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World J Gastroenterol 2005;11(43):6815-6822
World Journal of Gastroenterology ISSN 1007-9327

•*CLINICAL RESEARCH*•

Association between cag-pathogenicity island in *Helicobacter pylori* isolates from peptic ulcer, gastric carcinoma, and non-ulcer dyspepsia subjects with histological changes

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Received: 2005-03-24 Accepted: 2005-04-30

Abstract

AIM: To investigate the presence of the *cag*-pathogenicity island and the associated histological damage caused by strains with complete *cag*-PAI and with partial deletions in correlation to the disease status.

METHODS: We analyzed the complete *cag*-PAI of 174 representative *Helicobacter pylori* (*H pylori*) clinical isolates obtained from patients with duodenal ulcer, gastric ulcer, gastric cancer, and non-ulcer dyspepsia using eight different oligonucleotide primers viz *cag*A1, *cag*A2, *cag*AP1, *cag*AP2, *cag*E, *cag*T, LEC-1, LEC-2 spanning five different loci of the whole *cag*-PAI by polymerase chain reaction (PCR).

RESULTS: The complete screening of the genes comprising the *cag*-PAI showed that larger proportions of subjects with gastric ulcer (97.8%) inhabited strains with complete *cag*-PAI, followed by gastric cancer (85.7%), non-ulcer dyspepsia (7.1%), and duodenal ulcer (6.9%), significant differences were found in the percentage distribution of the genes in all the clinical groups studied. It was found that strains with complete *cag*-PAI were able to cause severe histological damage than with the partially deleted ones.

CONCLUSION: The *cag*-PAI is a strong virulent marker in the disease pathogenesis as it is shown that a large number of those infected with strain with complete *cag*-PAI had one or the other of the irreversible gastric pathologies and interestingly 18.5% of them developed gastric carcinoma. The presence of an intact *cag*-PAI correlates with the development of more severe pathology, and such strains were found more frequently in patients with severe gastroduodenal disease. Partial deletions of the *cag*-PAI appear to be sufficient to render the organism less pathogenic.

Key words: *Helicobacter pylori*; *cag*-pathogenicity island; Genetic diversity; Gastro-duodenal diseases

Ali M, Khan AA, Tiwari SK, Ahmed N, Rao LV, Habibullah C. Association between *cag*-pathogenicity island in *Helicobacter pylori* isolates from peptic ulcer, gastric carcinoma, and non-ulcer dyspepsia subjects with histological changes. *World J Gastroenterol* 2005; 11(43): 6815-6822
http://www.wjgnet.com/1007-9327/11/6815.asp

INTRODUCTION

Gastric cancer is the second most deadly malignant neoplasia worldwide. According to the presently available statistics, approximately 74% of those diagnosed succumb to this disease every year[1]; this is because of poor prognosis as it is often made when the disease has assaulted the muscularis propria. Evidences show that the pathogenesis of gastric cancer is a multistep process[2,3]. This 'cascade' is believed to be triggered by *Helicobacter pylori* (*H pylori*) infection, a Gram negative pathogen. Chronic infection with this gastric pathogen is known to be the major factor driving the precancerous process via mechanisms including direct transformation of cells, induction of immunosuppression with consequently reduced cancer immunosurveillance, or by causing chronic inflammation[4,5]. In 1994, the International Agency for Research on Cancer (IARC) declared *H pylori* a Class 1 (definite) carcinogen based on the epidemiological and interventional studies in human beings[6] and convinced that this bacterial infection indeed plays a key role in the initiation of the neoplastic process in the stomach.

Although many attempts in the past have been made to understand and associate the causal link between *H pylori* infection and the sequelae that leads to gastric carcinoma[7,8], there are conflicting data in the literature due

to differences in the study population and designs[9,10].

