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The Helicobacter Pylori Genotyping Among Mongolian Dyspeptic Patients

Tserentogtokh Tegshee[1], Gantuya Boldbaatar[1, 2], Khasag Oyuntsetseg[2], Davaadorj Duger[1, 2], Battulga Adiyasuren[3], Bolor Otgondemberel[4]

[1]Department of Gastroenterology, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Endoscopy Unit, Mongolia-Japan Teaching Hospital, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [3]Endoscopy Unit, Songdo Hospital, Ulaanbaatar, Mongolia, [4]Endoscopy Unit, Brilliant Hospital, Ulaanbaatar, Mongolia

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Corresponding Author
Tserentogtokh Tegshee MD
Department of Gastroenterology, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar 14910, Mongolia
Tel: 976-9907-0988
E-mail: tserentogtokh.doctor@gmail.com

Objective: The prevalence of *H. pylori* infection varies significantly and developing countries are found to be the highest in Asia. In the present study, we have aimed to perform a combined analysis of histochemical stains and IHC to confirm the *H.pylori* infection of patients in 5 different provinces and Ulaanbaatar city, Mongolia. **Method:** Five hundred and thirty-eight patients were enrolled in this study (142 gastric mucosal atrophy, 333 gastritis and 62 gastroesophageal reflux). **Results:** 67.1 % of participants had CagA positive and 69.3 % were immunohistochemically positive. All histological results showed that the gastritis group was significantly higher in patients who were positive for *H. pylori* than in antral-predominant gastritis. By the hematoxylin and eosin staining with May– Giemsa confirmation by IHC, the prevalence of *H. pylori* infection in the gastric mucosal atrophy group was 62.1 %, while in the gastritis and gastroesophageal reflux group, the rate was 74.3 % and 59.1 %, respectively. **Conclusion:** CagA sequence profile in each group of patients revealed that 22.6 % of the gastric mucosal atrophy group was ABC type, 43.4 % was ABCC type and 33.9 % was ABTC type. On the other hand, in the gastritis group, the dominant type was ABTC (41.3 %). In the gastroesophageal reflux group, the ABCC type was dominant while 23.1% was the ABC type.

Keywords: Atrophy, Bacteria, Immunohistochemistry, Histology, Reflux, Gastritis, Sequence

Introduction

Helicobacter pylori *(H. pylori)* is a spiral-shaped, gram-negative bacterium present in the gastric mucosa of humans. It most likely transmits via oral-oral and has been demonstrated that over 50% of the world population is infected with *H.pylori* [1 - 4]. *H.pylori* infection appears to be a risk factor for peptic ulcer disease and gastric adenocarcinoma. There are two important virulence factors in *H.pylori*, CagA and VacA, which each exhibit a high level of intraspecies genetic diversity. VacA is a pore-forming toxin secreted extracellularly, generates vacuole formation, and induces apoptosis. The structure of this

protein has similarities to other intracellular bacterial toxins such as anthrax and cholera toxins. On the other hand, CagA alters numerous signaling pathways in the cell, which further stimulates the malignant transformation of cells. The gene is located within a 40 kb chromosomal region known as the cag pathogenicity island (cag PAI) and CagA protein is translocated into the host cell through the type IV secretion system.

Strains with a cytotoxin-associated gene (Cag) secretion system have been reported to enhance cancer risk [5 - 7]. According to the C-terminal structure, CagA possesses polymorphisms and displays different functions among areas and races. Within the gastric cell, the C-terminal motif of the CagA, EPIYA (Glu-Pro-Ile-Tyr-Ala), undergoes phosphorylation by Src family protein tyrosine kinase and alters further the cell signaling pathways [8 - 9]. The EPIYA motif is subdivided into A, B, C and D based on the repeated number of sequences. Within these sequences, *H.pylori* strains are grouped into two subtypes: Western and East Asian. East Asian type of first repeat region (FR region) of cagA gene, a 57 bp region, is similar to Western-type, however, 162 bp region is absolutely different from western type second repeat region (WSR region). It has been demonstrated that the East Asian type is associated with more severe inflammation and gastric cancer mortality compared to that of the Western type.

In Asia, the prevalence of *H. pylori* is geographically different [10 - 12]. The South Asian region has a pooled prevalence of 56.5 %, ranging from 10.3 to 91.7 %. In the subgroup analysis by country, the highest prevalence rate was reported from Bangladesh (86.3 %, 95% CI: 0.806 – 0.921), whereas the lowest prevalence was from Sri Lanka (10.3 %, 95% CI: 0.072 – 0.135) [13]. In the case of Thailand, the overall infection rate was 34.1 % and the prevalence was different in the North and South regions (50.4 % vs. 17.6 %, $p < 0.001$). Low income, birth condition, employment as well as consumption of alcohol were risk factors for the infection. Of the 74 strains cultured, 56.8 % carried Western-type cagA, with a higher proportion in the South than in the North (76.2 % vs. 49.1 %, $p = 0.05$) [14].

