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# Analysis of *Helicobacter pylori* Genotypes and Correlation with Clinical Outcome in Turkey

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**The predominant *Helicobacter pylori* strains circulating among geographic locations differ in regard to genomic structure. The association of the *cagA*-positive, *vacA* s1 genotypes with peptic ulcer disease (PUD) and gastric cancer was reported in Western countries but not in East Asian countries. Strains from Western countries predominantly possessed *cagA* type 2a, *vacA* s1a or s1b/m1a, or *vacA* m2a genotypes, whereas strains from East Asia possessed *cagA* type 1a, *vacA* s1c/m1b, or *vacA* m2b genotypes. Whether the Turkish strains possessed such genotypes was investigated and correlated with the disease outcome. Seventy-three patients from Turkey were enrolled. *H. pylori* was detected in 65 (89%) patients (22 with gastritis, 33 with PUD, and 10 with gastric cancer) by any of the following tests: *Campylobacter*-like organism test, culture, or PCR. Among the *H. pylori*-positive patients, presence of the *cagA* gene (78%) was significantly associated with PUD ($P < 0.00001$), gastric cancer ($P < 0.001$), and *vacA* s1a genotypes ($P < 0.0001$). Multiple *vacA* genotypes were more prevalent in PUD and gastric cancer patients than in patients with gastritis. Restriction fragment length polymorphism analysis of the *cagA* gene revealed three different patterns with no significant association with clinical outcome. Turkish strains examined predominantly possessed *cagA* type 2a, *vacA* s1a/m1a, or *vacA* m2a genotypes, which were typical genotypes in strains from Western countries. This fact might be one of the reasons for the low prevalence of severe gastroduodenal diseases in Turkey compared to the East Asian countries.**

The *Helicobacter pylori* genotypes and the geographic distribution are linked to the severity of peptic ulcer disease (PUD) (9, 13, 27). *H. pylori* appears to be one of the most genetically diverse bacterial species, as evidenced by the presence, among different strains, of nonconserved DNA fragments such as the *cagA* gene in the *cag* pathogenicity island and allelic variation within the *vacA* gene (9, 27, 31). Such diversity was found to affect the function and antigenicity of virulence factors associated with bacterial infection and, ultimately, disease outcome (6, 14, 31).

The *cagA* gene, located at the right end of the *cag* pathogenicity island that contains approximately 30 genes, encodes the CagA protein, which varies in molecular mass between 120 and 140 kDa (8, 30). The *vacA* gene, which encodes the vacuolating cytotoxin, is the major toxin secreted by *H. pylori* that induces vacuolation in the human epithelial cells in vitro (3, 10, 23). Previous studies permitted a comprehensive description of the *vacA* signal (s) and middle (m) regions, which exist as s1 or s2 and m1 or m2, respectively (3). In Western countries, the presence of *vacA* s1 and *cagA* was reported to be significantly associated with PUD (4, 25, 28), whereas such association has not been reported in Asian countries (21, 31).

This discrepancy between Western countries and Asia might be explained by the fact that the predominant *H. pylori* strain circulating among geographic locations differs with regard to the genomic structure. The variation of the *cagA* gene was attributed to the presence of a variable number of repeated sequences in the 3′ region of the gene (8, 22, 30, 32). We previously reported that the sequence of the 3′ region of the *cagA* gene from isolates in East Asia (type 1a) differs markedly from the primary sequence of *cagA* genes from isolates in Western countries (type 2a) (30, 33). The *vacA* s1 region was subtyped into s1a, s1b, and s1c; the m1 region was subtyped into m1a, m1b, and m1c; and the m2 region was subtyped into m2a and m2b (4, 14, 20, 21, 25, 27, 28, 31). Previous studies showed that the *vacA* s1a or s1b genotypes were predominant in strains from Western countries, whereas s1c is highly prevalent in strains from East Asia (28, 33). The *vacA* m1a and m2a genotypes were predominant in strains from Western countries, the m1c genotype was predominant in strains from South Asia, and the m1b and m2b genotypes were predominant in strains from East Asia (28, 33). Overall, strains from Western countries predominantly possessed *cagA* type 2a; *vacA* s1a, s1b, or s2/m1a; or m2a genotypes. Strains from South Asia predominantly possessed *cagA* type 2a and *vacA* s1a/m1c genotypes, whereas strains from East Asia predominantly possessed *cagA* type 1a, *vacA* s1c/m1b, or m2b genotypes (28, 33). These variations in the global distribution of the *cagA* and *vacA* genotypes may reflect the diversity of reports associating the *cagA* and *vacA* genotypes with the clinical outcome from different geographic regions.

