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Distribution of *Helicobacter pylori* in north China

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Abstract

AIM: To compare the distribution of virulence-associated genotypes of *Helicobacter pylori* (*H pylori)* in two areas of north China with different gastric cancer risk and furthermore probe into the pathogenicity of the bacterium.

METHODS: Gastric biopsies were taken from 355 subjects from Zhuanghe, a high risk area of gastric cancer, and 136 subjects from Shenyang, a low risk area of gastric cancer. A total of 149 *H pylori* strains isolated from these patients were studied by PCR for differences in the genotypes of cagA, vacA, and iceA.

RESULTS: In patients with high risk for gastric cancer, higher frequencies of vacA s1 or s1m1b genotypes were found as compared to those from the low risk area.

CONCLUSION: There is significantly different distribution of *H pylori* genotypes between Zhuanghe and Shenyang areas in north China.

Key words: *Helicobacter pylori*; Gastric disease; cagA; vacA; iceA; Virulence genotype; High-risk area

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INTRODUCTION

Helicobacter pylori (*H pylori*) colonizes the human stomach and establishes long-term infection of the gastric and duodenal mucosa[1]. Although *H pylori* infects approximately half of the world's population, only a small proportion of infected subjects develop symptoms or clinically significant diseases. The reasons for this fact are unknown but may be related to the host's immunological defenses, environmental factors and/or the virulence of different strains of the bacteria[2,3]. Several genes have been identified that may play a role in the pathogenicity of *H pylori* genotypes such as cagA, vacA, and iceA[4].

Many researches have shown *H pylori* genotype not only has a different disease association but also has a particular geographic distribution. $CagA^+$ strains are more commonly associated with peptic ulceration, atrophic gastritis, and adenocarcinoma of the stomach than $cagA^-$ strains[5,6] in many northern countries. Subtype vacA s1a is predominant in populations of northern European ancestry, and is associated with duodenal ulcer. Subtype s1b is predominant in Africa and very frequently found in Portugal, Spain, and Central and South America. In France, Italy, and the USA, the frequency of s1a and s1b genotypes is similar[7]. In Guangdong and Zhejiang Provinces, and Shanghai of China, the main genotype is vacAs1m1[8,9]. IceA genotype also has a particular geographic distribution. IceA1 is predominant in Japan and Korea, iceA2 is predominant in America. In Columbia iceA2 is the predominant genotype in gastric cancer and gastritis. But the distribution of *H pylori* genotypes in north China is not reported.

Disease associations (e.g., with duodenal ulcers) have been proposed for the cag pathogenicity island (cagA for marker), vacA and iceA. However, these associations are not consistent in different geographic regions. Though genotyping of cagA, vacA, and iceA appears not to be useful for disease specificity in some regions, it may play a role in molecular epidemiological studies in terms of identifying the predominant *H pylori* strain that is circulating in a given geographic area.

Zhuanghe in Liaoning Province is a high-risk area of gastric cancer in north China. The mortality rate of gastric cancer is 50 per 100 000 persons, compared to 14.5 per 100 000 (1995) persons in Shenyang, a low risk area of north China[10]. The prevalence of *H pylori* infection in adults from Zhuanghe is more than 60%, compared to 12% from Shenyang. A previous research from the high-risk area in Zhuanghe reported that there is a significantly positive relationship between gastric diseases or precancerous lesions and *H pylori* infection[11], suggesting there may be some relationship between *H pylori* infection and gastric cancer incidence in these areas. But whether the high incidence of gastric cancer in the high-risk area has a relationship with specific *H pylori* genotype is still unclear. The aim of this study was to compare the distribution of *H pylori* genotypes in the two areas with different cancer risk and furthermore explore its geographic characteristics.

MATERIALS AND METHODS

Patients

A total of 491 cases were involved in this study including 136 cases from Shenyang (69 men and 57 women, 25-78 years, mean age: 48.61 years), 355 cases from Zhuanghe (174 men and 181 women, 21-79 years, mean age: 49.33 years). Their biopsies were obtained during endoscopy with informed consent. One antrum biopsy was taken for culture and stored at -70 ℃ in 0.5 mL of brucella broth (Difco) with 15% glycerol until incubation. Three biopsies were taken for pathology diagnoses, from gastric antrum, corpus, and angularis, respectively.

