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World J Gastroenterol 2003;9(7):1580-1583
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

Association of *CagA* and *VacA* presence with ulcer and non-ulcer dyspepsia in a Turkish population

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Received: 2003-01-14 **Accepted:** 2003-02-25

Abstract

AIM: The mostly known genotypic virulence features, of *H. pylori* are cytotoxin associated gene A (*cagA*) and Vacuolating cytotoxin gene A (*VacA*). We investigated the association of these major virulence factors with ulcer and non-ulcer dyspepsia in our region.

METHODS: One hundred and forty two dyspeptic patients were studied (average age 44.8±15.9 years, range 15-87 years, 64 males and 78 females). Antral and corpus biopsies were taken for detecting and genotyping of *H. pylori*. 107 patients who were *H. pylori* positive by histological assessment were divided into three groups according to endoscopic findings: Duodenal ulcer (DU), gastric ulcer (GU) and non-ulcer dyspepsia (NUD). The polymerase chain reaction (PCR) was used to detect *CagA* and *VacA* genes of *H. pylori* using specific primers.

RESULTS: *H. pylori* was isolated from 75.4 % (107/142) of the patients. Of the 107 patients, 66 (61.7 %) were *cagA*-positive and 82 (76.6 %) were *VacA*-positive. *CagA* gene was positively associated with DU and GU ($P<0.01$, $P<0.02$), but not with NUD ($P>0.05$). Although *VacA* positivity in ulcer patients was higher than that in NUD group, the difference was not statistically significant ($P>0.05$).

CONCLUSION: There is a significantly positive association between *CagA* genes and DU and GU. The presence of *VacA* is not a predictive marker for DU, GU, and NUD in our patients.

Bulent K, Murat A, Esin A, Fatih K, MMMurat H, Hakan H, Melih K, Mehmet A, Bulent Y, Fatih H. Association of *CagA* and *VacA* presence with ulcer and non-ulcer dyspepsia in a Turkish population. *World J Gastroenterol* 2003; 9(7): 1580-1583
http://www.wjgnet.com/1007-9327/9/1580.asp

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral shaped microaerophilic bacterium that colonizes in the gastric mucosa in humans[1]. In the majority of individuals, infection causes asymptomatic histological chronic gastritis. A significant minority subsequently develop peptic ulcer disease (PUD), and infection with *H. pylori* is a significant risk factor for gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma[2-4]. The process by which different disease patterns develop has not been fully elucidated. But two putative virulence determinants of *H. pylori* have been identified as markers of ulcerogenic strains, the cytotoxin associated gene A (*CagA*) and the vacuolating cytotoxin gene A (*VacA*) (Phenotype 1, ulcerogenic; *CagA* & *VacA*-positive, phenotype 2, non-ulcerogenic; *CagA* & *VacA*-negative). The *CagA* gene encodes a 120-140 kDa protein of unknown function in about 60-70 % of *H. pylori* strains. This gene is part of the cag pathogenicity island (PAI), a 40-kbp segment with several genes involved in cytokine production[5, 6]. Strains that do not produce the *CagA* protein generally lack the entire cag PAI. *H. pylori* strains produced by *CagA* have been detected in patients with PUD more frequently than in patients with chronic gastritis alone[7- 9]. Another virulence factor that injures epithelial cells is encoded by *VacA*. *VacA* is present nearly in all *H. pylori* strains and contains at least two variable parts[10,12]. The s-region (encoding the signal peptide) exists as s1 or s2 allelic types. Among type s1 strains, subtypes s1a, s1b, and s1c have been identified. The m-region (middle) occurs as m1 or m2 allelic types. Among type m2, two subtypes have been identified, and designated as m2a and m2b[11]. Production of the vacuolating cytotoxin is related to the mosaic structure of *VacA*. The *VacA* signal sequence type s1, but not type s2 is closely associated with *in vitro* cytotoxin activitiy, PUD, and the presence of *CagA* gene[9, 10, 12]. The m1 allele is associated with higher levels of toxin activitiy and more severe gastric epithelial damage than the m2 allele[10, 12].

MATERIALS AND METHODS

Materials

Patients and classification of endoscopic findings One hundred forty two dyspeptic patients (excluding those taking proton pump inhibitors [PPIs] and/or NSAIDs in the past month, and/or had previous *H. pylori* eradication) were studied (average age 44.8±15.9 years, range 15-87 years, 64 males and 78 females) and referred for routine endoscopy at the Department of Gastroenterology in the Turgut Özal Medical Center, Malatya, Turkey. Endoscopic findings were recorded and according to endoscopic findings, *H. pylori* positive patients were divided into three groups: Group 1: Patients with duodenal ulcer (DU); Group 2: Patients with gastric ulcer (GU); Group 3: Patients with non-ulcer dyspepsia (NUD). All the patients gave informed consent to participate in the study.

