BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2017
VOL. 74, NO. 3, 121–126
https://doi.org/10.1080/09674845.2017.1278887

Association of heterogenicity of *Helicobacter pylori* cag pathogenicity island with peptic ulcer diseases and gastric cancer

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ABSTRACT

Objective: To investigate the frequency and integrity of certain *cag* pathogenicity island genes (*cagPAI*) in *Helicobacter pylori* strains and their association with peptic ulcer disease (PUD) and gastric cancer.
Material and Methods: We enrolled 240 adult patients [120 with functional dyspepsia (FD), 50 with PUD and 70 with gastric cancer] undergoing upper gastrointestinal endoscopy. *H. pylori* infection was diagnosed when either culture or any two of the three tests (rapid urease test, histopathology and specific ureA PCR) were positive. DNA extracted from *H. pylori* isolates and positive gastric tissues were tested by PCR for the presence of different genes of *cagPAI* using specific primers.
Results: A total of 122 (51%) patients were *H. pylori* positive. Frequencies of *cagPAI* genes *cagA*, *cagE*, *cagT* and *cagM* in *H. pylori* strains from different groups of patients were as follows: functional dyspepsia 73, 83, 76 and 60%, PUD 70, 94, 91, 70% and gastric cancer 75, 95, 90 and 70%, respectively. Risk associated for the presence of PUD and gastric cancer with *cagPAI* genes *cagE*, *cagT* and *cagM* was 5.0-, 4.6- and 4.1- and 3.0-, 2.8- and 2.5-folds, respectively. Prevalence of intact *cagPAI* was significantly higher in PUD and gastric cancer compared to functional dyspepsia (PUD vs. functional dyspepsia, 71% vs. 38%, $P = 0.01$; gastric cancer vs. functional dyspepsia, 75% vs. 38%, $P < 0.01$). Intact *cagPAI* was associated with increased risk for the presence of PUD (odds ratio 5.2, 95% CI 2.4–11.3) and for the presence of gastric cancer (odds ratio 4.5, 95% CI 2.3–7.1).
Conclusions: *cagPAI* integrity and its different genes are linked to different forms of gastric disease and so may have a role in pathogenesis, diagnosis and management.

ARTICLE HISTORY
Received 7 September 2016
Accepted 19 December 2016

KEYWORDS
cagPAI; gastric cancer; gastric tissues; H. pylori; functional dyspepsia; peptic ulcer diseases

Introduction

Helicobacter pylor (H. pylori)-induced pathogenesis of gastric disease is multifactorial. Many putative *H. pylori* virulence factors have been recognised that may play a crucial role in determining disease outcome [1]. The infection remains asymptomatic in vast majority of population with only about 10–20% of infected individuals developing PUD and 1–2% gastric cancer. Determining factors for this disparity in clinical outcomes are yet not clear [2, 3]. So in order to understand these inconsistencies, studies on the impact of *H. pylori*-related potent virulence factors are required. The *cag* pathogenicity island (*cagPAI*) is a well-known virulent marker of *H. pylori* that encodes a type IV secretion apparatus(T4SS) which forms a syringe-like structure to translocate *cagA* oncoprotein as well as peptidoglycans directly into the host epithelial cells. This translocation results in the induction of potent proinflammatory cytokines such as interleukin-8 (IL-8) from gastric epithelial cells [4, 5]. Studies proved that the *cagA* protein is associated with an increased risk for subsequent development of atrophic gastritis, intestinal metaplasia and adenocarcinoma [6–8].

The *cagPAI* locus is approximately 40 kb long and divided into two parts: an upstream cagII region (14 genes) and a downstream cagI region (16 genes) [9]. The cagI harbours cytotoxin-associated genes *A* and *E* (*cagA* and *cagE)* and these genes are required for IL-8 induction from gastric epithelial cells [10]. Furthermore, *cagM* and *cagT* are the markers of the cagII region and the presence of these genes has been associated with PUD and gastric cancer [11, 12].

In earlier studies, *cagA* was thought to be the marker for integrity of *cagPAI* [13]. Many experimental studies demonstrated that the *cagA* protein was associated with severe form of gastric pathologies such as chronic gastritis, ulcers and gastric cancer [14, 15]. However, several studies cast doubt on the strength of association of *cagA* with severity of diseases [10, 16–19].

