding the EPIYA motif. EPIYA-A and EPIYA-B are found in both the Western and East Asian CagA type protein, whereas EPIYA-C is specifically found in the Western type and EPIYA-D in the East Asian type (Azuma, 2004).

Detection of *cag*A alone appears to be less important as a virulence marker than determining the type and pattern of EPIYA motif (Argent *et al*, 2004, 2005). The differences in *cag*A genotypes and CagA forms were reportedly associated with the severity of gastroduodenal diseases (Zhou *et al*, 2004; Basso *et al*, 2008; Li *et al*, 2009). The East Asian type of CagA is more virulent than the Western type and is highly related to the risk of gastric cancer (GCA) (Satomi *et al*, 2006; Jang *et al*, 2009). However, a number of research groups showed that the severity of disease not only depended on East Asian CagA type or the type and pattern of EPIYA, but also on the numbers of EPIYA motifs/segments, especially EPIYA-C that increases the risk of developing gastric cancer (Basso *et al*, 2008; Xia *et al*, 2009), although other researchers demonstrated no association between them (Occhialini *et al*, 2001).

The relationship among *cag*A genotypes, form of CagA, geographic distribution of *cag*A, and clinical outcome is still controversial. Thus, we evaluated these parameters for any epidemiological trends and relationship to disease in northeastern Thailand.

MATERIALS AND METHODS

Patients and gastric biopsy specimens

The subjects enrolled in this study comprised of 308 dyspeptic patients who underwent routine endoscopy at the Endoscopy Unit of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University in Khon Kaen, northeastern Thailand. One hundred and forty-seven patients (48%) were *H. pylori* positive; 89 determined using rapid urease test (RUT) (Pronto Dry Medical Instruments Corporation, Solothurn, Switzerland) and another 58 by isolation of *H. pylori* from biopsy specimen.

The 147 *H. pylori*-positive dyspeptic patients included 68 patients with non-ulcer dyspepsia (NUD) or gastritis (GT), 57 with peptic ulcer disease (PUD), 18 with gastric cancer (GCA) and 4 with other gastroduodenal diseases [including 1 with gastroesophageal reflux disease (GERD), 1 malignant melanoma and 2 duodenitis]. Among the 147 *H. pylori*-positive dyspeptic patients, 77 (52%) were males and 70 (48%) were females. The mean age was 50 years (range, 15-81). These patients included 121 Thais (82%), 6 Chinese (4%), 17 Thai-Chinese (12%), 2 Laotians (1%), and 1 Cambodian (0.5%). Patients taking antibiotics or omeprazole within the two weeks prior to sampling were excluded.

The study was approved by Institutional Human Ethics Committee (HE 521214) of Khon Kaen University and performed in accordance with the Helsinki Declaration. Informed consent was obtained from each patient prior to enrolling in the study.

Culture and identification of *H. pylori*

H. pylori culture was performed as described previously (Chomvarin *et al*, 2008). In brief, each antral and corpus biopsy specimen was separately homogenized in 200 ml of normal saline and cultured on 7% human blood agar (Difco, Detroit, MI), that contained 10 mg/l vancomycin, 5 mg/l trimethoprim, 5 mg/l amphotericin B and 5 mg/l cefsulodin. Plates were incubated at 37°C under microaerophilic condition for 7 days. *H. pylori* colonies were confirmed by Gram's staining, catalase, oxidase and urease tests. *H. pylori* colonies were used for PCR analysis.

RUT

Biopsy specimens were directly inoculated into a commercial RUT agar gel (Medical Instruments Corporation, Solothurn, Switzerland). A positive RUT was indicated when the color changed from yellow to pink within 24 hours. The method was performed according to the manufacturer's instructions.

Detection of *glm*M, *cag*A gene, *cag*A EPIYA motifs by PCR

DNA from 89 gastric biopsy positive by RUT and 58 *H. pylori* isolates was extracted using a genomic DNA purification kit (Gentra System, Big Lake, MN), according to the manufacturer's instructions.