H pylori culture

Culture was prepared by smearing single biopsy specimens on petri plates containing brain heart infusion (BHI) agar (Difco) supplemented with 7% sheep blood, 0.4% IsoVitale X, amphotericin B (8 μg/mL), trimethoprim (5 μg/mL), and vancomycin (6 μg/mL) and incubated at 37 ℃ in an atmosphere of 5% O_2-100 mL/L CO_2-85% N_2 for 3-6 d. *H pylori* colonies were identified based on their typical morphology, characteristic appearance on Gram staining, a positive urease test, and subsequent gene-specific PCR tests. *H pylori* cells that grew out from biopsy on the primary culture plate were collected as a pooled population, and preserved in sterile BHI broth with 15% glycerol at -70 ℃. In general, only one such a culture was analyzed per patient.

DNA extraction

The strain was centrifuged, the supernatant was removed, and then suspended in cell lysis, incubated at 37 ℃ overnight. The DNA was extracted with phenol-chloroformisoamyl alcohol by standard procedures and precipitated by the addition of 1/10 volume of ammonium acetate and 2.5 volume of cold ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol and dissolved in TE buffer (10 Mm Tris-HCl [pH 8.3], 0.1 mmol/L EDTA).

Analysis of vacA, cagA, and iceA by PCR

The integrity of the DNA was assessed by 0.7% agarose gels stained with ethidium bromide. Polymerase chain reaction (PCR) was performed in a volume of 20 μL containing 10 pmoL of primer, 0.5 μL genomic DNA, 2.5 mmol/L of each of 4 dNTPs (Takara Company), and 2.5 U of Taq DNA polymerase (Takara Company). PCR amplifications were performed in an automated thermal cycler (Biometra Co., Germany). Table 1 summarizes the primer sequences and the expected size of PCR products. The following cyclical conditions were used: for vacA: 35 cycles of 1 min at 94 ℃, 1 min at 52 ℃, and 1 min at 72 ℃; for cagA: 1 min at 94 ℃, 1 min at 50 ℃, and 1 min at 72 ℃; for iceA: 1 min at 94 ℃, 1 min at 55 ℃, and 1 min at 72 ℃. The amplified PCR products were resolved in 2% agarose gels containing 0.5*TBE, stained with ethidium bromide and visualized under a short wavelength ultraviolet light source.

Statistical analyses

Data were analyzed using SPSS for windows version 11.0. The χ^2 test or Fisher's exact test was used to assess the relationships between different areas. $P<0.05$ was considered statistically significant.

RESULTS

A total of 149 *H pylori* strains out of 491 biopsies from two geographic areas in China were obtained. The high-risk group comprised 102 strains from Zhuanghe. The low risk group comprised 47 strains from Shenyang. cagA, vacA, and iceA genotypes of *H pylori* strains were analyzed by PCR. Genotyping results are summarized in Table 2.

Detection of cagA gene in two areas

There was a high prevalence of the $cagA^+$ strain in the two areas. The cagA gene was detected in 101 of 107 *H pylori* isolates in Zhuanghe (94.4%) and 42 of 42 in Shenyang (100%). There was no difference in the distribution between the two areas. Six strains did not yield any PCR product for cagA (Table 2 and Figure 1A).