In the past few years, many *H pylori* virulence factors have been described that contribute to the survival of this pathogen in an extremely hostile acidic milieu of the stomach and its colonization in that organ[11,12]. Other putative virulence determinants such as vacuolating cytotoxin gene A (vacA), cytotoxin associated gene pathogenicity island (*cag*-PAI) and induced by contact with epithelium gene A (iceA) are not present in all *H pylori* strains or are known to exhibit different allelic variations[13]. The cytotoxin-associated gene island also referred to as the *cag*-PAI is an approximately 40-kb cluster of genes and is the most studied marker of the *H pylori*. In many studies[20,22,24], this large fragment has been a criterion of typing *H pylori* into pathogenic and non-pathogenic strains (Type I and Type II). Studies emphasizing on the functional importance of this island have reported that strains possessing *cag*-PAI induce more notable phenotypic changes *in vivo*, such as higher levels of IL-8 production than *cag*-PAI negative ones[30]. Recent studies made from within our institute[14,15] and other parts of the world[16,17] have mainly focused on the presence of this large fragment and its association with the disease status. Therefore, in a continuing attempt to further establish a strong epidemiologic relation between the *cag*-PAI and the disease conditions with reference to the histopathologic changes lead to the inception of the present study. As it is reported[18] that the presence of *cag* island and the consequent *cag* instability may produce differences in the pathogenicity and host adaptability within a bacterial strain, detailed analyses of the genes of the *cag* island in human beings isolates with reference to histologic damage and disease outcome would be essential. Therefore, compelled by these observations, the present study was designed to identify the distribution of different genes of the *cag*-PAI in clinical *H pylori* isolates by assessing the presence of representative genes located in different segments of the *cag*-island and its correlation with the histologic changes and disease status.

MATERIALS AND METHODS

Patients and sampling

The study population consisted of 174 patients (100 males and 74 females) with a mean age of 48.4 years (range 21-73 years). The patients were classified at the time of endoscopy into those suffering from active ulcers disease [duodenal ulcers (n = 58), gastric ulcers (n = 46), gastric carcinoma (n = 14)] and those with no evidence of mucosal ulcer and gastritis, but suffering from mild or severe dyspeptic symptoms, i.e. non-ulcer dyspepsia (n = 56). None of the patients included in the study had received NSAIDs or antibiotics within the previous 2 mo. Informed consents were taken from the patients who underwent upper gastrointestinal endoscopy at the department of gastroenterology, Deccan College of Medical Sciences, Hyderabad.

Four gastric biopsies were collected: one in urea solution for the rapid urease test (RUT), one in supplemented broth for isolating culture, one in phosphate-buffered saline (PBS) for testing by PCR assay, and one in 10% buffered formalin for histological examination by modified Giemsa stain for the presence of *H pylori*.

H pylori strains

A total of 174 clinical *H pylori* strains were screened for the presence of *cag*-PAI genes. These strains were recovered from individual subjects undergoing upper gastrointestinal endoscopy presenting with various symptoms. This included 43 live strains and 131 genomic DNA isolated from the gastric biopsy.

Extraction of genomic DNA

H pylori culture and DNA extraction from the culture and biopsy was carried out as described elsewhere[19].

PCR analysis of the *cag*-PAI genes

The genes of the *cag*-PAI were PCR amplified under the conditions described by Ikenoue *et al*[20]. One microliter of the extracted genomic DNA was used in a 20 μL reaction volume containing 1´ PCR buffer, 1 U Taq DNA polymerase, 1.5 mmol/L $Mg2^{+}$, 200 μmol/L each dNTP and 10 pmol/L of each primer.

The cycling parameters were optimized and are as follows: Initial denaturation at 95 °C for 5 min, followed by 40 cycles each of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1.5 min and finally after the last cycle, extension was continued for another 7 min.

Eight sets of oligonucleotide primers spanning the 40 kb *cag*-PAI were used in the study. Appropriate positive and negative controls were included in each set to avoid misinterpretation of results. The details of the primers used with their product sizes are enlisted in Table 1. Amplicons were separated by electrophoresis on 2% agarose gel and stained by ethidium bromide.