Turkey's geographic location, which has been under continuous influences from both Asian and Western countries, has made it an ideal site for epidemiological studies on *H. pylori* infection and genotyping. The prevalence of gastric cancer in

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Turkey is much lower than that in certain Asian countries (e.g., Japan) according to the World Health Organization world cancer report (29a) (annual rates of 4,440 cases [Turkey] versus 115,294 cases [Japan]/100,000 individuals); however, it is not known whether the difference is due to host, environment, or bacterial factors or a combination of these factors. Since no detailed studies in Turkey on the distribution and association of *H. pylori cagA* and *vacA* genotypes with PUD have been reported, our objectives were to detect whether the *cagA* and *vacA* genotypes of the Turkish strains were of the genotypes typically observed in Western countries or in Asia and to determine their association with clinical outcomes.

## MATERIALS AND METHODS

**Patients.** Seventy-three patients (42 males, 31 females; mean age, 46 years; age range, 17 to 80 years) (22 with gastritis, 33 with PUD, and 10 with gastric cancer) were enrolled in this study. Three biopsy specimens—for *Campylobacter*-like organism (CLO) test, culture, and PCR—were taken from the antral part of the stomach of each patient. Gastritis patients had chronic histological gastritis without peptic ulcer, gastric cancer, or esophageal disease. Peptic ulcers were defined endoscopically. Gastric cancer patients had no other primary malignancies or inflammatory diseases. Patients had received no treatment for *H. pylori* infection. Informed consent was obtained from all patients and approved by an ethics committee at Fatih University.

**Culture.** Biopsy specimens placed in 1 ml of normal saline (0.9% sodium chloride) were dissected and spread on Columbia agar plates containing 5% horse blood and then were incubated under microaerobic conditions in an anaerobic jar at 37°C for 5 to 7 days. The organisms were identified as *H. pylori* by colony morphology; Gram stain reaction; and positive reactions to oxidase, catalase, and urease activities. Cultures were harvested and stored in nutrient broth containing 20% glycerol at −80°C.

**DNA isolation and PCR.** DNA isolation was done using the QIAamp DNA kit (Qiagen Co., Hilden, Germany) according to the manufacturer's instructions. The presence of the *cagA* gene as well as the *cagA* genotype (type 1a, specific genotype in strains from East Asia, versus type 2a, typically observed in strains from Western countries) was performed as previously described (23, 32, 33). The *vacA* genotypes (s1a, s1b, s1c, m1a, m1b, m1c, m2a, and m2b) were identified as previously described (3, 28, 31, 33). Amplified PCR products were visualized by electrophoresis on 2% agarose gels, and imaging and analysis of bands were done using Gel Doc 2000 (Bio-Rad, Milan, Italy).

**RFLP analysis.** The restriction fragment length polymorphism (RFLP) analysis procedure described earlier (7) was followed. A 10-μl sample of the PCR product of the *cagA* gene was digested with 10 U of the restriction enzyme HaeIII (Sigma, St. Louis, Mo.) for 4 h at 37°C as recommended by the supplier. The digested fragments were then electrophoresed on 2% agarose gel.

**Statistical analysis.** The chi-square test with Yates correction was used. The *P* value set at <0.05 was regarded as statistically significant.