Methods

Endoscopy and detection of *H. pylori* by histological assessment During each endoscopic procedure (by Olympus

GIF XQ 240 videoendoscope), two antral and two corpus mucosal biopsy specimens were obtained by using biopsy forceps (FB-25 k; Olympus, Japan) which were cleaned with a detergent and disinfected after each examination. Two biopsy samples were transported to pathology laboratory and fixed in 10 % formalin overnight. Tissue processing was undertaken with graded ethanol solutions and clearing was made with xylene. Paraffin tissue blocks were cut into 4-5 μm sections with a rotary microtome. The sections were stained with hematoxylin-eosin and tissue Giemsa and assessed for the presence of *H.pylori* microorganisms. 107 (75.4 %) patients (average age 45.8±15.7 years, range 17-87 years, 50 males and 57 females) were found to be *H. pylori* positive and the other two biopsy specimens of those patients were transported to the microbiology laboratory for PCR examination to determine *CagA* and *VacA* status.

PCR examination Biopsy samples obtained from 107 patients with positive *H. pylori* were put into 20 % dextrose solution and stored at -20 ℃ until a sufficient number was reached for PCR assay. The transport media contained the tissue samples were discarded and the tissue samples were resuspended with 100-200 μg/ml of lysis buffer (10 mM Tris-HCL, 0.1 M EDTA, 5 % SDS, 100-200 μg/ml proteinase K). The mixture was incubated at 52 ℃ for 2 hours in a thermal cycler. 100 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added into the mixture. The mixture was then vortexed and centrifuged at 5 000×g for 5 minutes. The supernatant was discarded and isoamyl alcohol (24:1) was added as much as the taken volume. The mixture was vortexed and centrifuged as described above. The supernatant was discarded and 2.5 volume of cold ethanol (70 %) was added and stored at -20 ℃ overnight. On the following day, the mixture was centrifuged at 13 000×g for 13 minutes. The supernatant was discarded and the pellet was resuspended with TE buffer and used for PCR assay. The reaction mixture (50 μl) was prepared for *CagA* as described below. 2 μl dNTP mix (200 μM/ μl of each deoxynucleotide), 1 μl primer 1(0.5 μM/1 μl of each oligonucleotide), 1 μl primer II, 1 μl taq polymerase (2.5U/ μl), 4.5 μl 10×PCR buffer, 0.5 μl $MgCl_2$ (5 μM/ μl), 35 μl distilled water, 5 μl sample DNA. PCR reaction was performed in the thermal cycler (M.J. research) with the following incubation steps: at 94 ℃ **for** 4 min (Pre-heating), 35 cycles at 94 ℃ **for** 1 min (denaturation), at 57 ℃ 1.30 min (annealing) and at 74 ℃ **for** 2 min (elongation), 1 cycle at 74 ℃ **for** 5 min post elongation. *Cag A* primers: 5' -GAT AAC AGG CAA GCT TTT GAG G-3', 5' - CTG CAA AAG ATT ATT TGG CAA GA-3' targeting 349 bp fragment. The reaction mixture was prepared for *VacA* as described below. 2 μl dNTP mix, 1 μl primer 1, 1 μl primer II, 1 μl taq polymerase, 4.5 μl 10×PCR buffer, 0.5 μl $MgCl_2$, 35 μl distilled water, 5 μl sample DNA. Then at 94 ℃ **for** 1 min (denaturation), at 63 ℃ **for** 1.30 min (annealing), at 72 ℃ **for** 1 min (elongation), 30 cycles, and at 74 ℃ **for** 5 min 1 cycle for post elongation. *VacA* primers: 5' - CCG AAG AAG CCA ATA AAA CCC CAG-3', 5' - CAA AGT CAA AAC CGT AGA GCT GGC- 3' targeting 467 bp fragment. The PCR products were analysed by 2 % agarose gel with 0.5 % ethidium bromide via electrophoresis.

Statistical analysis

Normal χ^2 analysis and Fisher' s exact χ^2 method were used for statistical evaluation of data derived from the results of the procedures mentioned above.

RESULTS

Prevalence of H. pylori infection

H. pylori infection was found in 107 of 142 patients (75.4 %).

Endoscopic findings

35 of the 107 patients had DU (32.7 %), 24 (22.4 %) GU and 48 NUD (44.9 %).

