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RESEARCH REPORT

Carcinogenic *Helicobacter pylori* in gastric pre-cancer and cancer lesions: Association with tobacco-chewing

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Abstract

AIM: To investigate the low gastric cancer incidence rate relative to the highly prevalent *Helicobacter pylori* (*H. pylori*) infection; data relevant to *H. pylori* infection during gastric carcinogenesis in Indian patients is currently lacking.

METHODS: The present study examines the prevalence of *H. pylori* infection in DNA derived from 156 endoscopic gastric biopsies of different disease groups that represent gastric pre-cancer [intestinal metaplasia (n = 15), dysplasia (n = 15)], cancer [diffuse adenocarcinoma (n = 44), intestinal adenocarcinoma (n = 21)], and symptomatic but histopathologically-normal controls (n = 61). This was done by generic *ureC* polymerase chain reaction (PCR) and *cagA*-specific PCR that could specifically identify the carcinogenic *H. pylori* strain.

RESULTS: Our analysis showed the presence of *H. pylori* infection in 61% of symptomatic histopathologically-normal individuals, however only 34% of control tissues were harboring the *cagA*$^+$ *H. pylori* strain. A similar proportion of *H. pylori* infection (52%) and *cagA* (26%) positivity was observed in the tumor tissue of the gastric cancer group. In comparison, *H. pylori* infection (90%) and *cagA* positivity (73%) were the highest in gastric pre-cancer lesions. In relation to tobacco and alcohol abuse, *H. pylori* infection showed an association with tobacco chewing, whereas we did not observe any association between tobacco smoking or alcohol abuse with prevalence of *H. pylori* infection in the tissue of any of the patient groups studied.

CONCLUSION: High incidence of *H. pylori* infection and carcinogenic *cagA* positive strain in pre-cancer lesions during gastric carcinogenesis may be associated

with the habit of chewing tobacco.

Key words: *UreC*; *CagA*; Dysplasia; Intestinal metaplasia; Gastric adenocarcinoma; Tobacco chewing

Core tip: The manuscript deals with the role of *Helicobacter pylori* (*H. pylori*) infection and its carcinogenic *cagA* positivity during gastric cancer progression from normal to pre-cancer and pre-cancer to adenocarcinoma, as well as its association with tobacco and alcohol use in these patients. Results showed that *H. pylori* infection in general, and its carcinogenic *cagA* positive strains in particular, were associated with tobacco chewing.

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INTRODUCTION

With nearly one million new cases estimated in 2008, gastric cancer accounts for 7.8% of the total cancer burden and is the second leading cause of cancer deaths reported worldwide (738000/year)[1]. The distribution of gastric cancer is particularly high in Asia, Latin America, and some areas of Europe and Africa. Despite some of the high risk populations being in Asian countries such as Japan, Korea, and China, other Asian countries like India present relatively low rates of gastric cancer[2].

Like other malignancies, data addressing the process of multistage carcinogenesis for gastric cancer suggest that pathogenesis of gastric cancer is associated with a variety of environmental factors, like chronic gastritis[3] and consumption of tobacco and alcohol[4,5]. Excluding these, *Helicobacter pylori* (*H. pylori*) infection is considered the most prominent risk factor for onset of gastric cancer. Based on experimental evidence by Marshall *et al*[6] and subsequent clinico-epidemiological studies[7,8], in 1994 the International Agency for Research on Cancer classified *H. pylori* as a group I carcinogen[9]. Despite the fact that *H. pylori* colonization is a risk factor for gastric adenocarcinoma, various aspects such as association of *H. pylori* with different grades of gastric cancer lesions, necessity of its persistent infection in the carcinogenic progression of malignant disease, and its correlation with tobacco and alcohol habits of infected individuals, particularly understanding of their pathological effects, are poorly understood. Regardless of the prevailing and varied tobacco and alcohol habits[10,11] and reportedly high prevalence of pathogenic *H. pylori* strains accompanied with low socioeconomic conditions, the incidence of gastric cancer is low in India, which could be partly due to underreporting of this disease. In India, gastric cancer is ranked the 5th and 7th most common cancer in males and females, respectively[1,12]. Moreover, these cancers are detected very late due to misdiagnosis caused by the absence of necessary health infrastructure and endoscopic clinics.