PCR amplifications were based on published protocols (Hamlet *et al*, 1999; Argent *et al*, 2005; Yamazaki *et al*, 2005a; Schmidt *et al*, 2009) with modification of some primer sequences (*viz*. cagAP3E). The PCR primers, annealing temperatures and amplicon sizes are shown in Table 1.

PCR for detection of the EPIYA motifs was performed as previously described (Argent *et al*, 2005) using the forward primer cag2 with each of the three reverse primers, cagP1C, cagP2TA and cagAP3E, for the amplification of EPIYA-A, -B, and -C/D, respectively. As *cag*AP3E could not distinguish between EPIYA-C and -D, we applied four reverse primers, namely cagA-WR and cagA-ER, based on the sequences of Yamazaki *et al* (2005a) and cagA-West and cagA-East, based on the sequences of Schmidt *et al* (2009) in order to compare and determine the specificity of EPIYA-C and -D amplification, respectively.

PCR for *glm*M (verification of *H. pylori*), *cag*A and the *cag*A EPIYA motifs were performed in a total volume of 50 µl containing 100 ng of genomic DNA from *H. pylori* culture or 400 ng from urease-positive gastric biopsies, 200 µM of each dNTP (Gibco BRL, Gaithersburg, MD), 1 x PCR buffer (20 mM Tris-HCl, pH 8.4), 50 mM KCl, 1.5 - 2.5 mM $MgCl_2$, 0.1 µM of each primer (except 0.3 µM for *cag*A, and 0.5 µM for EPIYA-C,-D), and 1.5 U *Taq* polymerase (Gibco BRL, Gaithersburg, MD). Amplification was conducted using a thermal cycler (2400 Gene Amp PCR system, Perkin-Elmer, San Jose, CA) as follows:-all pairs of primers were programmed for 35 amplification cycles consisting of denaturation at 95°C for 1 minute, annealing temperature as shown in Table 1, and extension at 72°C for 1 minute.

DNA sequence analysis

We confirmed that the PCR detection methods were valid by using multiple primer pairs to detect the *cag*A genotypes (Western and East Asian types) and the TPMs patterns. Any discrepancies of the *cag*A genotypes were confirmed by DNA sequencing. Of 41 randomly selected *H. pylori* isolates, 20 were selected in agreement between two pairs of primers and 21 had ambiguity in the identification of *cag*A genotype (Western type and East Asian

Table 1
Primer sequences used for target gene amplification.

Gene	Primer	Sequence (5′→3′)	Size (bp)	AT°C[e]	Reference
*glm*M	*ure*C	F-AAGCTTTTAGGGGTGTTAGGGGTTT	294	55	(Lu *et al*, 1999)
		R-AAGCTTACTTTCTAACACTAACGC			
*cag*A	D008	F-ATAATGCTAAATTAGACAACTTGAGCGA	298	60	(Hamlet *et al*, 1999)
	R008	R-TTAGAATAATCAACAAACATCACGCCAT			
*cag*A[d]	cag2	F-GGAACCCTAGTCGGTAATG	550-800	57	(Argent *et al*, 2005)
	cag4	R-ATCTTTGAGCTTGTCTATCG			
cagA TPMs	cagA-P1C[a]	R- GTCCTGCTTTCTTTTTATTAACTTKAGC[b]	177	57	(Argent *et al*, 2005)
	cagA-P2TA[a]	R-TTTAGCAACTTGAGTATAAATGGG	222	57	
	cagA-P3E[a]	R-ATCAATYGTAGCRTAAATGGG[c]	380	57	
EPIYA-C	cagA28F	F-TTCTCAAAGGAGCAATTGGC	493	61	(Argent *et al*, 2005; Yamazaki *et al*, 2005a)
	cagA-WR	R-AAAGGTCCGCCGAGATCAT			(Schmidt *et al*, 2009)
	cagAWest[a]	R-TTTCAAAGGGAAAGGTCCGCC	501	56	
EPIYA-D	cag2	F-GGAACCCTAGTCGGTAATG	403	61	(Yamazaki *et al*, 2005a)
	cagA-ER	R-CCTGCTTGATTTGCCTCATCA			(Schmidt *et al*, 2009)
	cagAEast[a]	R-AGAGGGAAGCCTGCTTGATT	495	56	