Mongolian esophageal cancer incidence and mortality were 18.5 and 16.3 per 100,000 populations by 2018, which is 2nd highest in Asian countries [15]. Lonjid et al. demonstrated that the highest incidence was registered in Uvs (38.13), Bayan-Ulgii (24.15), and Zavkhan (18.18) provinces, respectively. The incidence of stomach cancer was 20.33 per 100,000 population and the highest incidences were registered in Uvs (53.01), Khovd (46.02), and Darkhan-Uul (40.50) provinces, respectively [24]. *H. pylori* infections were CagA positive in 100 % with cancer vs 81 % without cancer ($p = 0.02$). The majority of CagA was non-East Asian type (95 % with cancer and 94.1 % without cancer) [16]. Another team also demonstrated cagA gene characterization in strains isolated from patients with gastritis and ulcer. One *H. pylori* isolate carried the East Asian type and 52 isolates were Western type. Among them, 30 were ABC, 19 were ABCC type and one each of type ABCCCC, AAABC and AAAAB [17]. In our previous study, we have determined cagA and vacA subtypes in the Mongolian population. Among 368 patients examined, 16.8 % of strains were cagA-negative and 306 (83.1 %) were cagA-positive (293 Western-type, 12 East-Asian-type, and one hybrid type). In the gastritis group, 78.6 % had Western-type 2.9 % had East-Asian-type, and 18.2 % (61/341) were cagA-negative. However, all *H. pylori* from gastric cancer patients possessed Western-type CagA. This cross-sectional study was conducted on patients with or without gastric cancer and the seroprevalence result gained from this study had disagreed with the previous studies where patients infected with the East-Asian type strains have more severe inflammation scores [15].

There are several studies that have been conducted to determine the relationships between *H. pylori* cagA polymorphism and disease severity. In the study of Ferreira et al. the presence of cagA and the number and type of EPIYA motifs were determined by PCR. Infection with strains harbouring two or more CagA EPIYA C motifs was associated with the presence of surface epithelial damage, and with atrophic gastritis and gastric carcinoma. The magnitude of risk for atrophic gastritis and gastric carcinoma increased with increasing number of EPIYA C motifs [18]. On the other hand, the evaluation of Korean *H. pylori* strains did not find an association between disease and higher numbers of EPIYA-C motifs. PCR and DNA analysis of 79 patients (chronic gastritis, 15; duodenal ulcer, 27; benign gastric ulcer, 18; gastric cancer, 19) biopsy specimens revealed that 98.8% contained three EPIYA motifs and one strain (1.2 %) isolated from a patient with duodenal ulcer contained four EPIYA motifs [19]. Chen et al. also demonstrated no association between cagA status and peptic ulceration or chronic gastritis was established, likely due to the high presence of cagA in both patient populations [20]. The differences presented in these studies could be related to environmental or geographical

factors.

Therefore, in the present study, we have aimed to conduct a study on Mongolian patients with different pathological states of gastritis (gastric mucosal atrophy, gastritis and gastroesophageal reflux) and to analyze seroprevalence rates in each subgroup.