## RESULTS

Patients were considered infected with *H. pylori* when the gastric biopsy specimens gave positive results in any one of the objective tests: CLO test, culture, or PCR. Sixty-five (89%) of 73 patients were *H. pylori* positive. Among these, 49 (75%) were positive by the CLO test, 48 (74%) were positive by culture, and 65 (100%) were positive by PCR. PCR amplification of DNA sample directly from gastric biopsy specimens detected the *cagA* gene in 51 (78%) of 65 patients, including 12 (55%) of 22 patents with gastritis, 5 (100%) of 5 patients with gastric ulcer, 25 (89%) of 28 patients with duodenal ulcer, and 9 (90%) of 10 patients with gastric cancer (Table 1). All *cagA* genotypes detected were type 2a, which was typically observed in strains from Western countries and showed a significant association with PUD ($P < 0.00001$) and gastric cancer ($P < 0.001$). PCR amplification of DNA samples isolated from culture material (single colony or pool of colonies) gave similar results to the DNA isolated directly from gastric biopsy specimens.

TABLE 1. *H. pylori cagA* and *vacA* s1a genotype correlation with PUD

| Patient group[a] | No. (%) positive for: | |
|---|---|---|
| | *cagA* | *vacA* s1a |
| G ($n = 22$) | 12 (54) | 18 (82) |
| GU ($n = 5$) | 5 (100) | 5 (100) |
| DU ($n = 28$) | 25 (89) | 23 (82) |
| GC ($n = 10$) | 9 (90) | 8 (80) |
| Total ($n = 65$) | 51 (78) | 54 (83) |

[a] Abbreviations: G, gastritis; GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer; *n*, number examined.

The *vacA* s1a genotype was detected in 54 (83%) of 65 patients: 18 (82%) of 22 with gastritis, 5 (100%) of 5 with gastric ulcer, 23 (82%) of 28 with duodenal ulcer, and 8 (80%) of 10 with gastric cancer (Table 2). The *vacA* s1a genotype had no significant association with PUD. No other *vacA* s1 subtype was detected in this study except for one s1b subtype in a patient with gastritis. The *vacA* s2 genotype was found in three (5%) of the patients. The *vacA* m1 genotype was detected in 8 (12%) patients, while the m2 genotype was detected in 28 (43%) of the 65 patients. All m1 genotypes were m1a genotype, and those of the m2 genotype were m2a genotype (Table 2). Both genotypes have been thought to be specific to strains from Western countries. Table 1 shows the association between the *cagA* and *vacA* s1a expression and PUD. The expression of the *cagA* gene was strongly associated with that of the *vacA* s1a genotype ($P < 0.0001$) in patients with gastric ulcer, duodenal ulcer, and gastric cancer. No significant association was found between *cagA* and *vacA* m1 or m2 genotypes and PUD. Multiple *vacA* genotypes were significantly more prevalent in PUD and gastric cancer patients than in patients with gastritis (Table 2).

PCR-RFLP analysis performed on specimens from 30 randomly selected subjects revealed three different patterns following digestion of the amplified *cagA* gene with the HaeIII enzyme (Table 3). These patterns had no significant association with clinical outcomes.

TABLE 2. *H. pylori vacA* s and m regions subtyping in dyspeptic patients

| Patient group[a] | No. of patients with subtyping result | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | s1a | s1b | s2 | M | N | m1a | m2a | M | N |
| G ($n = 22$) | 18 | 1 | 1 | 2 | 0 | 2 | 12 | 8 | 0 |
| GU ($n = 5$) | 5 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 0 |
| DU ($n = 28$) | 23 | 0 | 1 | 3 | 1 | 3 | 10 | 15 | 0 |
| GC ($n = 10$) | 8 | 0 | 1 | 1 | 0 | 3 | 4 | 3 | 0 |
| Total ($n = 65$) | 54 | 1 | 3 | 6 | 1 | 8 | 28 | 29 | 0 |

[a] Abbreviations: G, gastritis; GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer; *n* number examined; M, multiple infections (either s or m subtypes); N, negative (either s or m subtypes).

TABLE 3. RFLP-PCR patterns of *cagA* gene in a representative group of patients[a]

| Pattern | No. of patients | | | | |
|---|---|---|---|---|---|
| | G | GU | DU | GC | Total |
| No cut | 4 | 1 | 5 | 1 | 11 |
| I | 4 | 1 | 7 | 1 | 13 |
| II | 0 | 0 | 4 | 0 | 4 |
| III | 1 | 1 | 0 | 0 | 2 |
| Total | 9 | 3 | 16 | 2 | 30 |

[a] Abbreviations: G, gastritis; GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer.