[a]used in combination with the forward primer cag2. [b]K is either guanine (G) or thymine (T). [c]Y is either thymine (T) or cytosine (C), [c]R is either quanine (G) or adenine (A). [d]used in DNA sequencing. [e]Annealing temperature.

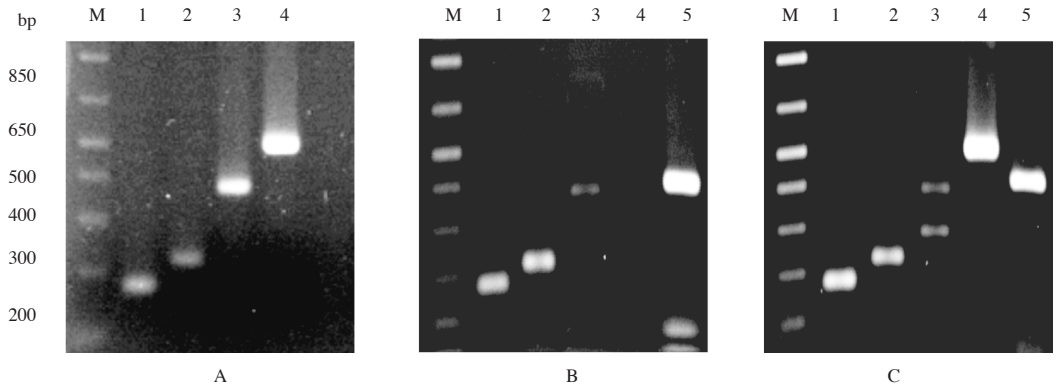

Fig 1–Typical examples of PCR amplification of four types of *cag*A EPIYA motifs. (A) a Western strain with EPIYA-ABC, (B) an East Asian strain with the EPIYA-ABD, (C) a mixed type with EPIYA-ABCD. Lane 1, molecular weight markers; lanes 2-5, EPIYA-A, -B, -C/D, -C, and -D, respectively.

type). The specificity of these primers was determined by DNA sequencing. The PCR amplicons of cag2 and cag4 primers were excised from gel and sequenced using DYEnamics™ET Dye Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences, Buckinghamshire, UK) in a DNA sequencer (according to MegaBace DNA Analysis Systems). Alignment of sequences was compared with the sequences of Western type CagA and East Asian type CagA in the public database.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL). The associations among *cag*A genotype, the number of EPIYA motifs, and clinical outcomes were analyzed using chi-square and Fisher's exact tests. A value of $p < 0.05$ is considered statistically significant.

RESULTS

Prevalence of *cag*A genotype

Of 147 *H. pylori* DNA amplicons, 138 were *cag*A positive. The prevalence of the Western type (54%) was higher than the East Asian type (32%). Seven (5%) were unclassified (A-B type) and 12 (9%) were the mixed type. The Western type is significantly higher in PUD patients (p = 0.0175) (Table 2).

Distribution of *cag*A EPIYA motif and clinical outcome

Examples of PCR amplification of TPM motifs and numbers and types of EPIYA motifs are shown in Fig 1. Examples of DNA sequence for EPIYA motifs are shown in Fig 2.