Histopathological analysis

The biopsy specimen collected from the gastric antrum was used for histopathologic examination to grade

Table 1 List of primers

Target gene	Primer	Sequence (5'- 3')	Product size (bp)
	CagA-F1	AACAGGACAAGTAGCTAGCC	
CagA1	CagA-R1	TATTAATGCGTGTGTGGCTG	701
	CagA-F2	GATAACAGGCAAGCTTTTGA	
CagA2	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	349
	CagAP-F1	GTGGGTAAAAATGTGAATCG	
CagAP1	CagA-R1	TATTAATGCGTGTGTGGCTG	730
	CagAP-F2	CTACTTGTCCCAACCATTTT	
CagAP2	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	1 181
	CagE-F1	GCGATTGTTATTGTGCTTGTAG	
CagE	CagE-R1	GAAGTGGTTAAAAAATCAATGCCCC	329
	CagT-F1	CCATGTTTATACGCCTGTGT	
CagT	CagT-R1	CATCACCACACCCTTTTGAT	301
	LEC-F1	ACATTTTGGCTAAATAAACGCTG	
LEC-1	LEC-R1	TCTCCATGTTGCCATTATGCT	384
	LEC-F2	ATAGCGTTTTGTGCATAGAA	
LEC-2	LEC-R2	ATCTTTAGTCTCTTTAGCTT	877

the severity of disease after they were embedded in paraffin and stained with hematoxylin and eosin. A single experienced pathologist (ZA), who was blinded to the patient's history and molecular data of the isolate, evaluated all histological data. The grade of gastritis was determined on the basis of the updated Sydney system[21].

Statistical analysis

The data were analyzed by means of χ^2 test and the Mann –Whitney *U* test.

RESULTS

In a total of 174 isolates screened, only 65 (37.4%) was found to carry the complete *cag*-PAI, while 109 (62.6%) carried the *cag*-PAI with partial deletions. No isolate was found with completely deleted *cag*-PAI (Table 2).

With reference to the clinical status, we found majority of the duodenal ulcer (DU) isolates and non-ulcer dyspepsia (NUD) isolates to possess partially deleted *cag*-PAI, while on the contrary we found ~97.8% of the gastric ulcer (GU) isolates and 85.7% of the gastric carcinoma (GC) isolates possessed the intact *cag*-PAI. The details of the results obtained are given in Table 2 and Figure 1. Statistically these differences were highly significant.

Table 2 Relationship between the presence of cag-PAI and the clinical status

Clinical status (*n*)	Intact PAI (%)	*cag*-PAI type Partially deleted PAI (%)	Completely deleted PAI (%)
DU (58)	4 (6.9)	54 (93.1)	0
GU (46)	45 (97.8)	1 (2.2)	0
NUD (56)	4 (7.1)	52 (92.9)	0
GC (14)	12 (85.7)	2 (14.3)	0
Total (174)	65 (37.4)	109 (62.6)	0

χ^2=130.71 .

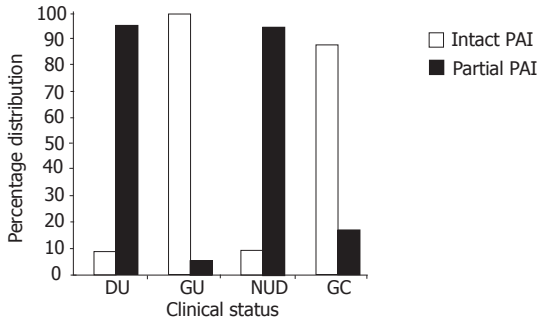

Figure 1 Distribution of *cag*-PAI in dyspeptic subjects.

When checked for the presence of each locus, we could find that the total *cag*A gene (i.e. with promoters) to be present in 69 (39.7%) of all the isolates screened, 4 (2.3%) isolates had total deletion of the gene and 101 (58%) were carrying partial deletions. Of the 69-*cag*A positive isolates, prevalence of *cag*A gene was predominant among gastric ulcer (GU) and gastric carcinoma (GC) strains (Table 3 and Figure 2) simultaneously *cag*A gene with partial deletions were more prevalent among *H pylori* isolated from duodenal ulcer (DU) and non-ulcer dyspepsia (NUD) subjects.

Table 3 *cag*A status of *H pylori* isolates

Clinical status (*n*)	*cagA* +ve (%)	Partial *cagA* +ve (%)	*cagA* –ve (%)
DU (58)	8 (13.8)	46 (79.3)	4 (6.9)
GU (46)	45 (97.8)	1 (2.2)	0
NUD (56)	4 (7.1)	52 (92.9)	0
GC (14)	12 (85.7)	2 (14.3)	0
Total (174)	69 (39.7)	101 (58)	4 (2.3)

DU *vs* GU, DU *vs* GC, GU *vs* NUD and NUD *vs* GC combinations are highly significant at 0.1% level. DU *vs* NUD and GU *vs* GC combinations are not significant at 0.1% level.

