Prevalence and Correlation with Clinical Diseases of *Helicobacter pylori* cagA and vacA Genotype among Gastric Patients from Northeast China

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*Helicobacter pylori* vacA and cagA genes have significant genetic heterogenicity, resulting in different clinical outcomes. Northeast part of China has reported high prevalence of *H. pylori* infections and gastric cancer. Hence, we investigated the *H. pylori* cagA and vacA genotypes with clinical outcomes in Northeast China. Gastric tissue samples (*n* = 169), chronic gastritis (GIs), gastric ulcer (GU), and gastric cancer (GC) were analysed for 16SrRNA, ureA, cagA, and vacA genotypes by PCR. A total of 141 (84%) cases were found positive for *H. pylori* by 16S rRNA and ureA. GC showed high *H. pylori* infection (93%) compared with GIs (72%) and GU (84%). The vacA s1am1 was highly found in GC (40%) and GU (36%), vacA s1bm1 (14%) and vacA s1bm2 (8%) in GU cases, and s2m1 in normal cases (33%), while vacA s1cm1 showed low frequency in GIs (2%) and GU (3%) and GC showed negative result. The East-Asian cagA strain was highly observed in GC (43%), as compared to GIs (41%) and GU (20%). The East-Asian cagA/vacA s1am1 was significantly higher in GC (23%) than in GU (22%) and GIs (14%) patients. The East-Asian type cagA with vacAsla and vacAml is the most predominant genotype in *H. pylori* strains of Northeast China.

1. Introduction

*Helicobacter pylori* is a causative agent of gastritis (GIs) and gastric ulcer (GU) diseases, which leads to the development of gastric cancer (GC). Hence it is classified as class I carcinogen by the World Health Organization. Developing countries have *H. pylori* prevalence rate of 80% as compared to developed countries (20–50%) [1, 2]. Genetic diversity may play an important role in genotypic variation of *H. pylori*, which makes it more virulent with diverse pathogenicity [3]. *H. pylori* carries different virulence factors, such as urease, flagellar, vacuolating cytotoxin A (VacA), and cytotoxin-associated gene A (CagA), that play an important role in invasion, colonization, and cell proliferation [4, 5]. High genetic variations of cagA and vacA gene are associated with more severe infections of *H. pylori* [6].

VacA toxin encoded by vacA gene induces cytoplasmic vacuoles and increases permeability, which leads to the damage of gastric epithelial cells [7]. The vacA gene exhibits significant allelic variation in the signal (s) and middle (m) regions. The s-region consists of two major subtypes (s1 and s2) and s1-region has further three subtypes (s1a, s1b, and s1c), whereas m-region designates m1 and m2 subtypes [8, 9]. A pleomorphic combination of s and m regions affects the vacuolating activity of vacA gene [10]. Different genotypic combination of vacA region results in different pathogenicity level as follows: slam1 and sibm1 produce high amount of toxin and are considered the most virulent as compared to slm2, which produces moderate vacuolating toxins [11, 12]. However, s2m1 and s2m2 are considered less toxic because of their inability to form vacuoles [9]. The slam1 and sibm1 subtypes are frequently reported in acute gastritis (GIs), peptic ulcer, and gastric cancer patients, while s2m1 and s2m2 have been reported in the gastric ulcer (GU) patients [9, 13].

Cytotoxin-associated gene A (CagA) is a cytotoxin-associated protein, linked with peptic ulcer and gastric cancer
2. Materials and Methods

2.1. Patients and Gastroendoscopy. All gastric patients underwent gastroendoscopy and examination at Dalian 1st & 2nd Affiliated Hospital, Dalian, China. Gastric tissues were obtained from the antral and corpus part of the stomach during gastrointestinal endoscopy. Gastric patient’s tissue samples were characterized as chronic gastritis (GIs), gastric ulcer (GU), and gastric cancer (GC). Clinical diseases were diagnosed by endoscopic appearance as "normal" in cases of intact mucosa, chronic gastritis, gastric ulcer, and gastric cancer. All the samples were processed and frozen at −20°C until tested. Fresh surgical gastric cancer tissues were collected in transport media and analyzed in laboratory. All gastric samples collected from the patients and the research protocols were in accordance with the Institutional Review Board of Dalian Medical University.