The distribution of *cag*A EPIYA motifs among patients with different clinical outcomes is shown in Table 3. The most frequently found EPIYA motif was EPIYA-ABC type (43%) followed by EPIYA-ABD type (28%). There is no significant association among EPIYA types, numbers of EPIYA-C motif and types of gastrointestinal diseases. Multiple EPIYA motifs were found in both the Western and East Asian types. For the Western type, the EPIYA motifs found included: -ABCC (7%) -AABC (2%), and 1 (0.5%) each of -ABCCC and -ABBC types. For the East Asian type, the EPIYA motif included ABDD (1.5%) and ABABD (0.5%).

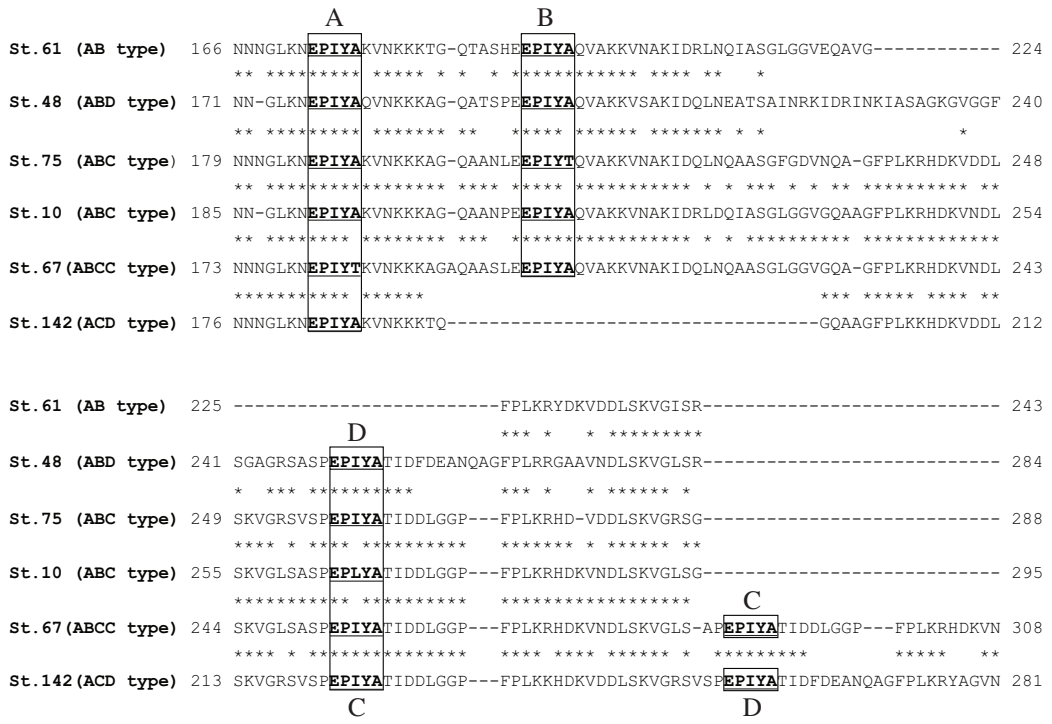

Fig 2–Alignment of amino acid sequences among CagA type strains. Numbers at the start and end of sequences represent the positions of the amino acid residues of each strain. Stars denote identity. A hyphen indicates the absence of an amino acid residue. St is strain.

The diversity of *cag*A genotypes among patients' ethnic groups is shown in Table 4. The Western type predominated among the Thai-Isan group, the major population in this region. There is an association between East Asian type *cag*A and ethnic Chinese ($p = 0.0057$), though the number of patients studied was too small to draw a more solid conclusion.

