RESEARCH ARTICLE

Helicobacter pylori vacA d1 Genotype Predicts Risk of Gastric Adenocarcinoma and Peptic Ulcers in Northwestern Iran

Zeinab Basiri[1], Reza Safaralizadeh[1]*, Morteza jabbarpour Bonyadi[1], Mohammad Hossein Somi[2], Majid Mahdavi[1], Saeid Latifi-Navid[3]

Abstract

Background: There is a close relationship between *Helicobacter pylori* (*H pylori*)-specific factors and different gastroduodenal diseases. The present study aimed to investigate the prevalence of *vacA* d1, d2 genotypes in the *H pylori* isolates from patients with gastric adenocarcinoma, peptic ulcer disease (PUD) and gastritis in East Azerbaijan region, where the incidence of gastric cancer (GC) is high. Strains isolated from this area are likely to be of European ancestry. Materials and Methods: In this study, genotyping of the vacA d region of 115 isolates obtained from patients with different gastrodoudenal diseases was accomplished by PCR methods. In addition to PCR amplification of *H pylori 16S rDNA*, rapid urease tests or histological examination were used to confirm the presence of *H pylori* in biopsy specimens. Data were collected and analyzed using SPSS version 19. Results: Of the total of 83 *H pylori* isolates, 36 (43.4%) contained the d1 allele and 47 (56.6%) were subtype d2. The results of the multiple linear/logistic regression analysis showed high correlation between allele d1 and gastric adenocarcinoma or PUD. Conclusions: This study suggests that the *H pylori vacA* d1 genotype helps predict risk for gastric adenocarcinoma and PUD in East Azerbaijan, Iran.

Keywords: East Azerbaijan - gastric adenocarcinoma - *H pylori* - *vacA* d1 genotype - Iran

Asian Pac J Cancer Prev, **15 (4)**, 1575-1579

Introduction

H pylori is a microaerophilic gram negative bacterium that is able to colonize and persist within the gastric mucosa layer (Atherton, 2006). Several studies have shown a close relationship between *H pylori* infection and gastroduodenal diseases, such as chronic active gastritis, PUD, MALT-lymphoma, and GC (Parsonnet et al., 1991). GC (90% adenocarcinomas) is the fourth most common cancer worldwide and the second leading cause of cancer-related deaths (700,000 deaths annually) (Coleman et al., 1993; Parkin et al., 2005). The 63% of all GC are related to chronic *H pylori* infection (Parkin, 2006). While *H pylori* infection plays a primary role, genetic and environmental factors modulate its outcome (Peek et al., 2010). Studies have shown that the elimination of *H pylori* may reduce the risk of PUD and GC but increase the risk of gastro- esophageal reflux (GERD), Barrett's esophagus, and esophageal adenocarcinoma (Blaser, 1999). Considering the significance of *H pylori* in certain diseases, it is important to identify which strains of *H pylori* have the potential to increase the risk of GC and PUD (Blaser, 1999). The vacuolating cytotoxin A (*vacA*) often present in every *H pylori* strain (Cover et al., 1994). Variation in levels of cytotoxicity is attributed to *vacA* allelic variation among *H pylori* strain, which occurs in the signal (s), middle (m) and intermediate (i) regions, each with two different alleles (Atherton et al., 1995; van Doorn et al., 1998): s1 or s2, m1 or m2,and 1i or i2, respectively (Rhead et al., 2007). Recently, a fourth disease-related region between the i and m regions has been indentified and named the deletion (d) region. The d region is divided into d1 (no deletion) and d2 (with a 69 to 81 bp deletion), with d1 identified a risk factor for GC and PUD in Western strains. Nearly all *H pylori* isolates from East Asia have been found to have a genotype of *vacA* d1 (Ogiwara et al., 2009).

Almost two-thirds of cases of GC occur in Asia (Nguyen et al., 2008). Iran with a high incidence of GC ranks fourth in Asia, after China, Japan and Korea respectively (Fujisawa et al., 1999; Wang and Wang, 2003; Derakhshan et al., 2004; Yim et al., 2007; Alizadeh et al., 2009).

