Articles

Helicobacter pylori virulence markers in gastroduodenal disorders

Detection of cytotoxin-associated gene A and vacuolating cytotoxin-associated gene A genes in Saudi patients

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ABSTRACT

الأهداف: الكشف والتعرف على جيني (كاق أي و ڤاك أي) في عينات من جدار المعدة والإثني عشر لمرضى إعتلال المعدة والإثني عشر.

الطريقة: لقد تم الكشف على جرثومة المعدة في العينات التي نُزعت من 118 مريض حيث تم إرسالها لمختبرات مستشفى الملك خالد الجامعي، الرياض، المملكة العربية السعودية خلال الفترة من مارس 2008م إلى فبراير 2009م. تم أخذ العينات المصابة بجرثومة المعدة من 81 مريض من الذكور و37 مريضة من الإناث بمعدل عمري (18±55 عاماً). كما تم التعرف والكشف على جيني كاق أي و ڤاك أي لجرثومة المعدة بواسطة طريقة مشتركة من البلمرة والإليزا.

النتائج: لقد تم التعرف على جيني كاق أي و ڤاك أي في 60 عينة (51%). كما تم رصد الملاحظات في 41 (35%) عينة من مرضى التهابات المعدة المزمنة النشطة والمصابة بجرثومة المعدة حيث 22 (54%) عينة منها احتوت على جين كاق أي، في حين احتوت 25 (61%) على جين ڤاك أي. كذلك 26 (22%) من مرضى قرحة الإثني عشر احتوت 14 (54%) منها على جين كاق أي، في حين 15 (58%) احتوت على جين ڤاك أي. وتمت ملاحظة 18 (15%) من حالات مرضى التهابات المعدة الحادة النشطة المصابة بجرثومة المعدة حيث احتوت 8 (44%) عينات على جين كاق أي و12 (67%) احتوت على جين ڤاك أي. في حين أن عينات مرضى سرطان المعدة الأدوني 17 (100%) احتوت على جينات كاق أي وڤاك أي معاً في نفس الوقت. لقد تم رصد تواجد جيني كاق أي وڤاك أي معاً في في 46% في مرضى قرحة الإثني عشر، وبنسبة 44% في مرضى التهابات المعدة الحادة النشطة وبنسبة 46% في مرضى التهابات المعدة المزمنة النشطة.

خاتمة: لقد تم التعرف وعزل الجينات كاق أي وڤاك أي بشكلٍ متكرر في عينات أمراض المعدة والإثني عشر، كما لوحظ تواجدهما معاً في عينات مرضى سرطان المعدة الأدوني مما يستوجب دراسة هذين الجينيين كمسببين مباشرين في سرطانات المعدة والإثني عشر.

Objectives: To detect the presence of virulence markers cytotoxin-associated (cagA) and vacuolating cytotoxin-associated (vacA) genes in gastric biopsy specimens of patients with gastroduodenal disorders.

Methods: This study was conducted at the Department of Pathology, King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia between March 2008 and February 2009. A total of 118 gastric biopsy specimens from 81 males and 37 females (mean age: 55 ± 18 years) with histological evidence for the presence of *Helicobacter pylori (H. pylori)* were included in the study. The *H. pylori* cagA and vacA genes were detected using polymerase chain reaction-enzyme-linked immunosorbent assay technique.

Results: Both *H. pylori* cagA and vacA genes were detected in 60 (51%) patients. Forty-one (35%) patients had active chronic gastritis, 22 (54%) harbored cagA, and 25 (61%) had vacA gene. Twenty-six (22%) patients with duodenal ulcer, 14 (54%) had cagA, and 15 (58%) had vacA genes. Eighteen (15%) patients with active acute gastritis, 8 (44%) carrying cagA gene, and 12 (67%) had vacA gene. The cagA and vacA genes co-existed in all the 17 (100%) patients with adenocarcinoma. These genes coexisted in 44% biopsies from active acute gastritis, and 46% each in duodenal ulcer and active chronic gastritis.

Conclusion: The cagA and vacA genes as *H. pylori* virulence markers were detected in gastroduodenal disorders, and their remarkably high co-existence in adenocarcinoma prompt further investigations for evaluating *H. pylori* as a direct carcinogen.