2.2. DNA Extraction. The SDS-PK method was adopted to extract DNA from gastric patient’s tissues. Gastric tissues were crushed manually and resuspended in 20 μL of 10% SDS, 80 μL of proteinase K buffer (0.5 M EDTA and 4 M NaCl, pH 7.5), 40 μL of proteinase K (10 mg/mL) and make final volume up to 380 μL with sterile water. The mixture was incubated at 55°C overnight. The following day, 100 μL of 6 M NaCl was added, followed by centrifugation at 14,000 rpm for 5 min and the supernatant was separated in new tube. To precipitate the DNA, 500 μL of absolute isopropanol was added, mixed well and centrifuged for 5 minutes at 9000 rpm. DNA pellet was washed with 70% ethanol and air dried. The pellet was resuspended in 50 μL of TE buffer (10 mM Tris and 1 mM EDTA; pH 8.0). Samples were stored at −20°C until used.

2.3. Polymerase Chain Reaction (PCR). PCR was performed to detect H. pylori by using specific primers. Target gene, amplicon size, primer names, and sequences are shown in Table 1. For PCR amplification, 1-2 μg of DNA samples was added to a PCR mixture containing 20 pmol forward and reverse primers, 1.5 mM MgCl2, 1.5 U of Taq polymerase (Takara, Japan), 2.5 μL PCR buffer, and 200 μM of dNTPs to the total volume of 25 μL. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s (Table 1), polymerization at 72°C for 30 s, and final polymerization at 72°C for 5 min (Bio-Rad Thermocycler). The PCR reaction products were electrophoresed on 1.5% agarose gel with 2000 bp DNA ladder (Takara, Japan) and the bands were visualized by ethidium bromide staining, followed by analysis with Quantity One software (Bio-Rad, USA). H. pylori strain was detected by using specific primers targeting 16S rRNA and ureA genes (Table 1). The cagA and vacA statuses were determined from H. pylori positive samples by PCR using their respective primers as described in Table 1.

2.4. Statistical Analysis. Categorical data were analysed by using chi-square test. P value of less than 0.05 was regarded as significant. The statistical software GraphPad prism 5.03 was used for analyzing the data.

3. Results

3.1. Gastric Patients History. This study was designed to determine the frequency of cagA and vacA genotypes in gastritis (GIs), gastric ulcer (GU), and gastric cancer (GC) patients from the northeast part of China. A total of 169 gastric patients, 63 males and 106 females ranging in age from 10 to 80 years (average age of 56.32 ± 2.394), were included in the study. Among 169 cases, 51 (30.17%) were diagnosed as GIs, 36 (21.30%) were diagnosed as GU, and 73 (43.19%) samples were regarded as GC, while 9 (5.32%) were normal cases.

3.2. Detection of H. pylori Infection. Helicobacter pylori specific genes (ureA and 16S rRNA) revealed that (141) 84% of samples were H. pylori positive. These included 6 (54%) normal, 37 (72%) GIs cases, 30 (84%) GU and 68 (93%) of the H. pylori positive GC cases (Table 2).

3.3. Detection of the H. pylori vacA Genotyping and Clinical Manifestations. Among 141 H. pylori positive gastric tissues, we determined six different allelic variants of the vacA gene in H. pylori positive gastric tissues. Among H. pylori positive gastric tissues, 51 (36%) were positive for vacAslaml1, 24 (17%) for vacAslaml2, and 7 (5%) for vacAslaml3, whereas 7 (5%) were vacAslaml2, 2 (2%) were vacAslaml1, and 12 (6%) were positive for vacAslaml2. As shown in Table 2, detailed analysis revealed that 29 (40%) of the GC cases had slaml1 genotype
Table 1: Primer sets used for genotyping *H. pylori* by PCR.