Detection of vacA gene in two areas

Specific primer was used to type allele of the vacA gene. The prevalence of vacA s1 strains was significantly higher in patients from Zhuanghe (95.33%), as compared to those from Shenyang (64.29%, $P<0.001$). The positive isolate

Table 1 PCR primers for amplification of cagA, vacA and iceA sequences

Gene and DNA region	Prime	Primer sequence (5′-3′)	Size of PCR product (bp)	References
cagA	CAGAF	GGCAATGGTGGTCCTGGAGCTAGGC	324	Pan[21]
	CAGAR	GGAAATCTTTAATCTCAGTTCGG		
vacAs1 and s2	VA1-F	ATGGAAATACAACAAACACAC	259/286	Atherton[22]
	VA1-R	CTGCTTGAATGCGCCAAAC		
m1a	VA3-F	GGTCAAAATGCGGTCATGG	290	Atherton[22]
	VA3-R	CCATTGGTACCTGTAGAAAC		
m1b	VAm-F3	GGCCCCAATGCAGTCATGGAT	291	Atherton[23]
	VAm-R3	GCTGTTAGTGCCTAAAGAAGCAT		
m2	VA4-F	GGAGCCCCAGGAAACATTG	352	Atherton[22]
	VA4-R	CATAACTAGCGCCTTGCAC		
iceA1	iceA1F	GTGTTTTTAACCAAAGTATC	247	Peek[10]
	iceA1R	CTATAGCCASTYTCTTTGCA		
iceA2	iceA2F	GTTGGGTATATCACAATTTAT	229/334	Peek[10]
	iceA2R	TTRCCCTATTTTCTAGTAGGT		

R = A/G.

yielded a 259-bp PCR fragment characteristic of the s1 (potentially more virulent) allele, and none yielded the 286-bp fragment characteristic of the s2 (less virulent) allele in both areas (Figure 1B). Twenty strains did not yield any PCR product for vacA s (Table 2).

Table 2 Distribution of *cagA,vacA and iceA* genotypes in Zhanghe and Shenyang

Gene	Zhuanghe area (%) n = 107	Shenyang area (%) n = 42
cagA$^+$	101 (94.4)	42 (100.00)
cagA$^-$	6 (5.6)	0 (0)
vacA s1	102 (95.33)[b]	27 (64.29)
vacA s2	0 (0)	0 (0)
vacA s$^-$	5 (4.67)[d]	15 (35.71)
vacA m1a	0 (0)	0 (0)
vacA m1b	24 (22.43)	5 (11.9)
vacA m2	26 (24.3)	11 (26.19)
vacA m1b/m2	48 (44.96)	22 (52.38)
vacA m$^-$	9 (8.41)	4 (9.52)
iceA1	10 (9.35)	1 (2.38)
iceA2	10 (9.35)	1 (2.38)
iceA1/iceA2	84 (78.5)	32 (76.19)
iceA$^-$	3 (2.8)	8 (19.05)

[b]P<0.001 *vs* vacA s1 in Shenyang area. [d] P<0.001 *vs* vacA s- in Shenyang area.