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Infection with *Helicobacter pylori* (*H. pylori)* is a worldwide problem. Although a marked regional variation in the prevalence of *H. pylori* infection exists, the infection rate, however, increases with increasing age.[1,2] Several epidemiological studies have shown an association of *H. pylori* infection with gastric inflammation and carcinogenesis.[3,4] Clinically, *H. pylori* has been implicated as an etiological agent in the pathogenesis of chronic gastritis,[5] and peptic ulcer[6]. Infection with *H. pylori* has also been shown to be a risk factor for gastric carcinoma.[7] Moreover, the organism has been classified as a class I carcinogen by the World Health Organization and International Agency for Research on Cancer Consensus Group.[8] Strains of *H. pylori* differ in their association with gastrointestinal diseases, and there is a tremendous genetic diversity in *H. pylori* species.[9,10] Several *H. pylori* genes, including cytotoxin-associated gene (cagA), vacuolating cytotoxin-associated (vacA) gene A, genotype s1a, and iceA1, have been shown to confer predisposition for the development of ulcer diseases.[11,12] The cagA encodes a 120-140 K protein of unknown function. This gene is believed to be a marker gene for the presence of the *H. pylori* pathogenicity island containing multiple virulence factors including those promoting inflammation.[13] It has been suggested that the cagA gene is more prevalent in *H. pylori* isolated from patients with duodenal ulcers compared to the symptomatic patients with histological gastritis without ulcer.[14-16] The vacA gene encodes a protein inducing vacuolation of epithelial cell cultures.[17] Within the vacA gene, 2 variable segments have been found, the signal (s) region (s1: subtype s1a, s1b, or s2) and the middle (m) region (m1, m2).[18-20] Specific mosaicism of these 2 regions of the vacA gene have been implicated with the pathogenicity of the bacterium.[19,21,22] On the contrary, there are data indicating that variation of vacA gene subtypes may not have any statistical correlation with the clinical disease.[22] Further investigations are needed to ascertain the pathogenic role of vacA gene. Utilizing enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) concurrently in this study, we aimed to correlate the presence of *H. pylori* cagA and vacA genes in gastric biopsies from patients with gastroduodenal diseases in the Kingdom of Saudi Arabia (KSA).

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Methods.

Patients. Paraffin wax-embedded gastric tissues from patients with gastroduodenal diseases from 152 patients was investigated at the Department of Pathology, King Khalid University Hospital, Riyadh, KSA between March 2008 and February 2009. Out of the total specimens examined, histological evidence for the presence of *H. pylori* was found in 118 patients. This group included patients with active chronic gastritis (n=41), duodenal ulcer (n=26), active acute gastritis (n=18), adenocarcinoma (n=17), gastric ulcer (n=10), and intestinal metaplasia (n=6). There were 81 (69%) male and 37 (31%) female patients with a mean age of 61.9 + 19 years (range: 19-90 years). The classification and grading of the gastroduodenal diseases was based on the revised Sydney-Houston System workshop.[23] Ethical approval was obtained from the Institutional Review Board, King Khalid University Hospital, Riyadh, KSA.

Detection of H. pylori cagA and vacA genes by PCR-ELISA. Ten micrometer thick formalin fixed-paraffin-embedded gastric and duodenal tissue sections were placed in sterile Eppendorf tubes for PCR. Paraffin was removed from the sections using n-octane, and deoxyribonucleic acid (DNA) was extracted according to instructions.[24] The PCR was performed to amplify targets of DNA using primers, and capture probe based on the *H. pylori* 26695 published sequence,[25] as described in the instructions.[24,26] Amplified targets of DNA were then hybridized to a specific capture probes for cagA, and vacA genes. Each probe was complementary to the inner part of the amplification product. Before hybridization, this specific capture probe was labeled with biotin to allow immobilization of the hybrid (DNA-probe) to a streptavidin-coated microtiter plate surface. The bound hybrid was detected by anti-DIG peroxidase conjugate using colorimetric substrate 2,2'-azino-d(3-ethyl-benz-thiazoline)-6-sulfonic acid (ABTS) (Roche Applied Science, Germany). The ELISA was performed as according to instructions.[24,26] The PCR positive control (*H. pylori* DNA) were tested at the same time. Positive results on PCR-ELISA were defined as those giving an optical density (OD) of greater than, or equal to the mean OD plus 2 times the standard deviation of a range negative controls.

Determination of sensitivity of the PCR-ELISA results. Sensitivity of the 2 PCR-ELISA were evaluated using tenfold dilutions of an overnight broth culture of *H. pylori* NCTC 11637. The highest dilution giving a positive PCR-ELISA tests was used to calculate the sensitivity of the PCR-ELISA reactions. Positive (DNA extract from a plate culture of *H. pylori* NCTC 11637) and negative (sterile water) controls were incorporated in each PCR run.