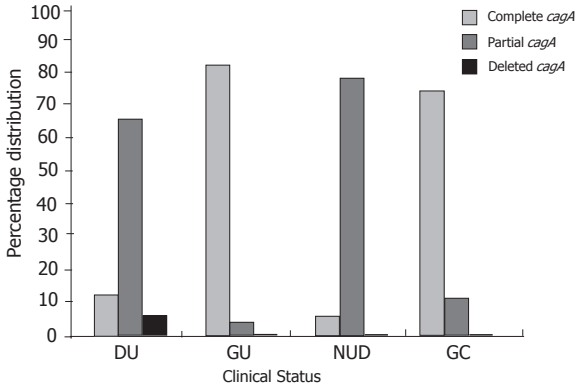

Figure 2 Distribution of *cag*A in clinical isolates.

Observing the prevalence patterns of *cag*A deletion mutant strains, we further analyzed the predominance of deletions in the promoter and body part of the *cag*A gene. The body part of *cag*A gene (i.e. A1+A2) was present in a maximum number of isolates i.e. 134 (77%), among which a considerable number of isolates lacked the promoter region (i.e. AP1+AP2) (Table 4).

Screening of the *cag*II region of the *cag*-PAI revealed *cag*E, *cag*T, LEC-1, and LEC-2 to be present in 143 (82.1%), 145 (87.3%), 142 (81.6%) and 111 (63.7%) of the total isolates, respectively. The distribution of these genes with reference to disease status is illustrated in detail in Table 5 and Figure 3. We found the difference between DU and GU, GU and NUD, GC and NUD to be highly significant statistically.

In the present study, histopathological examination of antral biopsies from a total of 174 subjects was carried out

Table 4 Distribution of *cag*A gene and *cag*A promoter in *H pylori* isolates

Clinical status (*n*)	A1+A2 (%)	AP1+AP2 (%)
DU (58)	43 (74.1)	8 (13.8)
GU (46)	45 (97.8)	46 (100)
NUD (56)	32 (57.1)	6 (10.7)
GC (14)	14 (100)	12 (85.7)
Total (174)	134 (77)	72 (41.4)

Table 5 Distribution of *cag*E, T, LEC1, and LEC2 in *H pylori* isolates

Clinical status (*n*)	*cag*E (%)	*cag*T (%)	LEC1 (%)	LEC2 (%)
DU (58)	47 (81)	56 (96.5)	44 (75.8)	33 (56.8)
GU (46)	46 (100)	46 (100)	46 (100)	46 (100)
NUD (56)	36 (64.2)	36 (64.2)	38 (67.8)	18 (32.1)
GC (14)	14 (100)	14 (100)	14 (100)	14 (100)
Total (174)	143 (82.1)	152 (87.3)	142 (81.6)	111 (63.7)

For *cag*E, GU *vs* NUD is highly significant at 0.1% level ($P<0.001$), DU *vs* GU, NUD *vs* GC combinations are significant at 1% level ($P<0.01$), DU *vs* NUD combination is significant at 5% level ($P<0.05$) and DU *vs* GC . For *cag*T, DU *vs* NUD, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$), NUD *vs* GC combination is significant at 1% level ($P<0.01$) and DU *vs* GU, DU *vs* GC, GU *vs* GC are not significant at 1% level. For LEC1, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$), NUD *vs* GC is significant at 1% level ($P<0.01$), DU *vs* GC is significant at 5% level ($P<0.05$). For LEC2, DU *vs* GU, GU *vs* NUD, NUD *vs* GC combinations are highly significant at 0.1% level ($P<0.001$), DU *vs* NUD, DU *vs* GC combinations are significant at 1% level ($P<0.01$) and GU *vs* GC is not significant at 1% level.