## DISCUSSION

The geographic distribution of distinct *H. pylori* genotypes remains largely unknown, and the prevalence of virulent bacterial genotypes in certain regions, particularly in Western countries, may have important epidemiological consequences that are linked to the presence of the *cagA* gene and the severity of *H. pylori*-related diseases (4, 9, 25, 27, 28). In Europe *cagA*-positive *H. pylori* is reported to account for 60 to 70% of *H. pylori* strains (1, 5, 23), while reports from East Asian countries have shown that more than 90% of *H. pylori* strains are *cagA* positive irrespective of the disease presentation (16, 18). Such differences in the prevalence of *cagA* positivity could not be explained precisely; however, they have been attributed to the genetic heterogeneity or to differences in the geographic locations (9, 32, 33). The present study revealed that the majority of PUD and gastric cancer patients were infected with *cagA*-positive strains as opposed to gastritis patients. These findings further substantiate the role of *cagA* as a marker for increased virulence and are in agreement with studies from Western countries (2, 24). More importantly, all *cagA* genotypes detected were type 2a, which was typically observed in strains from Western countries.

Analysis of *H. pylori* isolates from diverse geographic locations also showed high variability in the *vacA* gene (25, 28, 33). The *vacA* s1a and m2a genotypes predominant in our strains were also predominant in strains from Western countries. In the present study, the *cagA*-positive status was strongly associated with *vacA* s1a and PUD, which is in agreement with previous reports from Western countries (25, 28). In contrast, such relationships were not reported in studies from East Asia (16, 31). Overall, we may be able to conclude that only the *cagA* type 2a and *vacA* s1a genotypes typically observed in strains from Western countries, but not in strains from East Asia, are related to the clinical outcomes. Recently, Higashi et al. (15) have shown that the East Asia-specific CagA sequences (type 1a) conferred stronger Src homology-2 binding and morphological transforming activities to Western type CagA sequences (type 2a) and concluded that the presence of distinctly structured CagA proteins in Western and Asian *H. pylori* strains might underlie the striking differences in the incidence of gastric carcinoma between these two geographic regions. Thus, the *cagA* type 1a and *vacA* s1c genotypes typically observed in strains from East Asia might be the most virulent strains, followed by the *cagA* type 2a and *vacA* s1a genotypes observed in strains from Western countries, while the *cagA*-negative and *vacA* s2 genotypes are considered the least virulent strains. In this study, our findings that no Turkish strains examined possessed *cagA* type 1a and *vacA* s1c genotypes might be one of the reasons for the low prevalence of gastric cancer in Turkey.

Since it has been demonstrated that *H. pylori* carries only a single copy of the *vacA* gene, detection of multiple genotypes implies the presence of multiple strains in a clinical sample (27). The risk of coinfection or superinfection with multiple strains may be higher in countries with a high prevalence of *H. pylori* infection than in those with a low prevalence (11, 19, 27). Multiple *vacA* genotypes detected in this study were more prevalent in patients with PUD than in those with gastritis, which is in agreement with a study done by Gonzales-Valencia et al. (12), but not with that of Wang et al. (29). Further studies will be necessary to examine whether infection with multiple strains increases the risk of serious clinical outcomes or not.

Discrimination between closely related isolates of *H. pylori* is needed for epidemiological and clinical purposes and precise methods of strain characterization are necessary to monitor *H. pylori* infections. PCR-RFLP analysis has been widely used for *H. pylori* typing (17, 26). Li et al. (17) reported that RFLP analysis was a reliable method to detect multiple strains and suggested that it might be useful as a primary approach for the identification of specific *H. pylori* strains; however, we found no association between the RFLP pattern and PUD in the present study.

In conclusion, all Turkish strains examined possessed *cagA*/*vacA* genotypes typically observed in strains from Western countries. This finding might be one of the reasons for the low prevalence of severe gastroduodenal diseases such as gastric cancer in Turkey. The presence of the *cagA* gene was significantly associated with that of the *vacA* s1a genotype and the clinical presentations of PUD and gastric cancer. Multiple *vacA* genotypes were more prevalent in patients with PUD and gastric cancer than in those with gastritis.

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