Statistical analysis. The descriptive analysis and comparison of proportions was performed using MedCalc version 12.2.1 statistical software. A $p<0.05$ was considered statistically significant.

Results. Out of the total 152 specimens included in the study, 118 samples had the histological evidence for the presence of *H. pylori*. **Table 1** shows the data for detection of *H. pylori* cagA and vacA genes in the gastric tissue biopsies of 118 patients included in the study. Among them, 60 (51%) samples tested positive for the presence of *H. pylori* cagA or vacA genes. Most patients had active chronic gastritis (41), out these 19 (46%) tested positive for both cagA and vacA genes, 22 (54%) harbored cagA, and 25 (61%) had vacA gene. Of the 26 patients with duodenal ulcer, 12 (46%) had both cagA and vacA genes, where 14 (54%) had cagA, and 15 (58%) had vacA gene. Eight out of the 18 patients with active acute gastritis had both the virulence genes with 8 patients carrying cagA gene, and 12 tested positive for vacA gene. There were 17 patients with adenocarcinoma, and all of them had cagA and vacA genes tested positive 17/17 (100%) (p=0.00004). A relatively smaller number of patients with gastric ulcer (2/10), and intestinal metaplasia (2/6) had the evidence for the presence of both genes. **Table 2** describes the co-existence of both cagA and vacA genes of *H. pylori* in biopsy specimens from patients in all groups included in the study. Out of the total 118 specimens examined, only 28 patients tested negative for the presence of both genes despite the histological evidence of *H. pylori* on Hematoxylin-Eosin staining. The highest number (9) of such patients had active chronic gastritis, followed by 8 patients with duodenal ulcer, and 7 patients had active acute gastritis. Interestingly, all 17 (100%) patients with adenocarcinoma had co-existence of both the cagA and vacA genes. In patients with duodenal ulcer, co-existence of both cagA and vacA genes was found in 12 out of 26 patients (46%) followed by patients with active chronic gastritis (19/41) and active acute gastritis (8/18). Although a comparable percentage of cagA and vacA genes were also observed in patients with gastric ulcer and intestinal metaplasia, the number of patients in each group was insufficient. When compared, the proportion

Table 1 - Detection of *Helicobacter pylori* (*H. pylori)* cytotoxin-associated gene *(*cagA) and vacuolating cytotoxin-associated (vacA) genes in gastric tissue biopsies from patients with gastroduodenal diseases.

Conditions	Specimens per condition, n	Detection of *H. pylori* by histology prior to PCR	cagA		vacA	
			n	(%)	n	(%)
Active acute gastritis	26	18	8	(44)	12	(67)
Active chronic gastritis	53	41	22	(54)	25	(61)
Gastric ulcer	11	10	3	(30)	7	(70)
Duodenal ulcer	31	26	14	(54)	15	(58)
Intestinal metaplasia	11	6	2	(29)	3	(43)
Adenocarcinoma	20	17	17	(100)	17	(100)
Total	**152**	**118**	**65**	**(55)**	**79**	**(67)**

PCR - polymerase chain reaction

Table 2 - *Helicobacter pylori* (*H. pylori)* cytotoxin-associated gene (cagA) and vacuolating cytotoxin-associated (vacA) gene co-existence and comparison of proportions of the co-existing genes in adenocarcinoma with other conditions in biopsy specimens from patients with gastroduodenal diseases.

Conditions	Specimens per condition, n	Detection of *H. pylori* by histology	Both cagA & vacA present		cagA only		vacA only	
			n	(%)	n	(%)	n	(%)
Active acute gastritis	26	18	7*	(39)	2	(11)	2	(11)
Active chronic gastritis	53	41	19*	(46)	5	(12)	8	(20)
Gastric ulcer	11	10	3‡	(30)	0	(0)	4	(40)
Duodenal ulcer	31	26	12†	(46)	2	(8)	4	(15)
Intestinal metaplasia	11	6	2‡	(33)	0	(0)	3	(50)
Adenocarcinoma	20	17	17*	(100)	0	(0)	0	(0)
Total	**152**	**118**	**60**	**(51)**	**9**	**(8)**	**21**	**(18)**

*p=0.0004; †p=0.0008; ‡insufficient numbers

of cagA and vacA gene co-existence in adenocarcinoma was statistically higher than the co-existence of these genes in other gastroduodenal disorders.