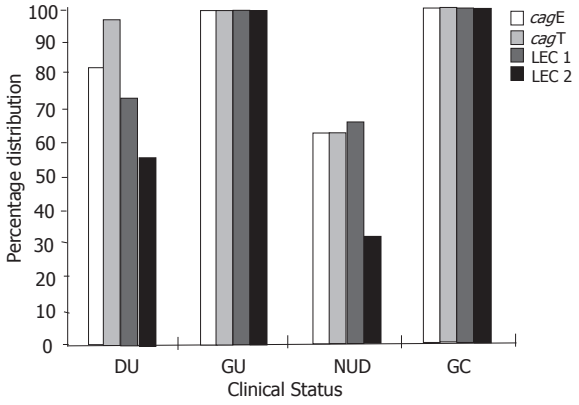

Figure 3 Distribution of *cag*E, T, LEC1, LEC2

to check the grade of gastritis and the differences between the pathologies of intact *cag*-PAI and partial *cag*-PAI infection. Of the 174 subjects included for the study, we screened 160 subjects as 14 of them had endoscopically proven gastric carcinoma and hence histopathology confirmed features of carcinoma. Out of the 160 subjects screened, all showed chronic gastritis and on further screening for any other advanced type of gastritis, we found 112 (70%) of the total 160 to possess topographic chronic superficial gastritis, 20 (12.5%) showed atrophic changes, whereas 18 (11.25%) and 10 (6.25%) of the subjects showed IM and dysplasia respectively (Table 6). When each of these histological lesions were scored individually in relation to their respective clinical status, we found that among the 58 duodenal ulcer cases, 52 (89.7%) showed chronic superficial gastritis, 2 (3.4%) showed atrophy and 4 (6.9%) showed intestinal metaplasia while none of the subjects had dysplasia (Table 6).

In the gastric ulcer category, we found that 9 (19.6%) showed chronic superficial gastritis, 13 (28.3%) showed atrophy, 14 (30.4%) showed intestinal metaplasia and 10 (21.7%) showed dysplasia. For atrophy, metaplasia and dysplasia, the difference between GU and DU was statistically highly significant ($P<0.001$, Table 6).

In the NUD category, we found that 51 (91.1%) showed chronic superficial gastritis and 5 (8.9%) showed atrophic gastritis while none had intestinal metaplasia and dysplasia. For metaplasia and dysplasia, the difference between GU and NUD, was statistically highly significant ($P<0.001$, Table 6).

Table 6 Frequency of atrophy, metaplasia, and dysplasia in chronic gastritis subjects

Clinical status (*n*)	Acute gastritis (%)	Chronic gastritis			
		Superficial gastritis	Gastritis with atrophy (%)	Gastritis with metaplasia (%)	Gastritis with dysplasia (%)
DU (58)	0	52 (89.7)	2 (3.4)	4 (6.9)	0
GU (46)	0	9 (19.6)	13 (28.3)	14 (30.4)	10 (21.7)
NUD (56)	0	51 (91.1)	5 (8.9)	0	0
Total (160)	0	112 (70)	20 (12.5)	18 (11.25)	10 (6.25)

For atrophy, DU *vs* GU combination is highly significant at 0.1% level ($P<0.001$), GU *vs* NUD is significant at 1% level ($P<0.01$) and DU *vs* NUD combination is not significant at 1% level. For metaplasia, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$) and DU *vs* NUD combination is significant at 5% level ($P<0.05$). For dysplasia, DU *vs* GU, GU *vs* NUD combinations are highly significant at 0.1% level ($P<0.001$) and DU *vs* NUD combination is not significant at any level.

When we analyzed the histological status, with reference to the strain infecting with either intact PAI or partially deleted ones, we found a significant difference among them. As evidenced from Table 7, we found that, of the 65 subjects infected with intact PAI, 8 (12.3%) showed chronic superficial gastritis, 17 (26.1%) showed atrophic changes, 18 (27.7%) showed intestinal metaplasia (all of them were Type III), 10 (15.4%) showed high grade dysplastic changes and 12 (18.5%) showed intestinal type gastric carcinoma. On the contrary, when we checked those subjects, who inhabited partially deleted strains, we found 104 (95.4%) to possess chronic superficial gastritis, 3 (2.8%) showed atrophy while 2 (1.8%) showed gastric carcinoma and these differences were statistically very significant ($P<0.001$, Table 7).