DISCUSSION

An average of 48% of dyspeptic patients in Thailand was found to be infected with *H. pylori* with *cag*A-positive rate of over 90% (Chomvarin *et al*, 2005, 2008). Nevertheless, the prevalence of gastric cancer in Thailand is quite low compared to other East Asian countries (Zhou *et al*, 2004; Choi *et al*, 2007). Most of *H. pylori* strains found in East Asia (*viz*, Japan, Korea and China) are the East Asian type (Zhou *et al*, 2004; Choi *et al*, 2007; Kanada *et al*, 2008). Predominance of East Asian type *H. pylori* also was noticed in northern Thailand (Yamazaki *et al*, 2005a). However, in this study we found that the prevalence of the Western type (54%) was higher than the East Asian type (32%) in northeastern Thailand. Hirai *et al* (2011) reported that the Western type (11.6%) occurred more frequently than the East Asian type (5.8%) among *cag*A-positive strains in asymptomatic healthy individuals in a general Thai population. They suggested that the low prevalence of the East Asian type *cag*A-positve strains may account for the low rates of gastric cancer

Table 2
*cag*A status and *cag*A genotype in *H. pylori* strains with different clinical outcomes.

*cag*A status	Clinical outcome (%)				Total (%)
	NUD ($n = 68$)	PUD ($n = 57$)	GCA ($n = 18$)	Other ($n = 4$)	
*cag*A					
*cag*A-	3 (4)	5 (9)	1 (6)	0 (0)	9 (6)
*cag*A+	65 (96)	52 (91)	17 (94)	4 (100)	138 (94)
*cag*A genotype					
East Asian type	24 (37)	10 (19)	8 (47)	2 (50)	44 (32)
Western type	29 (45)	35[a] (67)	9 (53)	2 (50)	75 (54)
Mixed type	10 (15)	2 (4)	0 (0)	0 (0)	12 (9)
Unclassified type	2 (3)	5 (10)	0 (0)	0 (0)	7 (5)

[a]Western type is significantly found in PUD patients ($\chi^2 = 5.65$; $p = 0.0175$; OR = 2.36; 95% CI 1.09-5.20). NUD, non-ulcer disease (gastritis); PUD, peptic ulcer disease; GCA, gastric cancer

Table 3
Diversity of *cag*A EPIYA motifs and clinical outcomes in *H. pylori cag*A positive infected patients.

*cag*A EPIYA motif	Clinical outcome (%)				Total (%) ($n = 138$)
	NUD ($n = 65$)	PUD ($n = 52$)	GCA ($n = 17$)	Other ($n = 4$)	
Western type					
A-B-C	22 (34)	28 (54)	8 (47)	1 (25)	59 (<100)
A-B-C-C	4 (6)	3 (6)	1 (6)	1 (25)	9 (6)
A-B-C-C-C	0 (0)	1 (2)	0 (0)	0 (0)	1 (1)
A-A-B-C	2 (3)	1 (2)	0 (0)	0 (0)	3 (2)
A-B-B-C	0 (0)	1 (2)	0 (0)	0 (0)	1 (1)
A-C	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)
B-C	0 (0)	1 (2)	0 (0)	0 (0)	1 (1)
East Asian type					
A-B-D	21 (32)	9 (17)	7 (41)	2 (50)	39 (28)
A-B-D-D	1 (1)	0 (0)	1 (6)	0 (0)	2 (2)
A-B-A-B-D	1 (1)	0 (0)	0 (0)	0 (0)	1 (1)
D	1 (1)	1 (2)	0 (0)	0 (0)	2 (2)
Mixed type					
A-C-D	1 (1)	1 (2)	0 (0)	0 (0)	2 (2)
A-B-C-D	9 (14)	1 (2)	0 (0)	0 (0)	10 (7)
Unclassiflied type					
A-B	2 (3)	5 (10)	0 (0)	0 (0)	7 (5)

NUD, non-ulcer disease (gastritis); PUD, peptic ulcer disease; GCA, gastric cancer

Table 4
Diversity of *cag*A genotypes according to ethnicity.