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This article was originally published with errors. This version has been corrected. Please see Erratum (https://doi.org/10.1080/09674845.2017.1347365).
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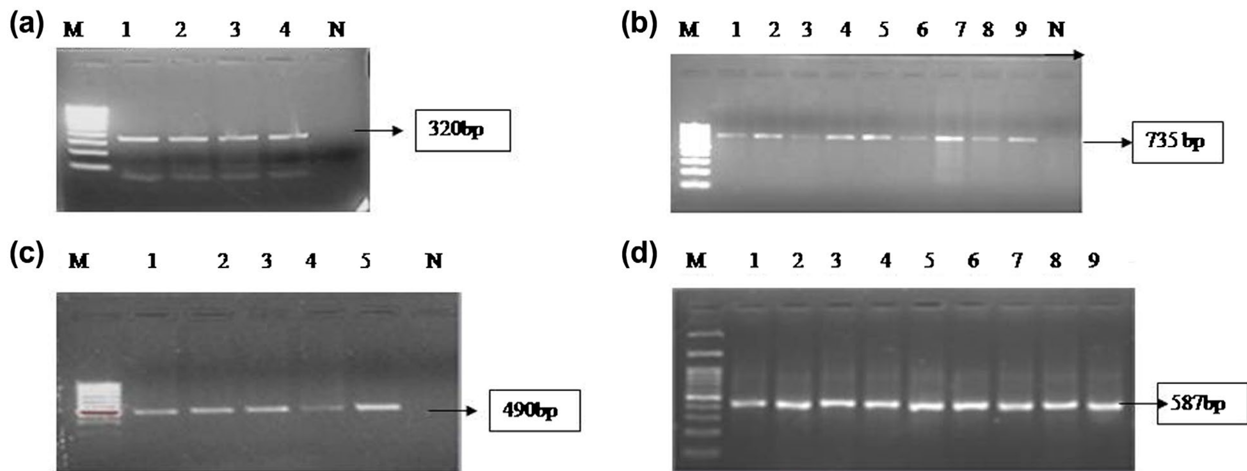

Figure 1. PCR of *Helicobacter pylori* showing amplified products of different *cagPAI* genes: (a) *cagA* (320 bp), (b) *cagE* (735 bp), (c) *cagT* (490 bp) and (d) *cagM* (587 bp). M-molecular weight maker (100 bp ladder), lane 1-positive control and lane N-negative control.

Table 1. PCR primer sequences used for the detection of different cagPAI genes of Helicobacter pylori.

cagPAI genes	Primers	Primer sequences	PCR product size	Reference
cagA	*cagA F*	AGATAACAGGCAAGCTTTTGA	349bp	29
	cagA R	TCTGCCAAACAATCTTTTGCAG		
cagE	*cagE F*	GTTACATCAAAAATAAAAGGAAGCG	735bp	30
	cagE R	CAATAATTTTGAAGAGTTTCAAAGG		
cagT	*cagT F*	TCTAAAAAGATTACGCTCATAGGCG	490bp	30
	cagT R	CTTTGGCTTGCATGTTCAAGTTGCC		
cagM	*cagM F*	ACAAATACAAAAAAGAAAAAGAGGC	587 bp	30
	cagM R	ATTTTTCAACAAGTTAGAAAAAGCC		
cagPAI empty site	Lunil	ACA TTT TGG CTA AAT AAA CGC TG	550bp	31
	R5280	GGT TGC ACG CAT TTT CCC TTA ATC		

Translocation of *cagA* into the host cell requires 17 genes of *cagPAI* [20]. *H. pylori* can induce proto-oncogenes c-fos and c-jun, an essential step in progress of *H. pylori*-associated neoplasia [21]. An intact *cagPAI* has therefore been thought to contribute towards full proinflammatory power of *H. pylori*. However, the island is not a conserved entity and is susceptible to various genetic rearrangements occurring within and outside the genes. In addition, integrity and deletion frequencies of individual genes and their association with development and progression of gastric diseases have still been questioned.

Investigating *cagA* alone does not assure that other virulent genes within the *cagPAI* are intact. However, studies comparing integrity of *cagPAI* and its association with gastric diseases are rare. So, we aimed to study the integrity and deletion frequencies of different *cagPAI* genes of *H. pylori* and their association with gastric pathologies such as PUD and gastric cancer.