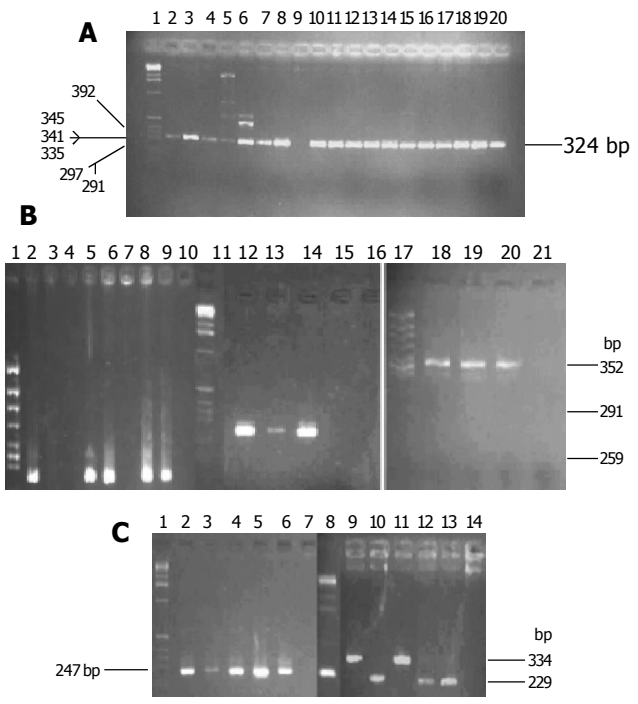

Figure 1 Two percent agarose gel electrophoresis of cagA(A), vacA(B) and iceA (C) PCR products. **A:** Lane 1: DNA maker; lane 9: negative cagA, other lanes: cagA (324 bp); **B:** lane 1, 11 and 17: DNA maker, lanes 2,5,6,8,9: vacA s1 (259 bp), lanes 12-14:vacA m1b (291 bp), lanes 18-21: vacA m2 (352 bp); **C:** lanes1, 8: DNA maker, lane 2-6: iceA1 (247 bp), lanes 9-14: iceA2 (229 or 334 bp).

The prevalence of vacA m1b and m2 strains had no difference in both areas (22.43% *vs* 11.95%, 24.3% *vs* 26.195%, respectively, P>0.05). VacA m1a was not found in either area. Forty-eight and twenty-two strains were found, respectively in the high and low risk areas, m1b and m2 were detected in one patient at the same time. Fourteen strains of vacA m gene could not be genotyped (Table 2 and Figure 1B).

Based on analysis of the vacA s and m region, we examined vacA s1m1b, vacA s1m2, vacA s1m1b/m2, vacA s1m$^-$, vacA s$^-$ different combinations in patients. The vacA genotype s1m1b was significantly higher in Zhuanghe than in Shenyang (20.56% *vs* 2.38%, P<0.01). The predominant vacA genotype in both areas was s1m1b/m2. However, the difference was not statistically significant (Table 3).

Detection of iceA gene in two areas

IceA gene could be genotyped as iceA1 and iceA2 with specific primers. The prevalence of iceA1 and iceA2 strains had no difference in both areas (9.35% *vs* 2.38%, 9.35% *vs* 2.38%, respectively, P>0.05). Eighty-four isolates (78.5%) in Zhuanghe and 32 isolates (76.19%) in Shenyang were positive for both iceA1 and iceA2, and 11 isolates (21.85%) did not yield any PCR product for iceA (Table 2 and Figure 1C).

Detection of cagA, vacA, and iceA combination genotypes in two areas

We examined several different combinations based on the analysis of the vacA s region (s1 and s2) and m region (m1a, m1b, m2), cagA (positive and negative), and the iceA type (iceA1 and iceA2) in patients (Table 4). The predominant combination genotypes in both areas were cagA$^+$, vacA s1/m1b/m2, iceA1/iceA2 (36.45% *vs* 33.3%, P>0.05, Table 5).

DISCUSSION

More than half of the people are infected with *H pylori* in the world, but not all individuals developed associated diseases[12]. This may be related to a complex of environmental factors, host characteristics and bacterial virulence determinants. Several virulence-associated factors of *H pylori* have been associated with clinical outcomes of the infection. In the early study we have reported that more than 60% individuals in the high-risk area of gastric cancer in Zhuanghe are infected with *H pylori*; and the infection has a significant association with gastric disease[11]. But the geographic characteristics of *H pylori* infection in north China have not been well described before. In the present research, we examined the distribution of *H pylori* genotypes in the high and low risk areas of gastric cancer in north China. The results indicate that vacA s1 or s1m1b genotype is more prevalent in Zhuanghe where gastric cancer incidence is high, whereas

Table 3 vacA genotypes of *H pylori* strains in Zhanghe and Shenyang

Area	n	s1$^+$				
		m1b(%)	m2(%)	m1b/m2(%)	m1b$^-$/m2(%)	S1$^-$ (%)
Zhuanghe	107	22 (20.56)[b]	25 (23.36)	46 (42.99)	9 (8.42)	5 (4.67)
Shenyang	42	1 (2.38)	9 (21.43)	13 (30.95)	3 (7.14)	15 (35.71)[d]

[b]P<0.01 *vs* vacA s1m1b group in Shengyang. [d]P<0.001 *vs* vacA s$^-$ group in Zhuanghe.