Materials and Methods

Research design and subjects

We conducted a cross-sectional study among dyspeptic patients aged >16 years, from 2014 to 2016. History of partial or total gastrectomy; treatment with bismuth containing compounds, H2-receptor blockers, or proton pump inhibitors within 2 weeks prior to the start of the study and history of previous eradication therapy for *H. pylori* infection were the exclusion criteria. We chose the places according to the geographical location and GC incidence rate in the 2012 Mongolian national data. The selected places were as follows: Ulaanbaatar City (central part of Mongolia, with an ASR of GC incidence of 31.3 per 100,000 population), Uvs Province (western; ASR, 80.7), Khuvsgul Province (northern; ASR, 37.0), Umnugovi Province (northern; ASR, 21.5), and Khentii Province (eastern; ASR, 11.5). Age and gender were used as non-modifable risk factors, and high salt intake and bad habits (tobacco smoking and alcohol drinking) were used as modifiable environmental risk factors. A previous nationwide study validated the questionnaire method by evaluating the excessive amount of salt using 24 h urine collection samples, revealing that the urine saline level was higher in daily salty tea drinkers than those who drink from other sources [16]. Therefore, to determine excessive amount of salt, we used the same questionnaire method to screen routine salty tea drinkers. For bad habits, tobacco smoking (daily smokers) and alcohol drinking (excessive amount of standard drink [350 mL glass of beer, 150 mL glass of 12% wine, or 44 mL glass of spirit] per monthly, weekly, or daily) statuses were used. During endoscopic examination, the Los Angeles (LA) classification was used to determine gastroesophageal reflux disease [17]. Gastric mucosal atrophy was evaluated using the Kimura-Takemoto classification [18] and was classified as closed type (mild atrophy, limited in the antrum and lesser curvature of the proximal corpus) and open type (advanced atrophy, extended into the corpus and cardia). Tree gastric mucosal tissues were taken from the antrum approximately 3 cm from the pyloric ring, which were used for rapid urease test (Mon-HP, developed at the Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia), histological examination, and bacterial culture. Two more biopsies were taken from the corpus and incisura angularis (angulus) for histological examination. If ulcer or suspected cancer lesions were detected, additional biopsy specimens was taken for histological diagnosis. Biopsy specimens for culture were immediately placed at – 20 °C on the day of endoscopic examination and stored at – 80 °C until used for culture testing. Blood samples from all participants were collected on the same day. The serum was separated and frozen at –80 °C until analysis.

Statistical Analysis

Statistical significance of the qualitative differences was calculated using the chi-square. For continuous variables, we carried out one-way ANOVA, followed by Tukey test as a multiple comparison. All statistical analyses were performed using the RStudio 4.2.0 software.

Ethical approval

Written informed consent was obtained from all participants, and the ethical permission was approved by the Mongolian Ministry of Health (accepted number N3, 2015), Mongolian National University of Medical Sciences (N13-02/1A, 2013), and Oita University Faculty of Medicine (Yufu, Japan) (P-12-10, 2013). We obtained four biopsies (three for histology and one for culturing *H. pylori*) from all patients.

Results

In total, 522 patients (176 women and 352 men; mean age, 42.85 ± 13.62 years) with gastritis were enrolled in this study (Table 1). The patients were from the Umnugovi (n = 169), Khentii (n = 128), Khovd (n = 146), Uvs (n = 79) provinces. Of 522 participants, most of them were male patients (66.7 %).

As for CagA sequence type, 30.5 % was ABC, 31 % was ABCC and 38.5 % was ABTC3 type.

In Table 2 we show the general characteristics of the study participants. The value of *H.pylori* IgG antibody titer was 15.27 ± 12.83 in the atrophy group, 18.87 ± 14.90 in the gastritis group and 15.93 ± 12.68 in the reflux group, respectively.

Hematoxylin and eosin staining with May–Giemsa

Table 1. Study participants by provinces.

Variables	Provinces					p-value
	Uvs (n = 79) Mean ± SD	Khuvsgul (n = 146) Mean ± SD	Khentii (n = 128) Mean ± SD	Umnugovi (n = 169) Mean ± SD	Total (n = 522) Mean ± SD	
Age, years	42.39 ± 12.28	41.48 ± 13.34	46.01 ± 12.98	43.48 ± 14.25	42.85 ± 13.62	0.081
IgG	17.29 ± 13.06	16.92 ± 14.04	17.55 ± 14.02	18.13 ± 15.50	17.47 ± 14.17	0.963
Gender	N (%)	N (%)	N (%)	N (%)	N (%)	
Male	44 (55.7)	84 (57.5)	97 (75.8)	115 (71.9)	352 (66.7)	0.001
Female	35 (44.3)	62 (42.5)	31 (24.2)	45 (28.1)	176 (33.3)	
CagA sequence type						
ABC	5 (17.9)	21 (35.0)	12 (25.0)	19 (37.3)	57 (30.5)	0.092
ABCC	12 (42.9)	19 (31.7)	19 (39.6)	8 (15.7)	58 (31.0)	
ABTC3	11 (39.3)	20 (33.3)	17 (35.4)	24 (47.0)	72 (38.5)	

Table 2. General characteristics of the study participants.