When a correlation between the histological status of the subjects infected with either intact *cag*-PAI or partially deleted strains isolated from subjects with varied disease status was made, we found that among 58 DU subjects, 4 had intact *cag*-PAI and all of them were Type III intestinal metaplasia, among the remaining 54, which had partial deletions in the *cag*-PAI, 52 (96.3%) showed chronic superficial gastritis and 2 (3.7%) showed atrophic changes. Among the GU subjects, of the 46 infected, 45 were known to possess the *cag*-PAI and 8 (17.8%) of them showed chronic superficial gastritis, 13 (28.9%) showed atrophic gastritis, 14 (31.1%) showed Type III intestinal metaplasia and 10 (22.2%) showed high grade dysplastic changes (Table 8). Among the 56 NUD subjects, 4 with intact *cag*-PAI showed atrophic changes and of the rest with partial deletions 51 (98.1%) showed chronic superficial gastritis and 1 (1.9%) showed atrophic gastritis (Table 8) while among the 14 isolated from

Table 7 Histological status of subjects with intact *cag*-PAI (*n* = 65) and partial *cag*-PAI (*n* = 109)

Cag-type	Chronic gastritis							Gastric carcinoma	
(*n*)	Superficial gastritis	Gastritis with atrophy	Gastritis with metaplasia (%)			Gastritis with dysplasia(%)		Intestinal (%)	Diffuse (%)
	(%)	(%)	Ty-1	Ty-2	Ty-3	Low grade	High grade		
Intact (65)	8 (12.3)	17 (26.1)[b]	0	0	18 (27.7)	0	10 (15.4)	12[b] (18.5)	0
Partial (109)	104 (95.4)	3 (2.8)	0	0	0	0	0	2 (1.8)	0

[b]*P*<0. 001 *vs* others.

Table 8 Correlation of the histological status of the subjects with intact and partially deleted *cag*-PAI from varied disease status

Clinical status	Chronic gastritis								Gastric carcinoma	
(*n*)	cag status	Superficial gastritis	Gastritis with atrophy (%)	Gastritis with metaplasia (%)			Gastritis with dysplasia (%)		Intestinal (%)	Diffuse (%)
	(*n*)	(%)		Ty-1	Ty-2	Ty-3	Low grade	High grade		
DU (58)	Intact (4)	0	0	0	0	4 (100)	0	0	0	0
	Partial (54)	52 (96.3)	2 (3.7)	0	0	0	0	0	0	0
GU (46)	Intact (45)	8(17.8)[a]	13 (28.9)	0	0	14 (31.1)	0	10 (22.2)	0	0
	Partial (1)	1 (100)	0	0	0	0	0	0	0	0
NUD (56)	Intact (4)	0	4(100)[a]	0	0	0	0	0	0	0
	Partial (52)	51 (98.1)	1 (1.9)	0	0	0	0	0	0	0
GC (14)	Intact (12)	0	0	0	0	0	0	0	12 (100)[b]	0
	Partial (2)	0	0	0	0	0	0	0	2 (100)	0

[a]*P* <0.05, [b]*P* <0.001 *vs* others.

gastric carcinoma 12 had intact *cag*-PAI while the other 2 partial deletions and when scored for the type of gastric carcinoma all of them showed intestinal type of carcinoma (Table 8).

DISCUSSION

The *cag*-PAI is an approximately 40-kb cluster of genes in *H pylori* chromosome, and a quite conservative entity. Censini *et al*[22] in 1996 first identified strains with partially deleted *cag*-PAIs. The molecular mechanism of these genetic rearrangements was explained by incorporation of an insertion element, IS605, in *cag*-PAI. Recently, the composition of the *cag*-PAI in clinical *H pylori* isolates has been studied in different populations by various methods, including PCR, dot blotting and long distance PCR[20,23,24].