Material and methods

Patient population: A total of 240 consecutive adult patients (mean [standard deviation] age 47.3 [14.7]; 153 male) undergoing upper gastrointestinal endoscopies at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow between March 2012 and 2016 were enrolled for the study. The patients were divided into three groups: gastric cancer 70 (age 54.2 [12.7]; 42 male), peptic ulcer diseases (PUD) 50 (age 48.1 [12.3]; 38 male) and functional dyspepsia 120 (age 42.1 [12.9]; 73 male). The diagnosis of gastroduodenal disease was based on clinical, endoscopic and histopathological examinations. Patients with functional dyspepsia were considered as controls. The ethics committee of the institute granted approval for the study and all the patients gave their consent to participate. Subjects who had received antimicrobial therapy, H2-receptor blockers, proton-pump inhibitors and non-steroidal anti-inflammatory drugs 30 days prior to endoscopy were excluded from the study.

Detection of Helicobacter pylori infection: The antral biopsies obtained during endoscopic examination were subjected to rapid urease test (RUT), culture, histopathology and *H. pylori*-specific ureA polymerase chain reaction (PCR) following the standard protocol as described elsewhere [22]. *H. pylori* infection was diagnosed if either culture or any two of the three tests was positive.

H. pylori genotyping: DNA was isolated from the bacterial growth and *H. pylori*-positive gastric tissues using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and subjected to PCR for the presence of different genes of cagI (*cagA* and *cagE)* and cagII (*cagT*, *cagM*) regions of the *cagPAI* locus using specific primers[23, 24]. The isolates were further analysed by an empty-site PCR to determine whether the *cagPAI* was deleted. Amplification of 550bp

Table 2. Detection of different cagPAI genes among Helicobacter pylori-infected individuals with gastroduodenal disorder.

Prevalence of different *cagPAI* genes (%)	FD/control (n = 120)	PUD (n = 50)	GC (n = 70)	Total (n = 240)	*P*-values		
					PUD vs. FD	GC vs. FD	PUD vs. GC
cagA	35 (73)	24 (70)	30 (75)	89 (73)	0.238	0.164	0.086
cagE	31 (65)	32 (94)	38 (95)	101 (83)	≤0.001	≤0.001	0.330
cagT	26 (54)	31 (91)	35 (90)	93 (76)	≤0.001	≤0.001	0.453
cagM	22 (46)	24 (70)	28 (70)	74 (60)	0.0012	0.0012	0.522
Intact *cagPAI*	18 (38%)	24 (71%)	30(75)	72 (59)	0.01	≤0.01	0.074
Partially deleted *cagPAI*	25 (52%)	10 (29%)	8 (26%)	43 (35)	0.067	0.052	0.342
≠Completely deleted	5 (10%)	–	2 (7%)	7 (6)	–	–	–

Notes: ≠Since the numbers of strains with complete *cagPAI* deletion were few, statistical analysis was not possible.

Table 3. Association of different cagPAI genes and their status (of intact, partially and completely deleted) with peptic ulcer disease (PUD) and gastric cancer (GC).

	PUD			GC		
cagPAI genes	OR	95% CI	*P*-value	OR	95% CI	*P*-value
cagA	0.3	1.51–2.42	0.077	0.24	681–2.34	0.089
cagE	5	2.31–8.22	0.001	3.0	1.82–6.13	0.002
cagT	4.6	1.56–5.67	0.012	2.8	1.04–5.37	0.013
cagM	4.1	1.17–4.86	0.014	2.5	1.82–4.26	0.032
cagPAI status						
Intact	5.2	2.42–11.3	0.001	4.5	2.34–7.12	0.001
Partially deleted	0.34	0.722–3.64	0.83	0.47	0.19–1.00	0.077
Completely deleted	0.31	1.00–2.56	0.96	0.61	1.23–0.328	0.634

fragment indicated the absence of whole *cagPAI* [25]. All the primer sequences used in this study are given in Table 1. *H. pylori* strain 26695 DNA was used as a positive control and DNA from single colonies of clinical isolates that were positive for empty-site PCR were used as negative controls. PCR was performed at least twice for each sample with basically identical results. Primers were designed on genome-based data of *H. pylori* strain 26695 [26]. All PCR mixtures were performed in a total volume of 25 µL containing 10 × PCR buffer, 500 nM of each primer, 200 M each dNTP, 1.5 U Taq DNA polymerase and 200 ng DNA sample. The total volume was made up with autoclaved Milli-Q water (Eppendorf AG 22331, Hamburg, Germany). Amplification conditions optimised in thermocycler were as follows: initial denaturation for 5 min at 94 °C was followed by 30 cycles of denaturation at 93 °C for 1 min, annealing at 58, 57 and 60 °C for *cagA* and *cagM*, *cagE* and *cagT*, respectively, for 30 s and extension at 72 °C for 1 min. After a final extension at 72 °C for 10 min, PCR products were visualised by electrophoresis in 1.2% agarose gel, stained with ethidium bromide and examined under UV illumination.