Table 4 Combination genotypes of *cagA*,*vacA* and *iceA* in Zhuanghe and Shenyang, *n* (%)

cagA	vacAs	vacAm	iceA	Zhuanghe (*n* = 107)	Shenyang (*n* = 42)
cagA	s1	m1b	A1	3 (2.8)	0 (0)
cagA	s1	m1b	A2	3 (2.8)	0 (0)
cagA	s1	m1b	A1+A2	13 (12.15)	0 (0)
cagA	s1	m2	A1	3 (2.8)	0 (0)
cagA	s1	m2	A2	1 (0.935)	1 (2.38)
cagA	s1	m2	A1+A2	18 (16.82)	5 (11.9)
cagA	s1	m1b+m2	A1+A2	39 (36.45)	14 (33.3)
cagA	s	m1b+m2	A1+A2	2 (1.87)	7 (16.67)
		Others		25 (23.36)	15 (14.02)

Table 5 Multiple strains infection in Zhuanghe and Shenyang, *n* (%)

Genotype	Zhuanghe (*n* = 107)	Shenyang (*n* = 42)
vacA m1/m2	48 (44.86)	22 (52.38)
iceA1/iceA2	84 (78.5)	32 (76.19)
vacAm1/m2 or iceA1/iceA	92 (85.98)	33 (78.57)

vacA s¯genotype is relatively more frequent in Shenyang where gastric cancer incidence is very low. Vacuolating cytotoxin encoded by vacA gene can aggregate into flower-shaped hexamers and heptamers, which represent the mature active toxin. The mature toxin is peculiarly suited to the gastric environment because it is activated by acid and its activated form causes more profound epithelial changes[13]. Infection with *H pylori* possessing vacA s1 is associated with a higher degree of neutrophil and lymphocytic infiltration of the human gastric mucosa, and the presence of cagA[14]. In human stomach, strains with vacA m1 allele are associated with severe epithelial damage compared to those with m2 allele[15]. The different m types seem to recognize different receptors on epithelial cells and may induce different intracellular responses. Previous studies have shown that vacA s1m1 strains produce large amounts of vacuolating cytotoxin and that these strains are associated with peptic ulcer disease (PUD)[16]. On the other hand, the s2m2 strains produce no or only small amounts of cytotoxin and are uncommon in patients with PUD. The s1m2 strains seem to take an intermediate position[16]. Miehlke *et al*[17], reported that the vacA s1, m1 genotypes are more frequently detected in *H pylori* from gastric cancer (GC) patients (70.6%) than from mucous associated lymph tumor (MALT), duodenal ulcer (DU), and functional dyspepsia (FD) patients ($P<0.05$) and may be used to identify infected patients at an increased risk for GC. A recent study showed that, when cocultured with AGS gastric epithelial cells, *H pylori* strain 60190, which expresses s1m1 VacA toxin, induces significantly higher levels of apoptosis than isogenic vacA null mutant strain[18]. The risk for developing gastric cancer is >90-fold higher in patients with severe multifocal atrophic gastritis than in patients with normal mucous[19]. Thus, patients infected with vacA s1m1 strains have a higher risk of carcinogenesis. In our study, distribution of the vacA s1m1 genotype was found in the high and low risk areas, this may be a factor contributing to the higher gastric cancer incidence in the high-risk area. Some conclusions may be drawn from the fact that patients infected with this strain are the high-risk persons for gastric cancer.

In addition, we did not find vacA s genotype in 20 isolates. This is currently unexplained and may be due to the existence of additional vacA. Pan *et al*[20], reported that 78 of 96 *H pylori* isolated from Shanghai and Guangzhou carry m2, they thought m1b alleles seem infrequent in China. But in our study 24 (22.42%) isolates from Zhuanghe and 5 (11.95%) isolates from Shenyang carried the canonical m1b allele that has a similar prevalence to m2 allele (24.3% and 26.9%, respectively). The distinct distribution of vacA alleles in different regions of China suggests that *H pylori* distribution has significant geographical characteristics. The studies of isolates from patients in different countries or regions give only a partial view of *H pylori* as a globally distributed human pathogen.