Discussion. This study investigating *H. pylori* associated virulence factors detected as significant expression of cagA (55%) and vacA (67%) genes in paraffin wax-embedded biopsy specimens from patients with gastroduodenal disorders. The frequency of detection of these virulence factors is considerably in agreement with previously reported figures.[27,28] Among the study population, a remarkable presence of cagA gene was observed in patients with active chronic gastritis (54%), duodenal ulcer (54%), and gastric adenocarcinoma (100%). A previous study from KSA reported cagA gene in 62% of the patients with gastritis, whereas 100% of patients with peptic ulcer had *H. pylori* cagA gene.[29] A recent study from Taiwan has reported a prevalence of cagA gene 50% in gastritis, 73% in gastric ulcer, and 73.3% in duodenal ulcer patients.[30] In a Brazilian study, the prevalence of cagA gene as high as 90.5% was found in patients with duodenal ulcer, and 60% in patients suffering from gastritis has also been reported.[31] A study from Korea, however, failed to show any association between the expression *H. pylori* cagA and vacA genes to the development of duodenal ulcer.[32] Despite conflicting reports, cagA gene has been extensively investigated as a virulence marker, and has shown to have a significant association with gastric and duodenal ulcers.[28] Furthermore, the historic perspective shows that gastroduodenal disease appears to be uncommon in Africa and *H. pylori* infection has only been associated with gastritis,[33] and may actually be providing protection against gastric carcinoma in African population.[34] These data indicate that the prevalence of *H. pylori* isolates appears to vary from one geographic region to another, and exhibit variable association with the prevailing pattern of gastroduodenal disorders.

The *H. pylori* vacA gene has also been associated with significant gastroduodenal disease. The gene has been detected in gastric biopsy specimens from a majority of patients (71%) with active chronic gastritis.[35] Infection with multiple strains of *H. pylori* as defined by different vacA genotypes has been shown to exhibit a strong association with peptic ulcer, where vacA gene was detected in 95% of adult patients.[36] A significant relationship of s1a allele of vacA gene with gastric cancer and s1a/m2 vacA genotype with gastric carcinoma and peptic ulcer disease has recently been established.[37] In agreement with these observations a notable expression of vacA gene was detected in the present study in patients with active chronic gastritis, duodenal ulcer, and adenocarcinoma. Despite the existence of a large body of evidence supporting a significant association of vacA gene with gastroduodenal disease there are a number of studies refuting these claims.[28,32] Although assigning a single gene to a particular disease condition may be challenging it is, however, possible that a coordinated interaction among different virulence genes may be important. This may be relevant in the context of *H. pylori* infection blamed as a major risk factor for gastric carcinoma, especially due to coexistence of cagA and vacA genes in patients with gastric cardiac intestinal metaplasia.[38]

Based on cagA and vacA gene co-expression and production of CagA protein and vacA, *H. pylori* strains have been classified into type I and type II. Type I strains are not only in majority, but they also express gene products, whereas type II strains lack both the genes and the expression of products.[39] It is therefore possible that the 28 patients with histological evidence of *H. pylori* infection who tested negative for the presence of cagA and vacA genes were infected with type II strains of the organism. Co-existence of both cagA and vacA genes was found in 46% of both duodenal ulcer and of active chronic gastritis in this study, however, the most striking observation was found in patients with gastric adenocarcinoma where 100% patients exhibited co-existence of cagA and vacA *H. pylori* genes. Strains of *H. pylori* with different cagA and vacA subtypes may be present in one individual and their coexistence has already been linked with the development of peptic ulcer.[40] It is possible that both cagA and vacA genes are functionally linked. This is evident from the fact that among cagA positive strains those carrying vacA s1a genotype are more likely to be associated with ulcers compared to the strains harboring vacA s1b genotype.[41] In addition, increased virulence of *H. pylori* strains has been attributed to the presence of more than one virulence markers,[41] which could possibly explain the significantly high coexistence of cagA and vacA genes in patients with adenocarcinoma in the present study. High levels of both cagA and vacA antibodies in sera of patients with gastric cancer even after the eradication of *H. pylori* infection reinforces the hypothesis that their coexistence may have a pivotal role in *H. pylori* associated gastric cancers.[42]

In conclusion, although the number of patients suffering from adenocarcinoma in this study was small, a remarkably high co-existence of cagA and vacA genes in patients with adenocarcinoma was observed. Large-scale studies are recommended to evaluate *H. pylori* as

a direct carcinogen with regard to co-existence of cagA and vagA genes. In addition, prospective studies are also recommended to monitor patients with gastric disorders other than malignancy harboring both cagA and vacA genes for the risk of developing gastric cancer. Moreover, the vacA gene typing and sub-typing that was not performed in the present study should also be assessed to determine the predominant sub-types in the region and their association with gastroduodenal disorders.

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