Histopathology: Sections of 5 µm were cut from formalin-fixed biopsies, embedded in paraffin wax. The sections were stained with hematoxylin and eosin for light microscopy to detect the presence of *H. pylori* infection. Patients with gastric cancer were confirmed by histopathology and classified into intestinal, diffuse and mixed according to the Lauren classification [27].

Statistical analysis: Data were analysed using SPSS software (version 12.0, SPSS, Chicago, IL., USA). Statistical differences in demographic characteristics among the study groups were analysed by χ^2 test. The univariate association between genotype status and gastroduodenal diseases was quantified by student *t*-test and χ^2 test. All the *P*-values were two sided and considered significant when less than 0.05.

Results

Fifty-one per cent (122/240) of the gastric biopsies were positive for *H. pylori*. Distribution of *H. pylori* infection among different groups of patients was as follows: functional dyspepsia 40% (48/120), PUD 68% (34/50) and gastric cancer 57% (40/70), respectively. *H. pylori* infection had a significant association with gastric cancer and PUD (gastric cancer vs. functional dyspepsia: 57% vs. 40%, $P = 0.024$; PUD vs. functional dyspepsia; 68% vs. 40%, $P < 0.01$).

The frequencies of *cagPAI* locus genes (*cagA*, *cagE*, *cagT and cagM*) were determined using DNA extracted from either *H. pylori* cultures or *H. pylori*-positive gastric biopsies by PCR (Figure 1). The most frequent *cagPAI* gene of cagI region was *cagE* followed by *cagA* and of cagII region was *cagT* and followed by *cagM* (Table 2). The distributions of *cagA* and *cagE* in different groups of patients were functional dyspepsia 73 and 65%, PUD 70 and 94% and, gastric cancer 75 and 95%, respectively (Table 2). Significant association of *cagE*, *cagT* and *cagM* with PUD and gastric cancer was observed (Table 3).

The integrity of *cagPAI* [i.e. an intact *cagPAI* locus] was defined when all the four genes of cagI (*cagA*, *cagE)* and cagII (*cagT* and *cagM*) regions were present. Intact *cagPAI* was detected in 71, 75 and 38% patients with PUD, gastric cancer and functional dyspepsia,

respectively (Table 2). *H. pylori* strains with completely deleted *cagPAI* (i.e. *cagA−/cagE−/cagT−/cagM−*) were detected in 7% patients with gastric cancer and in 10% patients with functional dyspepsia (Table 2). None of the *H. pylori*-infected patients with PUD had completely deleted *cagPAI*. Partially deleted *cagPAI* was detected in 35% of *H. pylori* strains and their disease wise frequencies were as follows: gastric cancer 26%, PUD 29% and functional dyspepsia 52% (Table 2). *H. pylori* infection with an intact *cagPAI* was associated with 5.2 and 4.5-fold increased risk of PUD and gastric cancer, respectively (Table 3).

Discussion

H. pylori is considered as one of the most genetically diverse bacterial species [28]. *H. pylori* genome's heterogeneity is attributable to inter-strain gene translocation, recombination and difference in epidemiology along with pressure created by host immune defences in long-term infection [2]. These factors collectively lead to continuous evolution in terms of mutational diversity and facilitates in colonising diverse niches [29]. The *cagPAI* is a 40 kb long stretch of DNA fragment that encodes T4SS containing 32 genes. The insertion element IS605 divides *cagPAI* into two parts, upstream (cagI) and downstream (cagII), consisting of 14 and 16 open reading frames [9]. T4SS exports bacterial protein and peptidoglycan including *cagA* oncoprotein into the host epithelial cells [5, 30, 31]. Studies from Europe had presumed *cagA* to be a virulence marker associated with severe pathology but this has not be confirmed in other populations such as East Asian countries including India. Despite the well-defined role of *H. pylori* virulence factors, it is still unclear why a substantial proportion of patients infected with *H. pylori* strains that possess virulence factors such as *cagA*, *babA* and *vacA* do not develop severe pathology like PUD and gastric cancer throughout their life. Hence, to resolve this paradox, the genotyping of *cagPAI* genes seems to be a useful tool. Furthermore, these findings also help us in understanding the *H. pylori*-mediated disease pathology and also enhance the present knowledge available regarding the host–parasite interactions.