Variables	Pathologies				p-value
	Atrophy (n = 142) Mean ± SD	Gastritis (n = 318) Mean ± SD	Reflux (n = 62) Mean ± SD	Total (n = 522) Mean ± SD	
Age, years [a, b]	42.71 ± 12.97	42.82 ± 13.86	47.60 ± 12.96	42.85 ± 13.62	*0.055
IgG [c]	15.27 ± 12.83	18.87 ± 14.90	15.93 ± 12.68	17.47 ± 14.17	*0.009
Gender	N (%)	N (%)	N (%)	N (%)	
Male	80 (56.3)	224 (72.8)	36 (58.1)	352 (66.6)	0.001
Female	62 (43.7)	85 (27.2)	26 (41.9)	176 (33.3)	
Provinces					
Umnugovi	22 (15.5)	136 (40.8)	11 (17.7)	169 (31.4)	0.000
Khuvsgul	7 (4.9)	112 (33.6)	9 (14.5)	128 (23.8)	
Khentii	58 (40.8)	58 (17.4)	30 (48.4)	146 (27.1)	
Uvs	55 (38.7)	12 (3.6)	12 (19.3)	79 (14.7)	

*One-way ANOVA: multiple comparison; [a]Atrophy vs. Reflux, p = 0.045; [b]Gastritis vs. Reflux, p = 0.028. Atrophy vs. Gastritis, p = 0.039 ; [c]Gastritis vs. Atrophy, p = 0.011.

Table 3. Summary of histology, Cag A test results by endoscopic diagnosis.

Variables	Pathologies				p-value
	Atrophy (n = 142) N (%)	Gastritis (n = 318) N (%)	Reflux (n = 62) N (%)	Total (n = 522) N (%)	
Histology					
Yes	49 (37.4)	77 (24.1)	25 (42.4)	151 (29.7)	0.001
No	82 (62.5)	242 (75.8)	34 (57.6)	358 (70.3)	
CagA					
Negative	59 (47.9)	53 (21.5)	26 (50.9)	138 (32.8)	0.000
Positive	64 (52.1)	193 (78.5)	25 (59.1)	282 (67.1)	

Continued

IHC					
No	52 (37.9)	83 (25.7)	25 (40.9)	160 (30.7)	0.006
Yes	85 (62.1)	240 (74.3)	36 (59.1)	361 (69.3)	
HP result last					
No	34 (23.9)	43 (12.9)	17 (27.4)	94 (17.5)	0.001
Yes	108 (76.1)	290 (87.1)	45 (72.6)	443 (82.5)	

Table 4. Serology, culture and urease assay test results.

Variables	Pathologies				p-value
	Atrophy (n = 142) N (%)	Gastritis (n = 318) N (%)	Reflux (n = 62) N (%)	Total (n = 522) N (%)	
Serology					
No	61 (44.2)	103 (33.5)	29 (48.3)	193 (38.2)	0.023
Yes	77 (55.8)	204 (66.5)	31 (51.7)	312 (61.8)	
Culture					
No	103 (72.5)	143 (42.9)	46 (74.2)	292 (54.3)	0.000
Yes	39 (27.5)	190 (57.1)	16 (25.8)	245 (45.7)	
H.pylori subtypes					
S1	64 (78.0)	178 (84.4)	23 (67.6)	265 (81.0)	0.000
S2	18 (22.0)	33 (15.6)	11 (32.4)	62 (19.0)	
Urease assay					
No	57 (40.1)	117 (35.1)	30 (48.3)	204 (37.9)	0.117
Yes	85 (59.8)	216 (64.9)	32 (51.7)	333 (62.0)	

Table 5. H.pylori genotyping results.

Variables	Pathologies				p-value
	Atrophy (n = 142)	Gastritis (n = 318)	Reflux (n = 62)	Total (n = 522)	
CagA sequence type	N (%)	N (%)	N (%)	N (%)	
ABC	12 (22.6)	42 (34.7)	3 (23.1)	57 (30.4)	0.082
ABCC	23 (43.4)	29 (23.9)	6 (46.2)	58 (31.0)	
ABTC	18 (33.9)	50 (41.3)	4 (30.8)	72 (38.5)	

confirmation by IHC showed in Table 3. 67.1 % of the participants were CagA positive and 32.8 % were negative. Immuno-histological analysis revealed the prevalence of *H. pylori* infection in the gastric mucosal atrophy group with 62.1 %, in the gastritis group with 74.3 % and the gastroesophageal reflux group with 59.1 %.

According to the histological analysis, the infection rate was 62.5 % in the gastric mucosal atrophy group, 75.8 % in the gastritis group and 57.6 % in the gastroesophageal reflux group.

In Table 4, serology, culture and urease assay tests were shown. 59.8 % of the atrophy group, 64.9 % of the gastritis group and 51.7 % of the reflux group was urease positive. Moreover, among the 245 culture-positive patients were gastritis in 190 (57.1 %), followed by atrophy in 39 (27.5 %) and reflux in 16 (25.8 %).

We next determined the CagA sequence profile in each

group of patients. As shown in Table 5, 22.6 % of the gastric mucosal atrophy group was ABC type, 43.4 % was ABCC type and 33.9 % was ABtC type. On the other hand, in the gastritis group, the highest type was the ABTC type (41.3 %) and the lowest was the ABCC type. The gastroesophageal reflux group was similar to the gastric mucosal atrophy group.