Prevalence of CagA and VacA among H. pylori positive patients

While 66 of 107 *H. pylori* strains were *CagA* positive (61.7 %), 82 of the patients were *VacA* positive (76.6 %), and 62 of the patients were both *CagA* and *VacA* positive (57.9 %).

Relation between CagA-VacA status and DU, GU, and NUD

28 of 35 patients with DU (80 %), 20 of 24 patients with gastric ulcers (83.3 %) and 18 of 48 patients with NUD (37.5 %) were *CagA* positive. The presence of *CagA* in the patients with DU and GU was significantly higher than that in the patients with NUD, respectively (P=0.007, P=0.013). *CagA* positivity was statistically lower in patients with NUD (P<0.001). 29 of 35 patients with DU (82.9 %), 21 of 24 patients with gastric ulcer (87.5 %), and 32 of 48 patients with NUD (66.7 %) were *VacA* positive. *VacA* positivity was both higher in the patients with DU and GU than that in the patients with NUD, but this difference between the groups was not statistically significant (P>0.05). We detected phenotype 1 *H. pylori*, characterized by the expression of both *CagA* and *VacA*, in 57.9 % (62 of 107) of the patients (71.4 % DU, 79.2 % GU, 37.5 % NUD patients). The prevalence of phenotype 1 was significantly higher in patients with duodenal or gastric ulcer, than that in the patients with NUD (P<0.0004, P<0.0002). Phenotype 2 *H. pylori* characterized by a lack of expression of either *CagA* or *VacA*, was found in 19.6 % of the patients (21 of 107) in our study (8.6 % DU, 8.3 % GU, 33.3 % NUD). The prevalence of phenotype 2 in the patients with NUD was significantly higher than that in patients with duodenal or gastric ulcer (P<0.01, P<0.04). The remaining 22.4 % of the total number of patients studied (24 of 107) had an intermediate phenotype, which expressed either *CagA* independent of the presence of *VacA* (CagA-positive and *VacA*-negative, 3.7 %) or vice versa (CagA-negative and *VacA* positive, 18.7 %). There was not any significant difference between the groups according to intermediate phenotypes (P>0.05) (Table 1).

DISCUSSION

The most common interaction between *H. pylori* and human is asymptomatic bacterial colonisation in the gastric mucosa, which can be continued lifelong. However, the presence of this bacterium in an individual increases the risk of serious

Table 1 Distribution of endoscopic findings according to *CagA* and *VacA* status

	CagA+	*VacA*+	*CagA*+*VacA*+ (Phenotype 1)	*CagA*+ *VacA*-	*CagA*-*VacA*+	*CagA*-*VacA*- (Phenotype 2)
DU(35)	28(80%)	29(82.8%)	25(71.4%)	3(8.6%)	4(11.4%)	3(8.6%)
GU(24)	20(83.3%)	21(87.5%)	19(79.2%)	1(4.2%)	2(8.3%)	2(8.3%)
NUD(48)	18(37.5%)	32(66.6%)	18(37.5%)	0(0.0%)	14(29.2%)	16(33.3%)
Total(107)	66(61.6%)	82(76.6%)	62(57.9%)	4(3.7%)	20(18.6%)	21(19.6%)

gastroduodenal diseases such as gastritis, GU, DU, gastric cancer and MALT lymphoma[3, 4, 13, 14]. It has been suggested that *H. pylori* may induce more or less severe gastroduodenal diseases according to the strain virulence. Two major markers of virulence, *CagA* and *VacA*, have been described in *H. pylori*. The association between putative virulence markers with ulcer and NUD was investigated in a Turkish population.

In our study, the presence of *CagA*, and both *CagA* and *VacA* was significantly more prevelent in patients with DU and GU, than those in patients with NUD ($P<0.05$). The positivity of *VacA* was higher in the patients with DU and GU than that in the patients with NUD, but difference was not statistically significant ($P>0.05$). Previous studies from different countries showed that CagA-positive strains were more common in patients with ulcer disease. CagA-positive strains were found in 79 % to 100 % of DU patients[7,8,15,18-31], 71 % to 100 % of GU patients [7-9,19,20,27,28], as compared with 37 % to 89.7 % of NUD patients[8,18-24,26-31]. In the present study, we found a significantly higher prevalence of CagA-positive strains in DU and GU than that in NUD patients (80 %, 83.3 %, and 37.5 %, respectively). It was reported from different centers that in patients with duodenal ulcer, the positivity rates of cagA, *VacA*, and both *Cag A* and *VacA* were 79-100 %, 47.5-92 %, 37-75 %, respectively[7,8,15,18-31]. The positivity rates of *Cag A*, *VacA*, and both *CagA* and *VacA* in the patients with gastric ulcer, have been reported to be 71-100 %, 40.8-75 %, and 38.8-56.6 %, respectively[7-9,19-30]. In all of these studies, the positivity of *CagA* and *VacA* was higher in the patients with DU or GU, however some was statistically significant[7-10,12,15-18,24-26,28-31] and some not[19-21,23,32,33,35- 37,39,42,43] when it was compared to patients without ulcer.