The prevalence of *H pylori* infection is 69% in Iran (Nouraie et al., 2009). Phylogenetic analysis performed in Iran has shown that Iranian strains have probably

*[1]Department of Animal Biology, Faculty of Natural Sciences, University of Tabriz, [2]Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, [3]Department of Biology, Faculty of Sciences, University of Mohaghegh Ardabili, Ardabil, Iran *For correspondence: safaralizadeh@tabrizu.ac.ir.*

been influenced by genetic exchange from neighboring countries, so that some researchers have suggested Iranian *H pylori* strains to be a subset of *HpEurope* population (Latifi-Navid et al., 2010). Another study in Iran has demonstrated that the prevalence of the *vacA* d1 genotypes in strains representing *European* ancestry were significantly higher in areas with high incidence of gastric cancer. (Latifi-Navid et al., 2013). East Azerbaijan Province is located in northwestern Iran, where the incidence of GC is high. GC is the leading cause of cancer-related deaths in males (ASRs=26.0) and the fourth type of cancer after breast, skin and esophagus cancers in females (ASRs=11.6) in this area (Somi et al., 2008).

The purpose of this study was to investigate the association between the *H pylori vacA* d1 genotype and disease outcome in infected patients, and to determine whether the *H pylori* strains in this study mimicked the isolates described in western countries.

Materials and Methods

Study location and patients

All patients Over 16 years of age referred to the department Endoscopy at Imam Reza Hospital in Tabriz, Iran were given a questionnaire to collect demographic data (age, gender, nationality and language). Individuals who had received non-steroidal anti-inflammatory drugs or anti-Helicobacter therapy at least 3 months prior to endoscopy were excluded (Willen et al., 2000), as were those meeting the criteria listed in Table 1 (Westbrook et al., 2005). Antral gastric biopsies were collected from 115 patients with different gastroduodenal diseases. Informed consent was obtained directly from each patient under protocols approved by the hospital's ethics committee. The sample consisted of 58 males and 57 females with a mean age of 52.9 years (range, 19 to 90 years).

Endoscopy and biopsy sampling

For each patient, at least two biopsy specimens for the rapid-urease test and histology examination, and an additional biopsy specimen for PCR analysis were taken from the antral mucosa Biopsy specimens for PCR analysis were frozen at -80°C until processing.

Table 1. The Exclusion Criteria and Number of Patients

Patients questioned	157
Female	84
Male	73
Patients excluded	42
Had received nonsteroidal anti-inflammatory drugs or-antihelicobacter therapy at least 3 months prior to endoscopy	41
Had a GI bleed	1
Had a previous history of peptic ulcer disease or gastric cancer	1*
Had undergone a previous endoscopy or gastric surgery	0
Patients remaining in the study	115

*This patient had also received nonsteroidal anti-inflammatoy drug

Histological examination

The 10% formalin-fixed biopsy specimens were embedded in paraffin, and then the tissue sections were prepared for histopathological examinations. Classification and grading of gastric cancer were done using the Sydney system (Dixon et al., 1996).

DNA extraction

First, the biopsy specimens were introduced with a sterile needle on the slides and crushed by other slides carefully. The DNA extraction was then performed from all specimens according to the protocol for extraction by DNGTM-Plus kit (CinnaGen, Iran). Briefly, specimens were washed into tubes with 400 or 500 microliters (depending on sample size) of DNGTM-Plus solution. The tubes were vortexed until a completely homogenous suspension was obtained. Next, 300 microliters of isopropanol was added and vortexed for 3-5 second ,and held at -20°C temperature for 20 min. Tubes were centrifuged for 10 min at 12000rpm (revolutions per minute). The supernatants were discarded. The precipitates were re-suspended in 1 ml of 75% Ethanol and vortexed slowly and then centrifuged for 5 min at 12000rpm. The ethanol was poured off completely. This step was repeated once more. The pellet was dried at 65°C for 5min. DNA pellet was dissolved in 50 microliters of sterile distilled water by gentle shaking and placed at 65°C for 5min. Any residual pellets on the tube walls were dissolved by softly pippeting with distilled water. Finally, through a 30sec centrifuge at 12000rpm, the supernatant containing purified DNA was removed.