We used PCR to screening different *cagPAI* genes in *H. Pylori* strains, finding that the overall prevalence of *H. pylori* infection had a significant association with gastric cancer and PUD, an observation concordant with other studies [19, 32]. The frequencies of these genes were significantly higher in PUD and gastric cancer compared to functional dyspepsia (Table 2). Detection rates of *cagE* and *cagT* were almost similar in PUD and gastric cancer. This observation is supported by a study from England that majority of *H. pylori* strains associated with severe gastric pathologies retained the *cagE and cagT* [33]. We also calculated the odds ratio of the risk of disease associated with the presence of these genes, finding significantly increased odds linked to PUD and gastric cancer by the presence of *cagE*, *cagT and cagM,* respectively, compared to functional dyspepsia (Table 3). *cagA* had similar frequency in *H. pylori* strains from PUD, gastric cancer and functional dyspepsia. Rohde et al. [34] revealed that deletion of *cagE and cagT* resulted in impaired 'molecular syringe' and their presence was associated with severe form of gastric pathologies [35].

Of 122 *H. pylori* positive gastric biopsies, 59% harboured the intact *cagPAI* and the distributions of intact *cagPAI* in different groups of patients were twice as high in gastric cancer and PUD compared to functional dyspepsia. This may be pathogenic. Keteas et al. [36] had shown that intact *cagPAI* gene products were necessary for activation of mitogen-activated protein kinases in gastric mucosal cells responsible for cell proliferation, differentiation, inflammatory responses, stress and apoptosis and induction of chronic inflammation, ulceration and finally neoplasia. Our observations reflect those of Maeda et al. [37], who have shown that the clinical isolates with intact *cagPAI* were associated with more severe form of gastroduodenal diseases like PUD and gastric cancer; partially deleted *cagPAI* strains were more common in non-ulcer dyspepsia. Similarly in our study, partially deleted *cagPAI* strains were more common in functional dyspepsia compared to PUD and gastric cancer. We found that more than twice as many functional dyspepsia samples had completely deleted *cagPAI* as compared to gastric cancer, corroborating a Swedish study which showed 15% of the isolates from non-ulcer dyspepsia lacking the *cagPAI* and 5% of the isolates from severe pathology, i.e. gastric cancer and PUD [38]. The results suggest that *cagPAI* is apparently not uniform and conserved but is undergoing continuous rearrangement and mutations corroborating earlier studies [25, 35, 39]. In addition, Naumann et al. [40] demonstrated that the intactness of *cagPAI* was a prerequisite for induction and activation of early transcription factor AP-1, which was associated with various immune stimulatory functions in the host epithelial cells.

Our data support the view that knowledge of *cagPAI* genotypes may be useful in supporting the firm diagnosis of a particular form of gastric disease (i.e. malignant or non-malignant), and so this form of molecular genetics may become a routine laboratory marker in this field. This work represents an advancement in biomedical science because it shows that the *cagPAI* integrity and its different genes play an important role in determining the disease outcomes.

Summary table

What is known about this subject

- cagA was thought to be the marker for integrity of cag PAI.
- cagPAI genes (cagE, cagT and cagM) and their relation with in gastric pathologies were documented.
- Contrary studies were documented on integrity and deletion frequencies of cagPAI genes and their association with development of peptic ulcer diseases and gastric cancer.

What this paper adds

- cagA alone was not found to be the marker for integrity of cag PAI.
- Risk associated with cagPAI genes cagE, cagT and cagM for peptic ulcer diseases was 5, 4.6, 4.1 and for gastric cancer 3, 2.8, 2.5 folds, respectively.
- Intact cagPAI was associated with 5.2 and 4.5 folds increased risk of PUD and GC respectively.

Acknowledgements

Jahanarah Khatoon and Ravi Prakash Rai gratefully acknowledge Indian Council of Medical Research (ICMR) for junior research fellowship through grant numbers 3/1/3/3/JRF-2011/ HRD-87(32606) and 3/1/3/JRF-2012/HRD-162(80220), respectively.

Disclosure statement

There is no conflict of interest among authors.

Funding

This work was supported by Indian Council of Medical Research (ICMR) [grant number 3/1/3/3/JRF-2011/ HRD-87(32606)], [grant number 3/1/3/JRF-2012/HRD-162(80220)].

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