Gastric cancer associated with *H. pylori* infection evolves as a consequence of histological changes in the mucosa due to chronic inflammation, which culminates initially into intestinal metaplasia (IM), dysplasia (Dys), and, at later stages, intestinal (IC) or diffuse-type adenocarcinoma (DC)[13]. Not all strains of *H. pylori* are equally pathogenic. The pathogenicity and virulence of *H. pylori* is primarily determined by a set of genes (denoted as pathogenicity island or PIA), among which the presence of the cytotoxin associated gene *cagA* codes for protein that confers the pathogenic character to *H. pylori* strains[14]. *CagA*$^+$ *H. pylori* strains have strong association with gastric cancer[15]. The CagA protein of *H. pylori* has been shown to translocate into the cytoplasm of gastric cells, where it mediates a number of cellular events including: rearrangement of the cytoskeleton, induction of inflammatory mediators through the specific induction of proliferative and oncogenic proteins (*via* induction of oncogenic transcription factors like nuclear factor κB), activating protein-1, phosphatidylinositol 3 kinase, signal transducer and activator of transcription (STAT)-3[16,17] that promote tumorigenic transformation. Despite disparity in the high rates of *H. pylori* infection, a low incidence of gastric carcinogenesis in India[2,18], and reports of *H. pylori* prevalence in different types of gastric ailments (primarily non-malignant conditions like gastritis, duodenal ulcers and gastric ulcer)[19-22], in India there is a strong need for a comprehensive analysis of the prevalence of *H. pylori* infection and carcinogenic *cagA* positivity in gastric pre-cancer and cancer lesions, along with its correlation with habits of tobacco and alcohol use that are considered potential risk factors.

In the view of the above, the present study aided to estimate the prevalence of carcinogenic *cagA*$^+$ *H. pylori* infection in different grades of pre-cancer and cancer lesions, as well as to assess its association with clinico-epidemiological variables, particularly the habits of tobacco and alcohol abuse.

MATERIALS AND METHODS

Clinical specimens and reagents

A total of 156 gastric tissues comprising 61 histologically normal, 30 pre-cancers [IM (n = 15) and Dys (n = 15)], and 65 adenocarcinomas [DC (n = 44) and IC (n = 21)] prospective, but selectively-collected, endoscopic biopsies were obtained prior to any chemo/radiotherapy from the patients attending the Gastroenterology Out-Patient Department of the Swaroop Rani Hospital (affiliated with the Motilal Nehru Medical College, Allahabad,

Table 1 Clinico-epidemiological details of subjects enrolled in study *n* (%)

Clinico-epidemiological characteristics	Normal	Pre-cancer	Cancer (adenocarcinoma)
Total subjects	61	30	65
Gender			
Male	42 (68.9)	17 (56.7)	42 (64.6)
Female	19 (31.1)	13 (43.3)	23 (35.4)
Histopathological classification of lesions			
Intestinal metaplasia	-	15 (50)	-
Dysplasia	-	15 (50)	-
Diffuse adenocarcinoma	-	-	44 (67.7)
Intestinal adenocarcinoma	-	-	21 (32.3)
Age (yr) (median)	7-85 (40)	13-78 (41.5)	22-90 (55)
Habits[1]			
Tobacco chewers	28 (45.9)	15 (50)	24 (37)
Tobacco non-chewers	33 (54.1)	15 (50)	41 (63)
Tobacco smokers	22 (36.1)	11 (36.7)	20 (30.8)
Tobacco non-smokers	39 (64)	19 (63.3)	45 (69.2)
Alcoholic	15 (24.6)	8 (26.7)	16 (24.6)
Non-alcoholic	46 (75.4)	22 (73.3)	49 (75.4)
Multiple habits	22 (36.1)	9 (30)	16 (24.6)

[1]Tobacco chewing habits include betel quid, areca nut, and/or pan masala use; multiple habits include two or more of any tobacco and/or alcohol habits.