Discussion

Chronic infection-induced with *H.pylori* strains carrying the CagA gene cluster is the strongest risk factor for gastric cancer. The encoded CagA protein is then delivered into gastric epithelial cells and undergoes tyrosine phosphorylation at the EPIYA motifs. *H.pylori* is divided into 2 isoforms according to the structural diversity within EPIYA motifs. The EPIYA repeat region of Western CagA has an arrangement of EPIYA-A, EPIYA-B and EPIYA-C segments, and the EPIYA-C segment variably multiplies in tandem. On the other hand, the East Asian variant has the same EPIYA- A and EPIYA-B segments but not the repeatable EPIYA-C segment. This variant has a distinct EPIYA-containing segment, termed EPIYA-D [16 - 20]. It has been demonstrated that the vast majority of gastric cancers are caused by *H. pylori* infection. The prevalence of the infection greatly varies geographically and significantly depends on the economic development of countries. Meta-analysis conducted with Zamani et al. presented that disease prevalence varied by development status (51 % in developing vs. 35 % in developed countries) but not by gender [21]. The prevalence of *H. pylori* infection varies significantly. Developing countries are found the highest and developed countries are lower in Asia. Moreover, the seroprevalence rates are different within the countries. The previous study showed that the prevalence of stomach and esophageal cancers in Mongolia is highest in Asia Pacific region [22].

CagA seropositivity and disease severity associations have been reported in several studies. Jones et al conducted a large-scale molecular epidemiologic analysis of South Korean strains and reported that East Asian CagA EPIYA-ABD genotype did not strictly correlate to expression and delivery of CagA in all noncancer strains [23]. Our research team previously showed that among 368 patients (341 gastritis, 10 peptic ulcer, and 17 gastric cancer), 16.8 % strains were CagA-negative and 83.1 % were CagA-positive (293 Western-type, 12 East-Asian-type, and one hybrid type). Further, all *H. pylori* from gastric cancer patients possessed Western-type CagA [15]. We have performed, in the present study, a combined analysis of histochemical stains and IHC in order to confirm the *H.pylori* infection of patients in 5 different provinces and Ulaanbaatar city, Mongolia. 67.1 % of participants had CagA positive and 69.3 % were immunohistochemically positive. Our study confirmed that, by the hematoxylin and eosin staining with May–Giemsa confirmation by IHC, the prevalence of *H. pylori* infection in the gastric mucosal atrophy group was 62.1 %, while in the gastritis and gastroesophageal reflux group, the rate was 74.3 % and 59.1 %, respectively. The infection rate according to the histological analysis was 62.5 % in the gastric mucosal atrophy group, 75.8 % in the gastritis group and 57.6 % in the gastroesophageal reflux group. CagA sequence profile in each group of patients revealed that 22.6 % of the gastric mucosal atrophy group was ABC type, 43.4 % was ABCC type and 33.9 % was ABtC type. On the other hand, in the gastritis group, the dominant type was ABtC (41.3 %). In the gastroesophageal reflux group, the ABCC type was dominant while 23.1 % was ABC type. In our previous study, 32 % of gastritis patients had ABCC type, which is lower than our present study. In the study of Chowdhury et al. among *H. pylori*-positive cases, the majority of gastroduodenal cases were ABC type (57.9 %), and 26.3 % had ABCC type [24 - 27]. Another study also presented that, the intestinal metaplasia scores in the antral of patients in Myanmar infected with the ABC and ABCC types of CagA were significantly higher than those of patients with AB- type CagA [28 - 32].

Our study has some limitations. First, our present study was limited to patients from five provinces in Mongolia and the results cannot certainly be generalized to other provinces. Second, we did not include covariates such as age in the analysis. Numerous studies demonstrated that age is an important determinant of gastric cancer risk therefore, it would be important to apply different covariates to the analysis. Third, even though CagA is an extremely known *H.pylori* virulence marker, it is not sufficient to conclude the relationship between the prediction of clinical outcomes and *H.pylori* serotypes. Further study may be necessary to elucidate other virulence factors on the prevalence of the infection and to confirm the same immunohistochemical outcomes using other patients from various provinces.

Conclusions

This is the first study using immunohistochemistry to confirm *H. pylori* infections of patients across different regions in Mongolia. Five hundred and thirty-eight patients were enrolled in this study (142 gastric mucosal atrophy, 333 gastritis and 62 gastroesophageal reflux). All histological results showed that the gastritis group was significantly higher in patients positive for *H. pylori* than in antral-predominant gastritis.

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