Primers and PCR conditions

In this study, genotyping of the 16S *rDNA* gene (for the confirmation of *H pylori* isolates) (Engstrand, 1992) and the *vacA* d region were determined by PCR methods. All primer sets were selected from published literature (Lu et al., 2002; Ogiwara et al., 2009). The PCR assay was performed in a reaction volume of 25μL using a commercially available kit (CinnaGen, Iran). The PCR conditions for *vacA*d1 and 16S *rDNA* were 95°C for 5min followed by thirty-seven cycles at 95°C for 45 s, 53°C for 60 s, and 72°C for 30 s; these 37 cycles were followed by one cycle at 72°C for 5 min. Finally, PCR products were separated by gel electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator. Strains J99 and Tx30a were used as a positive control for d1 and d2 alleles respectively.

Table 2. Oligonucleotide Primers Used for Genotyping *H pylori*

Genes		Primer Sequences (5'→3')	Optimized annealing temperature (ºC)	products	References
16S rDNA		HP1 GCAATCAGCGTCAGTAATGTTC HP2 GCTAAGAGATCAGCCTATGTCC	53	519 pb	(Westbrook et al., 2005)
vacA	d1/-d2	VAS-5 F ACTAATATTGGCACACTGGATTTG VAGF-R CTCGCTTGATTGGACAGATTG	53	d1: 367-379 pb d2: 298 pb	(Ogiwara et al., 2009)

Statistical analysis

All data were analysed using SPSS version 19. The chi-square and Fisher's exact tests were used to evaluate the relationship between the frequency of each allele with the risk of gastric cancer and other gastroduodenal diseases.

The multiple linear regression analysis, after controlling for age and sex variables, was carried out to examine which allele (s) was related to gastroduodenal diseases. A stepwise method was used and variables were selected by the f value out and f value, where F and f values were 3.84 and 0.05, respectively.

Logistic regression analysis was used to investigate the effect of each allele in gastric adenocarcinoma and gastroduodenal diseases, and the selection of variables was by the Enter method. A p value of less than 0.05 indicated significance. In all comparative analysis, patients with gastritis were considered as controls.

Results

Presence of H pylori in gastric biopsy specimens and classification of patients

None of the test methods for detecting *H pylori* infection has been entirely ideal. Therefore, in addition to PCR amplification of *H pylori* 16S *rDNA*, rapid urease test or histological examination was used to confirm the presence of *H pylori* in biopsy specimens.

Eighty three patients tested positive for *H pylori*. Of these, 51 were classified as gastritis, 14 as peptic ulcer, and 18 as gastric adenocarcinoma. In this study, approximately 72% of patients were infected with *H pylori* and of these, 60% had gastritis.

Prevalence of H pylori vacA d1/-d2 genotypes and its influence on clinical outcome

This study showed that the frequencies of the *vacA* in d1, d2 were 43.4% and 56.6%, respectively. The frequency of the allele d1 (Table 3) was significantly higher in *H pylori* isolates from patients with gastric adenocarcinoma (66.6%) and peptic ulcer (71.4%) than in those with gastritis (27.4%).

Table 3. Fequency of the *vacA* d1, d2 alleles in *H pylori* Isolates from Patients with Gastric Adenocarcinoma, Peptic Ulcer and Gastritis

	No. (%) of patients				
Genotype	Gastritis	Peptic ulcer	Adenocarc inoma	Total	p value*
d1	14 (27.4)	10 (71.4)	12 (66.6)	36 (43.37)	0.003
d2	37 (72.6)	4 (28.6)	6 (33.4)	47 (56.62)	0.003

*A p values less than or equal to 0.05 were accepted as statistically significant

Table 4. Correlation between Allele d1 and Gastric Adenocarcinoma and Peptic Ulcer Obtained by Multiple Linear Regression Analysis

	Adenocarcinoma		Peptic ulcer	
Genotype	Partial regression correlation	p	Partial regression correlation	p
d1	0.309±0.102	0.01	0.375±0.100	0.02

Table 5. Association between the Allele d1 and Gastric Adenocarcinoma and Peptic Ulcer in Comparison with Gastritis, Obtained by Logistic Regression Analysis