In the present study, we used simple PCR for structural screening of *cag*-PAI in clinical isolates of *H pylori*. Out of the 174 clinical isolates, we found only 37.4% were carrying the complete *cag*-PAI (Table 2), whereas Mukhopadhyay *et al*[25] reported more than 96% in Calcutta strains of peptic ulcer and non-ulcer dyspepsia. In our study, 97.8% of gastric ulcer and 85.7% (Table 2 and Figure 1) of the gastric carcinoma strains were carrying complete PAI, which are considered to be severe forms of the gastro-duodenal diseases. Even though duodenal ulcer is also considered to be a severe form of the gastro-duodenal disease, the proportion of DU strains that carried was just 6.9%. Jenks *et al*[23] reported that the presence of certain genes (*cag*A, *cag*E, *cag*M, T, ORF 6, 10, 13) in the *cag*-PAI is highly associated with duodenal ulcers. We too found a similar type of observation, but not for all the seven genes which they selected. We observed the same kind of correlation with only two genes i.e. *cag*E and *cag*T, where DU cases carried *cag*E with 81% and *cag*T with 96.5% (Table 5 and Figure 3). Whereas NUD isolates were carrying the genes with an average of 65% (Table 5 and Figure 3). Further, Day *et al*[26] revealed that isolates containing *cag*E were associated with duodenal ulceration.

Among non-ulcer dyspepsia strains, we found 7.1% to carry the complete *cag*-PAI, which is statistically almost equal to DU percentage, and a report from Sweden[27] showed 58% of *cag*-PAI positivity in NUD isolates. In the same report, the authors showed that 5% of isolates from severe pathology i.e. gastric carcinoma and duodenal ulcer, and 15% of the isolates from NUD lacking the *cag*-PAI. Not only the data from other continents, but from the same Indian sub-continent showed total deletions from ulcer group and non-ulcer group[25], whereas we could not come across a single isolate with entirely deleted *cag*-PAI from any of the disease condition indicating strain diversity. This kind of diversity, i.e. absence of completely deleted PAIs and presence of just 6.9% complete *cag*-PAIs in duodenal ulcer cases, might be particularly true for the south Indian of Telugu linguistic group who are mainly Dravidian and married consanguineously for millennia[28]. Their genetic separation from other Indian communities during much of the human history has already been reported[29].

Strains with intermediate genotypes, lacking parts of the *cag*-PAI, were found in 62.6% (Table 2) and more frequently found in patients with non-ulcer dyspepsia and duodenal ulcer (Figure 1). A probable mechanism for the establishment of these internal deletions within the *cag*-PAI would be that the short repeated sequences found by Nilsson *et al*[27] may serve as homologies enabling slipped strand mispairing and consequently excision of the enclosed DNA fragment especially in DU and NUD subjects. Moreover, in our observation, the partially deleted *cag*-PAI represented a genotype more common than a complete *cag*-PAI and no strain was found with completely

deleted *cag*-PAI.

Conventionally *cag*A was used and is still used as a marker for the presence of an intact *cag*-PAI and for virulence. Recently, Backert *et al*[30] showed a good correlation of *cag*A with the presence of *cag*-PAI. In this study, we found that 39.7% of the strains carried the complete *cag*A gene (Table 3) and the presence of *cag*A gene did not correlate with the genetic presence of complete *cag*-PAI. Further, for - 4 kb *cag*A gene, we used four sets of primers i.e. two primers for body of the gene and two primers for promoter, designed from various locations of the body region and promoter, whereas other studies taken up earlier had used a single set of primer for complete *cag*-PAI. This might be the reason for the correlation, which they obtained. The typical observation was when 77% of the isolates were carrying the body region (A1+A2) only 41.4% of them carried promoter region (Table 4), which means that in our isolates even though the strains carried the *cag*A gene, most of them lacked the promoter of the gene, without which *cag*A is not functional. Further, four isolates i.e. 2.3% completely lacked the *cag*A gene and all the four isolates belonged to duodenal ulcer cases.

Among many virulence markers present in the *H pylori* genome, *cag*-PAI is the major virulence factor and is associated with severe gastroduodenal pathology[31,32] that includes both duodenal and gastric ulcers along with carcinomas. Some studies have identified a correlation between an intact *cag*-PAI and development of disease[20,23,24,27], as we are trying to show with this present study in Indian scenario, whereas others could not find such a relationship[33,34].