| Target site | Amplicon size (bp) | Primer names and sequences | Annealing temperature | References |
|-------------|--------------------|---------------------------|-----------------------|------------|
| 16S rRNA    | 138                | HP-F (5-GCGACCTGCTGGAACATTAC-3) | 60°C                  | Gramley et al., 1999 [18] |
|             |                    | HP-R (5-CGTTAAGCTCATTGAGGAGA-3) |           |            |
| *UreA*      | 411                | HP1-F (5-GCCAATTGATATAATGGTT-3) | 45°C                  | Smith et al., 2004 [19] |
|             |                    | HP1-R (5-CTTCTAAATGGTTTTGCA-3) |           |            |
| *vacAsla*   | 190                | AA1-F (5-GGGCAGATCAGCGCAAC-3) | 56°C                  | Atherton et al., 1995 [9] |
|             |                    | AA1-R (5-TCGCTTGAATGCGCCAAAC-3) |           |            |
| *vacAslb*   | 187                | SS3-F (5-AGCGCAGCAGCGCAAGAG-3) | 56°C                  | Atherton et al., 1995 [9] |
|             |                    | SS3-R (5-TCGCTTGAATGCGCCAAAC-3) |           |            |
| *vacAslc*   | 213                | SIc-F (5-CTCATATGCTGCTGCA-3) | 56°C                  | Yamaoka et al., 1999 [20] |
|             |                    | SIc-R (5-CTGCTTGAATGCGCCAAAC-3) |           |            |
| *vacAs2*    | 199                | SS2-F (5-GCTAATGCAGGGCTAATGAG-3) | 56°C                  | Atherton et al., 1995 [9] |
|             |                    | SS2-R (5-CTGCTTGAATGCGCCAAAC-3) |           |            |
| *vacAm1/m2* | 570/645           | VAG-F (5-GATTCAAGCAACACCAAGAG-3) |           | Yamaoka et al., 1999 [20] |
|             |                    | VAG-R (5-GCGTCTAATAATTCGAAGAG-3) |           |            |
| *cagA*      | 189                | CAGA-F (5-TGAGCAGCAGGCAACAGA-3) | 62°C                  | van Doorn et al., 1998 [21] |
|             |                    | CAGA-R (5-TCCTCCTTTATGCGCGAG-3) |           |            |
| Western type *cagA* | Variable | CAGT-F (5-ACAGCTGAGCTGAGG-3) | 61°C                  | Yamaoka et al., 1999 [22] |
|             |                    | CAGW-R (5-TCCGTTACAGCCCAAGAC-3) |           |            |
|             |                    | CAGW-F (5-AAAAAATGACGACCTCAATC-3) |           |            |
|             |                    | CAGT-R (5-CTGCTTGAATGCGCCAAAC-3) |           |            |
| East-Asian type *cagA* | Variable | CAGT-F (5-ACAGCTGAGCTGAGG-3) | 52°C                  | Yamaoka et al., 2000 [14] |
|             |                    | CAGJ-R (5-CTGCTTGAATGCGCCAAAC-3) |           |            |
|             |                    | CAGJ-F (5-GCTTCTTGAATGCGCCAAAC-3) |           |            |
|             |                    | CAGT-R (5-CTGCTTGAATGCGCCAAAC-3) |           |            |

Table 2: Distribution of *cagA* and *vacA* genotypes with diseases outcomes.

| Description | Total n = 169 | Normal n = 9 | Chronic gastritis n = 51 | Gastric ulcer n = 36 | Gastric cancer n = 73 |
|-------------|--------------|-------------|--------------------------|----------------------|-----------------------|
| *H. pylori* positive | 141 | 84 | 6 | 54 | 37 | 72 | 30 | 84 | 68 | 93 |
| *cagA* positive | 86 | 61 | 1 | 11 | 21 | 41 | 18 | 50 | 46 | 63 |
| (i) Western type *cagA* | 27 | 19 | 1 | 11 | 2 | 4 | 11 | 31 | 13 | 18 |
| (ii) East-Asian type *cagA* | 59 | 42 | 0 | 0 | 21 | 41 | 7 | 20 | 31 | 43 |
| *vacAslam1* | 51 | 36 | 0 | 0 | 9 | 18 | 13 | 36 | 29 | 40 |
| *vacAslam2* | 24 | 17 | 1 | 11 | 17 | 33 | 2 | 6 | 4 | 6 |
| *vacAslbm1* | 7 | 5 | 0 | 0 | 1 | 2 | 5 | 14 | 1 | 1 |
| *vacAslbm2* | 7 | 5 | 0 | 0 | 2 | 4 | 3 | 8 | 2 | 3 |
| *vacAslcm1* | 2 | 2 | 0 | 0 | 1 | 2 | 1 | 3 | 0 | 0 |
| *vacAslcm2* | 12 | 6 | 3 | 33 | 9 | 18 | 0 | 0 | 0 | 0 |

(P < 0.05), which was significantly higher than GIs (9) (18%), GU (13) (36%), and normal cases (P < 0.05). Respective percentages in GIs, GU, and GC tissue samples of slam2 were high (33%, 6%, and 6%) as compared to slm1b2 (2%, 14%, and 1%) and slbm2 (4%, 8%, and 3%) *vacA* genotype. slcm1 was observed in GIs and GU, while s2cm1 was found only in normal and GIs cases (Table 2).

3.4. *H. pylori cagA* Genotyping with Clinical Association. We found that the prevalence of *cagA* gene was 86 (61%), out of which 27 (19%) were Western types and 59 (42%) were East-Asian types (Table 2). All negative tissues for 16S rRNA gene were also negative for *cagA* and *vacA* genes. In detailed analysis, Western-type *cagA* gene was observed in 4% of the patients with GIs, 31% with GU, and 18% with GC (P < 0.05), whereas 11% of the normal cases were also positive. East-Asian type strains were observed in 41% of the patients with GIs, 20% with GU, and 43% with GC (P < 0.05) (Table 2).