	Adenocarcinoma			Peptic ulcer		
Genotype	OR	95%CI	p value	OR	95%CI	p value
d1	4.662	1.345-16.164	0.015	6.275	1.629-24.167	0.008

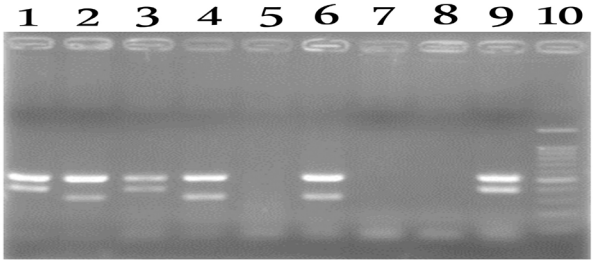

Figure 1. Agarose gel (1.2%) Contains PCR Products from Allele d1 and Allele d2 of *vacA* Gene and *16S rDNA* Gene: lane 1-2: Positive Control, Lanes 3-7 and 9: clinical Positive and Negative Strains, lane 8: Negative Control and Lane 10: *100-bp DNA ladder*

The results of multiple linear/logistic regression analysis confirmed the high association of the *vacA* d1 genotype with gastric adenocarcinoma and Peptic ulcer rather than with gastritis.

Discussion

Many reports have shown an association between *H pylori* infection and the development of gastroduodenal diseases (Suerbaum and Michetti, 2002). However, the incidence of gastric cancer is enigmatically low in Africa and South Asia, where frequency of *H pylori* infection is high. Furthermore, several studies from East Asia and Iran have indicated a tendency towards reduction of GC incidence from north to south within these areas (Suzuki et al., 2012; Latifi-Navid et al., 2013). *H pylori* virulence factors have been shown to play an important role in determining the course of *H pylori* associated disease (Suerbaum and Michetti, 2002). Strains with the particular *vacA* genotypes have been found to be good markers for gastroduodenal disease (Atherton et al., 1995). Ogiwara et al. (2009) showed that the *vacA*d1 allele could be considered a more sensitive independent biomarker in the development of gastroduodenal disease, and Latifi-Navid et al. (2013) have also recently reported that the *vacA* d1/-i1 alleles from strains with *European* ancestry could be considered good markers for gastric cancer in Ardabil and Mazanderan (high incidence areas of GC in Iran) (Ogiwara et al., 2009; Latifi-Navid et al., 2013). In the present study, the genotype of the *vacA* d region was evaluated in *H pylori* isolated from patients with gastric adenocarcinoma, PUD and gastritis (control group) in East Azerbaijan in the Northwest of Iran. First, the frequency of *vacA* d1 was determined and compared with that reported in two previous studies (Ogiwara et al., 2009; Latifi-Navid et al., 2013). In this study, the frequencies for the *vacA* d1 and d2 alleles were 43.37% and 56/63% respectively in patients with gastric adenocarcinoma, peptic ulcer and gastritis. The prevalence of d1 allele is almost similar

to the pattern observed in a previous study (d1=39.9%) (Latifi-Navid et al., 2013). However, it is different from studies from western countries (d1=74.1%) and East Asian countries where almost 100% of the strains carry the *vacA* d1 genotype regardless of clinical outcomes (Ogiwara et al., 2009). In this study, the relationship between the *vacA* d region and different gastroduodenal diseases was also investigated. In agreement with findings from western countries, the strains carrying the *vacA* d1 genotype are significantly associated with gastric adenocarcinoma (66.6%) and peptic ulcer (71.4%), and these results were confirmed by multiple linear/logistic regression analysis (Ogiwara et al., 2009). In contrast, there is no significant correlation between the *vacA* genotypes and gastroduodenal disease in East Asian countries. The results of several studies have indicated that some Asian strains are influenced by genetic exchange with neighboring countries and are similar to other isolates from Western countries (Yamaoka et al., 2002; Azuma et al., 2004; Yamazaki et al., 2005; Satomi et al., 2006; Latifi-Navid et al., 2010), Whereas none of the Western strains have an East-Asian-type gene sequence (Xia et al., 2009). Since East Azerbaijan, like Ardabil and Mazanderan could be considered as the high incidence areas of GC in Iran (Somi et al., 2008; Latifi-Navid et al., 2013), *H pylori* strains from this region also might be similar to other isolates from Western Eurasia and placed in the hpEurope population (Latifi-Navid et al., 2010).