Detailed molecular analyses[16,21] have shown that each *H pylori* strain only contains a single vacA s region, m region and iceA allele. VacA s1 and s2, m1 and m2, as well as iceA1 and iceA2 can be considered as mutually exclusive genotypes of a single strain. Consequently, if multiple genotypes are found, this is a strong indication of the presence of multiple strains. In our study, 48 cases in Zhuanghe (44.86%) and 22 cases in Shenyang (52.38%) showed evidence of multiple vacA genotypes. In the iceA locus, 84 cases (78.5%) and 32 cases (76.19%) in the high and low areas were positive for iceA1 and iceA2. Considering vacA and iceA genes, the presence of multiple *H pylori* strains was found respectively in 92 (85.98%) and 33 (78.57%) patients from the two areas. It may be speculated that more than one strain may be acquired in childhood, especially in countries with a very high prevalence of *H pylori*. It is not known whether multiple strains colonize simultaneously (co-infection) or at different time points (superinfection). The co-existence of more than one strain in the same individual may reflect the adaptation of multiple bacterial genotypes to different, non-overlapping microniches in the same stomach. The dynamics of co-colonization by multiple strains has been studied in animal models[22]. Non-human primates experimentally challenged with a mixture of strains show only a transient infection by more than one strain, and then a single strain becomes predominant over the others[22]. The early study from Mexico found there is some association between multiple strain infections and peptic ulceration[18]. One possible reason for such an association may be that multiple strain infection increases the chance of infection with a more pathogenic strain or mixed *H pylori* strains may act synergistically to persist in the stomach and cause damage[23]. Whether multiple strain infection has some practical significance in north China is to be discussed further.

Combined analysis of vacA, cagA, and iceA genotypes may permit identification of high-risk patients infected with more pathogenic *H pylori* strains. Eventually, patients infected with such strains could be selected for prophylactic anti-*H pylori* treatment to prevent associated gastric diseases later in life. For example, Figueiredo *et al*, reported that the s1/m1/cagA+/iceA1 and s1/m1/cagA+/iceA2 strains are more predominant in patients with duodenal ulcer (27.6% and 17.2%), gastric ulcer (50% and 37.5%) and gastric carcinoma (36.8% and 36.8%), whereas s2/m2/cagA-/iceA2 strains are predominant in patients with gastritis only (31.3%). Yamaoka *et al*[4], used PCR to examine iceA, vacA, and cagA status of 424 *H pylori* isolates obtained from patients of four different countries and found that the cagA+/iceA1/vacAs1cm1 genotype is predominant in Japan and Korea, the cagA+/iceA2/vacAs1bm1 genotype is predominant in the USA, and the cagA+/iceA2/vacAs1am1 genotype is predominant in Columbia. But there is no association between the iceA, vacA, or cagA status and clinical outcome. In our study, the predominant genotype in both high- and low-risk areas was cagA+/vacAs1m1bm2/iceA1/iceA2 and had no correlation with other reports. This may be ascribed to the high percent of multiple strain infection in both areas. Whether this result has some relationship with associated gastric diseases is to be discussed further.

In our study, the cagA gene was found in all the 42 isolates from Shenyang and 101 (94.4%) isolates from Zhuanghe. There was no difference in the distribution of *H pylori* genotypes in the two areas. This high prevalence contrasts with the 30% cagA- frequency of *H pylori* in Western countries. Bravo *et al*[12], also reported that higher frequency of cagA is in patients from high risk areas of gastric cancer than in those from low risk areas of gastric cancer. Some persons showed that cagA positive strains are associated with higher gastric cancer risk. But in our study, there was no significant difference in cagA status between the two areas. This provides further evidence on *H pylori* genotypes circulation in west and north China.

In conclusion, the present study showed the distinct distribution of *H pylori* virulence genotypes in two areas of north China with different gastric cancer risk. Further studies are required to determine the epidemiological and clinical importance of *H pylori* virulence-associated genotypes in different geographic areas in China.

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