3.5. Combinational Study of *H. pylori vacA* with *cagA* Genotype. In combinational analysis of *vacA* strain with Western type of *cagA*, as shown in Table 3, we found that 17 (20%) of the *H. pylori* strains have *vacAslam1/cagA* combination, while 7 (8%) of *H. pylori* strains have *vacAslam2/cagA*. In
contrast, vacAsb1m1/cagA and vacAsb2m2/cagA showed low number of H. pylori strains of 2 (2%) and 1 (1%), respectively. The vacAsclm1/cagA and vacAs2cmlm1/cagA were not found in H. pylori positive strains. In detailed analysis, 6 (17%) of GU patients have vacAslml1, 2 (6%) vacAslml2, 1 (3%) vacAsblml1, and 1 (3%) vacAsblml2. Gastric cancer patients showed a number of H. pylori strains as follows: 9 (12%) have vacAslml1, 5 (7%) vacAslml2, and 1 (1%) vacAsblml1, and vacAsblml2 has no case reported. GIs patients showed only 1 (2%) vacAslml1 combinational H. pylori strain (Table 3). In contrast, combinational analysis of East-Asian type of cagA with vacA genes demonstrated more frequently H. pylori strains of vacAslml1/cagA 32 (37%) and vacAslml2/cagA 19 (22%) than vacAsblml1/cagA 6 (7%) and vacAsblml2/cagA 2 (2%) (P < 0.05). However, vacAsclm1/cagA and vacAs2cmlm1/cagA were not found in H. pylori positive strains. Distribution analysis further showed that H. pylori strains have 7 (14%) slaml1/cagA, 4 (18%) vacAslml2/cagA, and 3 (6%) vacAsblml1/cagA H. pylori positive strains. In GU patients, we found 8 (22%) vacAslml1/cagA and 2 (6%) vacAslml2/cagA combinations in H. pylori positive strain. In contrast, GC patients showed high number of vacAslml1/cagA 17 (23%) and vacAslml2/cagA 13 (18%) H. pylori positive strains. However, combinations of vacAsblml1/cagA and vacAsblml2/cagA in GC were found in low percentages as 3 (4%) and 2 (3%), respectively (P < 0.05) (Table 3).

### 4. Discussion

Several studies have focused on the diversity of the H. pylori vacA and cagA virulence genotypes. However, there is little information available related to the frequency of H. pylori vacA and cagA genotypes in Northeast China. In the present study, we determined the vacA and cagA statuses among gastritis (GIs), gastric ulcer (GU), and gastric cancer (GC) patients' samples. The prevalence of H. pylori was found to be considerably high in the Pacific Asian countries: for example, China has high prevalence of H. pylori infection and gastric cancer compared to the rest of the world [23]. In this study, we observed high prevalence of H. pylori infection (84%) in gastric patients from 2011 to 2013 (Table 2). Previous studies from the northern and central part of China reported H. pylori prevalence rate of 58% from 1990 to 2002 [24]. In comparison, other countries in the same region such as Singapore, Malaysia, Taiwan, and Vietnam showed low H. pylori prevalence [23]. These results indicate that H. pylori infections have epidemiological diversity, which show different prevalence in different geographical locations.