Coleman et al. showed that almost 90% of cases of gastric cancer are adenocarcinomas (Coleman et al., 1993). In the present study, among the 34 gastric cancer patients, 27 (nearly 80%) had adenocarcinoma.

In agreement with other studies, the PCR technique possessed high sensitivity and specificity for the detection of *H pylori* in this study (Weiss et al., 1994). Sharp bands in PCR products of samples collected from urease test suggest that PCR is useful for diagnosing *H pylori* in gastric biopsy specimens.

In agreement with previous studies (Ogiwara et al., 2009; Latifi-Navid et al., 2013) we have proposed that the *H pylori vacA* d1 genotype might be a new risk marker for gastric adenocarcinoma and peptic ulcer in the Northwestern region of Iran. Regarding the strains carrying the virulence factors, *vacA* and *cagA* are particularly associated with the risk of gastric cancer (Huang et al., 2003; Atherton, 2006). We suggest that future studies on *CagA* EPIYA polymorphisms will also be required in these regions. We selected samples with sufficient size in this study. However, this study might be limited due to the number of samples collected. Future studies with a sufficient number of samples will also be required.

Acknowledgements

This work was funded by the Animal Biology Department, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran. Our gratitude also goes to Tabriz Imam Reza Hospital staff and patients for their helpful collaboration. We would also like to thank doctors Yaghob Moadab, Kamal Boostani, Saleh Azad Bakht and Amir-Taher Eftekhar Sadat for their kind help with the collection of the biopsy specimens.

References

Alizadeh AH, Ansari S, Ranjbar M, et al (2009). Seroprevalence of Helicobacter pylori in Nahavand: a population-based study. *East Mediterr Health J*, **15**, 129-35.

Atherton JC (2006). The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu Rev Pathol*, **1**, 63-96.

Atherton JC, Cao P, Peek RM, et al (1995). Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J Biol Chem*, **270**, 17771-7.

Azuma T, Yamakawa A, Yamazaki S, et al (2004). Distinct diversity of the cag pathogenicity island among Helicobacter pylori strains in Japan. *J Clin Microbiol*, **42**, 2508-17.

Blaser MJ (1999). Hypothesis: the changing relationships of Helicobacter pylori and humans: implications for health and disease. *J Infect Dis*, **179**, 1523-30.

Coleman MP, Esteve J, Damiecki P, et al (1993). Trends in cancer incidence and mortality. *IARC Sci Publ*, **121**, 1-806.

Cover TL, Tummuru MK, Cao P, et al (1994). Divergence of genetic sequences for the vacuolating cytotoxin among Helicobacter pylori strains. *J Biol Chem*, **269**, 10566-73.

Derakhshan MH, Yazdanbod A, Sadjadi AR, et al (2004). High incidence of adenocarcinoma arising from the right side of the gastric cardia in NW Iran. *Gut*, **53**, 1262-6.

Dixon MF, Genta RM, Yardley JH, et al (1996). Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol*, **20**, 1161-81.

Engstrand L (1992). Helicobacter pylori. New diagnostic tools. Clinical and experimental studies on local and systemic immune response. Minireview based on a doctoral thesis. *Ups J Med Sci*, **97**, 1-26.

Fujisawa T, Kumagai T, Akamatsu T, et al (1999). Changes in seroepidemiological pattern of *Helicobacter pylori* and hepatitis A virus over the last 20 years in Japan. *Am J Gastroenterol*, **94**, 2094-9.

Huang JQ, Zheng GF, Sumanac K, et al (2003). Meta-analysis of the relationship between *cagA* seropositivity and gastric cancer. *Gastroenterology*, **125**, 1636-44.

Latifi-Navid S, Ghorashi SA, Siavoshi F, et al (2010). Ethnic and geographic differentiation of *Helicobacter pylori* within Iran. *PLoS One*, **5**.

Latifi-Navid S, Mohammadi S, Maleki P, et al (2013). *Helicobacter pylori vacA* d1/-i1 genotypes and geographic differentiation between high and low incidence areas of gastric cancer in Iran. *Arch Iran Med*, **16**, 330-7.