In patients with NUD, the positivity rates of *CagA* and *VacA* were reported to be 37-89.7 %[8,18,19,21-24,26,28,29] and 33.3-73 %[8,19,21-23,28-30], respectively. Nearly in all of these studies, *CagA* and *VacA* positivity rate in the patients with NUD was found to be low compared to that in the patients with ulcer, however, this difference was statistically significant in some studies[7-9, 15-18, 24-26, 28-30], but not in some others[19-21,23,27,33,35-38,41,42]. In our study, the *CagA* positivity in the patients with NUD was significantly lower than that in the ulcer patients ($P<0.01$). This supported the results of DU and GU mentioned above. Although *VacA* positivity was higher in the patients with ulcer than that in the patients with NUD, this was not statistically significant and did not seem to be an important risk factor for the development of ulcer in our patients. However, determination the *VacA* genotypes and the presence of *CagA* gene together may contribute to potential clinic determination of patients who have different levels of risk. It has been shown that *VacA* type s1/m1 strains produce more cytotoxins than type s1/m2, and that type s2/m2 strains do not produce active cytotoxins[10]. Many studies have confirmed these findings[9,12,24,25,31,38]. In this study, we couldn' t detect the *VacA* subtypes for not having their primers. Also, we had no information on the *in vitro* cytotoxin production of our strains, so we could not compare our results directly with those from other studies. If we could have determined these factors, perhaps we would find an association between *VacA* and ulcer disease. It was reported that the presence of *CagA* and *VacA* genes in *H. pylori* isolates increased the risk of gastric cancer[22,40] but some studies refused this[37,41,42]. In two studies, no statisticallly significant difference between the presence of *CagA* or *VacA* in patients with MALT lymphoma and NUD was found[8]. In another study which interrogated the importance of the presence of *CagA* for developing resistance to metronidazole, which was used in eradication therapies of *H. pylori*, an association between resistance and the presence of *CagA* was not shown. It was investigated that if the patients could be selected for gastroscopy adequately only by looking for anti *H. pylori* and anti-*CagA* serologically, and it was observed that the method was not adequate for screening, since many serious pathology and malignancy could not have been noticed by just a selection of this method[43].

Gastroduodenal lesions developed in the patients infected with *H. pylori* isolates that had *CagA* and *VacA* gene and showed differences according to regions, countries and ethnic groups. In the literature, it has been controversial that *CagA* and *VacA* positive isolates cause more serious gastroduodenal lesions[7-42]. In our study, it was seen that gastric and duodenal ulcer incidence increased in the patiens with *CagA*, and both *CagA* and *VacA* positive. Recently, it was reported similar results for *CagA* in ulcer patients from Turkey abtained by using an ELISA method[46]. Many risk factors have been determined for *H. pylori* infection (CagA, *VacA*, *IceA* etc.), but none of them is specific for disease. It has been put forward that *CagA* plays a partial role in increased mucosal inflammation, increased density of *H. pylori* in antrum, and causes more profound inhibition of mucin synthesis, DU, GU and gastric cancer[7-9,39,44], and has a protective role in Barret' s esophagitis[16,17]. However direct association was found only with IL-8 induction[32,34,45]. The *IceA* gene, considered as a virulence factor for *H. pylori* infection recently, has no disease specific features, and there is no biologic and epidemiologic evidences that *IceA* gene is a virulence factor associated with *H. pylori*[33,34]. The opinion that *VacA* genotyping may be useful clinically (for example, predicting the presence of DU) is controversial from now on[19,32, 34].

As a result, the association between, the virulence factors in *H.pylori* positive patients, clinical course and gastroduodenal lesions that develop subsequently has not been understood yet. For determining these interactions, it needs great scale and multicenter studies which examine the structural features of *H. pylori* (virulence factors), host features and environmental features together. To have definite results, study must be large enough and include different diseases and ethnic groups. Also, in our country, multicenter and large scale studies would reveal the virulence differences between different regions.

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Edited by Xu XQ, Wang XL and Zhu LH