Genetic variations of the vacA genotype make the requirement to investigate clinical outcomes, which is directly linked with the virulence status of H. pylori. vacA gene shows variation in the signal and midregion that determine H. pylori cytotoxin activity. In our study, we predominantly found vacA subtypes of sl, m1, and m2, while slb and scl were found in low frequency (Table 2). Our results showed comparable vacA frequency rates in China with those from other reported data; for example, Hou et al. reported that sl and m2 were more prevalent vacA genes in China [25], while Mishra et al. found that vacAsla and vacA m1 were major subtypes in India [26]. Previously, studies carried out on the Chinese population have reported high prevalence of sla subtype, while slb subtype is rarely found in East-Asian countries [27–29]. In another study, slb subtype was not detected in Hong Kong vacA positive H. pylori infected patients, while m2 subtype was found in high frequency of 67%. Similarly, we also rarely observed scl subtype, while scl was exclusively found from East-Asian isolates [30]. In our study, we found that vacAslml1 (36%) is a more prevalent genotype as compared to the vacAslml2 (17%), while slbml1 (5%), slbml2 (5%), sl clm1 (2%), and s2 ml (6%)
genotypes were found in low frequency (Table 2). Previous study showed high prevalence of \textit{vacA} genotype; Ahmad et al. reported the prevalence of \textit{slb}m2 (54.5%) in adult dyspeptic patients from Pakistan [8]. According to Mishra et al., \textit{vacAsl}a/m1 (53.2%) has predominant genotype in India [26]. However, Hou et al. reported high (90.5%) frequency of \textit{vacA} genotype in China [25]. \textit{vacAsl}a and \textit{slb} genotypes are prominently associated with high toxin activity and linked to clinical outcome of the diseases. In a detailed analysis of \textit{vacA} genotype, we found high frequency of \textit{slam1} in GU (36%) and GC (40%) patients, while \textit{slam2} was highly found in GIs (33%) and normal cases (11%). \textit{slb}m1 and \textit{slb}m2 showed high frequency in GU (14%) and (8%), respectively, while \textit{slam2} was found only in normal (33%) and GIs patients (18%) (Table 2). Our results indicate diverse epidemiology of gastritis patients containing \textit{vacAs2} m1 genotype in the northeast part of China. The \textit{vacAs1} and \textit{vacAm1} bearing \textit{H. pylori} strains have been associated with increased virulence capability and higher gastric epithelial damage and ulceration than \textit{slb} and \textit{m2} strains [31].

\textit{H. pylori} CagA induces pathological alterations, which is closely associated with development of gastritis, gastric ulcer, and gastric cancer. \textit{H. pylori} \textit{cagA}-positive strains are more virulent causing higher levels of gastric mucosal inflammation in gastritis and gastric cancer [32, 33]. In present study, about 61% of strains were \textit{cagA} positive, which comprises 19% Western-type \textit{cagA} and 42% East-Asian type \textit{cagA} strain. Conversely, our results showed low prevalence of \textit{cagA} gene in gastric patients from Northeast China (Table 2). Previous studies reported high prevalence (93.9%) of \textit{cagA}-positive \textit{H. pylori} strain in China [34–36]. According to Hou et al., \textit{H. pylori} \textit{cagA} has high prevalence of 93.2% in Shanghai (the southern part of China) [25]. The neighboring countries of China were also reported to have high prevalence of \textit{cagA}; for example, India has high \textit{cagA} prevalence of 96.2% [26]. Rasheed et al. reported that 52% of \textit{H. pylori} strains carried \textit{cagA} gene with the positivity rate of 80% in GC, 74% in GU, 63% in duodenal ulcer (DU), and 11% in normal cases from Pakistan [37]. Conversely, in Western Europe, \textit{cagA}-positive strains are less prevalent and more frequently found in GU or GC patients [38].

In combinational analysis of \textit{vacA} and \textit{cagA} genotypes, we found that \textit{H. pylori} strains have high frequency of \textit{vacAs1}m1 (37%) or \textit{vacAs1}m2 (22%) with East-Asian type \textit{cagA} genotype (Table 3). Rasheed et al. reported high frequency of \textit{cagA} (61.9%) with predominant \textit{vacAsl}a/m2 genotype in \textit{H. pylori} infected gastric tissues of Pakistani children [37]. A previous study reported significantly higher percentages of \textit{cagA}-positive and \textit{vacAsl} and \textit{vacAm1} genotypes with high risk for GC [39]. A recent study conducted in China found no differences in the distribution of \textit{cagA}-positive and \textit{vacAm} genotypes [40]. Our results showed high prevalence of \textit{vacAs1}m1 and East-Asian type \textit{cagA}-positive \textit{H. pylori} strain in gastric cancer patients.

These results might have useful roles in clinical appreciation so as to categorize the specific and most prevalent biomarkers of \textit{H. pylori} strains in the northeast part of China that will be helpful to precisely diagnose large population of gastric cancer patients. \textit{H. pylori} has high capability of developing antibiotic resistance, which make difficult to treat \textit{H. pylori} by conventional antibiotics; however, targeted marker therapy leads to effective \textit{H. pylori} treatment and helps reduce antibiotic resistance.

In conclusion, the present study showed high diversity of the \textit{H. pylori} \textit{vacA} and \textit{cagA} genotyping. We have reported high frequency of East-Asian type \textit{cagA} with prevalent \textit{vacAs1}m1 and \textit{vacAs2}m2 genotypes in gastric ulcer, gastritis, and gastric cancer patients. This study helps to effectively diagnose and treat gastric patients by understanding the trend of \textit{H. pylori} infection in Northeast China.

**Ethical Approval**

The project was approved by the Dalian Medical University Ethics Committee in China.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

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