"Infection with *H pylori* always causes chronic active gastritis"[35]. This phrase has become true in our observation. As it is observed in Table 6, there are no acute gastritis subjects. Moreover, the subjects infected with *cag*-PAI positive strains were found to show severe forms of histopathological changes, like atrophic gastritis, intestinal metaplasia, and neoplasia. This might be the reason for IARC-WHO to designate *H pylori* a class I (definite) carcinogen[6].

Out of the 174 isolates, 65 (37.4%) had complete *cag*-PAI and 109 (62.6%) had partial deletions in the *cag*-PAI (Table 2). As evident from Table 7, it can be observed that subjects infected with *H pylori* strains with intact *cag*-PAI had many remarkable histopathological changes, when compared to those who had partially deleted *cag*-PAI. It is quite clear from the statistics ($P<0.001$) that among 65 *H pylori* strains with intact *cag*-PAI, 18.5% subjects had advanced cancerous lesions, while only 1.8% of the 109 subjects, who harbored *H pylori* with partial *cag*-PAI had advanced to carcinoma thus allowing us to delineate that persons with intact *cag*-PAI are 10-fold more prone to develop carcinoma in comparison to the partially deleted *cag*-PAI strains.

Parallelly, a small group of population, i.e. 12.3% with intact *cag*-PAI, were shown to have only chronic superficial gastritis, but according to Ohkuma *et al*[36] *H pylori* positive cases with chronic gastritis have increased risk of atrophy and intestinal metaplasia. On the other hand, partial *cag*-PAI subjects were also shown to have chronic gastritis (Table 6), but the percentage of disease progression was very high among *cag*-PAI positive subjects (Table 7) than those with partially deleted ones. Moreover, Type-3 metaplasia and high-grade dysplasia were seen only in *cag*-PAI positive subjects, where Type-3 metaplasia is closely linked to carcinoma[37] and dysplasia is nothing but a non-invasive type of neoplasia[38]. The results of this study are in contrast to those obtained by Keates *et al*[39] who determined that gene products of the *cag* pathogenicity island are required for maximal activation of mitogen-activated protein kinases (MAPK) in gastric epithelial cells, which regulate cell proliferation, differentiation, inflammatory responses, stress, and programmed death, leading to induce gastroduodenal inflammation, ulceration and neoplasia. Further, Naumann *et al*[40] stated that the integrity of whole *cag*-PAI is also a pre-requisite for efficient activation of early transcription factor AP-1, which is known for its immuno-stimulatory function.

In relation with disease status, among DU, GU, and NUD, gastric ulcers are considered to be more prone to the gastric carcinoma[41]. The observations of this study are in correlation to that obtained by Hansson *et al*[41]. When we compare GU subjects with DU and NUD, the percentage of predisposing factors was much more among GU subjects. Recently, Wanatabe *et al*[42] proved this in animal models. In their study at 26 wk, Mongolian gerbils developed chronic gastritis, ulceration and metaplasia. At 62 wk, 31% of them developed adenocarcinoma. Interestingly the inoculum used for the infection was obtained from a patient with gastric ulcer. Moreover, there have been reports that gastric cancer mortality rates bear an inverse relationship to duodenal ulcer disease rates[12], suggesting that they are directly relating with gastric ulcer in ulcer groups.

Further, in partial *cag*-PAI subjects, 2.8% showed atrophy, 1.8% showed carcinoma (Table 7) suggesting the role of other virulence genes and risk factors. Parallelly high incidence rate of gastric carcinoma among the gastric ulcer cases might be true, but it should not be assigned to a single determinant such as *cag*-PAI, but it is a result of many factors such as host genetic factors, environment, low socio-economic status, irregular dietary habits in addition to *H. pylori* with complete *cag*-PAI[43].

Hence, we can suggest that the *cag*-PAI is a strong virulent marker in the disease pathogenesis because more than 85% of the *cag*-PAI positive subjects were shown to have one or the other of the irreversible gastric pathologies and interestingly 18.5% of them developed gastric carcinoma and GU is the major risk/predisposing factor for the gastric carcinoma. Moreover, duodenal ulcer is not at all a risk factor for severe gastric pathologies and it is not a severe kind of disease, like gastric ulcer in our population.

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Science Editor Pravda J and Guo SY **Language Editor** Elsevier HK