Lu Y, Redlinger TE, Avitia R, et al (2002). Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. *Appl Environ Microbiol*, **68**, 1436-9.

Nguyen LT, Uchida T, Murakami K, et al (2008). *Helicobacter pylori* virulence and the diversity of gastric cancer in Asia. *J Med Microbiol*, **57**, 1445-53.

Nouraie M, Latifi-Navid S, Rezvan H, et al (2009). Childhood hygienic practice and family education status determine the prevalence of Helicobacter pylori infection in Iran. *Helicobacter*, **14**, 40-6.

Ogiwara H, Sugimoto M, Ohno T, et al (2009). Role of deletion located between the intermediate and middle regions of the *Helicobacter pylori vacA* gene in cases of gastroduodenal diseases. *J Clin Microbiol*, **47**, 3493-500.

Parkin DM (2006). The global health burden of infection-associated cancers in the year 2002. *Int J Cancer*, **118**,

3030-44.
Parkin DM, Bray F, Ferlay J, et al (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.
Parsonnet J, Friedman GD, Vandersteen DP, et al (1991). Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med*, **325**, 1127-31.
Peek RM, Jr, Fiske C, et al (2010). Role of innate immunity in Helicobacter pylori-induced gastric malignancy. *Physiol Rev*, **90**, 831-58.
Rhead JL, Letley DP, Mohammadi M,et al (2007). A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology*, **133**, 926-36.
Satomi S, Yamakawa A, Matsunaga S, et al (2006). Relationship between the diversity of the *cagA* gene of Helicobacter pylori and gastric cancer in Okinawa, Japan. *J Gastroenterol*, **41**, 668-73.
Somi MH, Farhang S, Mirinezhad SK, et al (2008). Cancer in East Azerbaijan, Iran: results of a population-based cancer registry. *Asian Pac J Cancer Prev*, **9**, 327-30.
Suerbaum S, Michetti P (2002). Helicobacter pylori infection. *N Engl J Med*, **347**, 1175-86.
Suzuki R, Shiota S, Yamaoka Y (2012). Molecular epidemiology, population genetics, and pathogenic role of *Helicobacter pylori*. *Infect Genet Evol*, **12**, 203-13.
van Doorn LJ, Figueiredo C, Sanna R, et al (1998). Expanding allelic diversity of Helicobacter pylori *vacA*. *J Clin Microbiol*, **36**, 2597-603.
Wang KJ, Wang RT (2003). Meta-analysis on the epidemiology of Helicobacter pylori infection in China. *Zhonghua Liu Xing Bing Xue Za Zhi*, **24**, 443-6 (in Chinese).
Weiss J, Mecca J, da Silva E, et al (1994). Comparison of PCR and other diagnostic techniques for detection of *Helicobacter pylori* infection in dyspeptic patients. *J Clin Microbiol*, **32**, 1663-8.
Westbrook JI, Duggan AE, Duggan JM, et al (2005). A 9 year prospective cohort study of endoscoped patients with upper gastrointestinal symptoms. *Eur J Epidemiol*, **20**, 619-27.
Willen R, Carlen B, Wang X, et al (2000). Morphologic conversion of Helicobacter pylori from spiral to coccoid form. Scanning (SEM) and transmission electron microscopy (TEM) suggest viability. *Ups J Med Sci*, **105**, 31-40.
Xia Y, Yamaoka Y, Zhu Q, et al (2009). A comprehensive sequence and disease correlation analyses for the C-terminal region of *CagA* protein of Helicobacter pylori. *PLoS One*, **4**, 7736.
Yamaoka Y, Orito E, Mizokami M, et al (2002). *Helicobacter pylori* in North and South America before Columbus. *FEBS Lett*, **517**, 180-4.
Yamazaki S, Yamakawa A, Okuda T,et al (2005). Distinct diversity of *vacA*, *cagA*, and cagE genes of Helicobacter pylori associated with peptic ulcer in Japan. *J Clin Microbiol*, **43**, 3906-16.
Yim JY, Kim N, Choi SH, et al (2007). Seroprevalence of Helicobacter pylori in South Korea. *Helicobacter*, **12**, 333-40.