Ethnic group	*cag*A genotype				Total (%) *n*=138
	East Asian type	Western type	Mixed type	Unclassified type	
Thai-Isan	31 (27)	65 (57)	11 (10)	7 (6)	114 (83)
Chinese	5 (83)[a]	1 (17)	0	0	6 (4)
Thai-Chinese	6 (40)	8 (53)	1 (7)	0	15 (11)
Loa	1 (50)	1 (50)	0	0	2 (1)
Cambodian	1 (100)	0	0	0	1 (1)

[a]The East Asian type is significantly found in the ethnic Chinese ($\chi^2 = 7.65$; $p = 0.0057$; OR 11.9231; 95% CI 1.2519 - 569.7096).

in Thailand. Similarly, in the Philippines, although its population is considered to have originated in East Asia, the major strain of CagA is the Western type in concordance with the low prevalence of gastric cancer (Cortes *et al*, 2010).

In the present study, the Western *cag*A type predominated among the Thai-Isan ethnic group who are considered of Thai-Lao origin. In Bangkok, located in central Thailand, the *cag*A typing is that of the South/Central Asian type and mixed types, which are predominant in the Thai group, while the East Asian type is predominant in the ethnic Chinese and Thai-Chinese (Vilaichone *et al*, 2004). The predominance of the East Asian type in dyspeptic patients in northern Thailand has been reported (Yamazaki *et al*, 2005b). These results suggest that there are distinct *H. pylori cag*A strains circulating within each ethnic group and that there is also a geographical diversity (Vilaichone *et al*, 2004). Additionally, Western residents have been increasing in northeastern Thailand, it remains to be solved whether this could account for the predominance of the Western *cag*A type in northeastern Thailand. We need to conduct a nationwide cross-sectional survey for this clarification.

Although the numbers examined were too small to draw a solid conclusion, the number of ethnic Chinese patients infected with the East Asian type is significantly ($p = 0.0047$) higher than those with the Western type. Similarly, the high prevalence of East Asian type among ethnic Chinese patients has been reported in central Thailand (Vilaichone *et al*, 2004). Related to this, Schmidt *et al* (2009) reported that EPIYA-C motif occurs more frequently than EPIYA-D in ethnic Malay and Indian population in Malaysia and Singapore, although no significant association between EPIYA-D and GCA was observed. A recent study showed that the variation in the CagA sequence, particularly in the variable C-terminal region, is an important immune evasion mechanism of the bacteria (Mohamed *et al*, 2009). The linkage study between the genetic variations of CagA structure of *H. pylori* strains to the ethnicity and the severity of diseases may, therefore, be an interesting question to be solved in the future.

The multiplicity of EPIYA segments, especially EPIYA-C, is thought to be related to the development of gastro-

duodenal diseases (Naito *et al*, 2006; Basso *et al*, 2008; Nguyen *et al*, 2008; Shokrzadeh *et al*, 2009; Salih *et al*, 2010), although conflicting results were shown by other researchers (Occhialini *et al*, 2001). The current study showed that the majority of *cag*A EPIYA motifs were EPIYA-ABC (43%) and EPIYA-ABD (28%). EPIYA-ABCC was not frequently found and there is no correlation with clinical outcomes. In this study, the Western type is significantly found in PUD patients but not in NUD and GCA, in agreement with previous reports (Yamazaki *et al*, 2005b; Salih *et al*, 2010). These EPIYA motif variations can cause an effect in the CagA structure and can be correlated with its interaction inside the gastroepithelial cells (Acosta *et al*, 2010). Therefore, the biological activity of CagA proteins in the development of carcinogenesis of the epithelial cells should be further elucidated.

In summary, this study showed that the major type of CagA in northeastern Thai (Isan) patients was the Western type with significant association to PUD. No association is found between the increased numbers of EPIYA-C or EPIYA-D motif and clinical outcomes. The low incidence of GCA in this study area may be related to the low number of East Asian CagA type and the low prevalence of repeated EPIYA-C motif. The association of the type and structure of *cag*A EPIYA motifs with clinical outcomes should be elucidated further to help in the prediction of disease outcome.

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