India) during the period of 2007-2012. IM and Dys were grouped as gastric pre-cancer lesions, while DC and IC were grouped as cancer lesions. Symptomatic, clinically-suspected cases that were found to be histologically normal (non-malignant) were used as controls. Patients taking antibiotics, with a bleeding ulcer, suffering acute hemorrhage from other sites in the upper gastrointestinal track, or having undergone stomach surgery were excluded. Written informed consent was obtained from all subjects. The study was carried out in accordance with the principles of the Helsinki Declaration. The Institutional Ethics Committees of the Motilal Nehru Medical College, Allahabad and the Institute of Cytology and Preventive Oncology, Noida, India approved this study prior to its commencement. Clinico-epidemiological and demographic details were taken from clinical record of patients and captured on a pre-designed study *pro forma*. The study questionnaire/case report form included details of tobacco use (chewing or smoking) and alcohol use habits. Any person having these habits at the time of examination or using tobacco or alcohol daily (at least one cigarette or beedi/tobacco chew/alcohol shot) and regularly (at least 3 d/wk) for 6 mo or more in the past were grouped as tobacco users or alcoholics. A person having more than one habit was grouped under multiple habits. A summary of clinico-epidemiological characteristics included in the different disease groups is presented in Table 1.

Thorough endoscopic examination was done after obtaining patient consent. Two biopsies from the lesion were taken. One piece was immediately fixed in formalin for routine histopathological examination, whereas the other piece was collected in chilled 1xPBS for DNA isolation. Formalin-fixed tissues were processed routinely, and 3 micron thick sections from paraffin blocks were stained with hematoxylin and eosin. The histopathological grading of cancer lesions was done as per Laurén's classification[23,24]. All reagents used in the study were of analytical or molecular biology grade and procured from Sigma Aldrich (St Louis, MO, United States) unless otherwise specified.

Detection of *H. pylori* infection in gastric samples

DNA from gastric endoscopic biopsies was extracted by commercially available DNA isolation kits for tissues (Roche Diagnostics GmbH, Germany) as per the manufacturer's protocol. *H. pylori* diagnosis was performed by conventional *ureC* and *cagA* gene polymerase chain reaction (PCR) using a pair of pre-published primers derived from the highly conserved ureC region[25] and *cagA* intragenic region[26], while β-globin amplification was used as an internal control as previously described[27]. PCR assays based on *ureC* and *cagA* pre-standardized primer could detect at least 3.6 fg of bacterial DNA, which corresponds to approximately two *H. pylori* genomes[28]. PCR was performed in a 25 μL reaction mixture containing approximately 100 ng DNA, 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 125 μmol/L of each dNTPs [dATP, dGTP, dCTP, and dTTP (Applied Biosystems, CA, United States)], 5 pmol of oligonucleotide primers, and 0.5 U Taq DNA polymerase (Merck, Bangalore Genei, India). Details of PCR primers with their respective target region, primer sequence, amplicon size, and amplification program are provided in Table 2. DNA from known *ureC* and *cagA* gene positive *H. pylori* strains and water blank were assayed invariably in each PCR run as positive and negative controls, respectively. After amplification, PCR products were electrophoresed on 2% agarose gel along with 100 bp DNA ladder as a molecular weight marker, visualized under an UV transilluminator, and documented in AlphaDigidoc (Alpha Innotech Corp. CA, United States).

Table 2 Primer sequences, primer annealing positions, expected lengths of amplified DNA products, and amplification cycle of polymerase chain reaction performed in the study

Primer	Sequence	Annealing position	Product length (bp)	Amplification cycle
Hp ureC-F	5′-AAG CTT TTA GGG GTG TTA GGG GTT T-3′	1293-1318	294	94 ℃, 10′, 36 cycle (94 ℃, 2′; 55 ℃, 2′; 72 ℃, 2′), 72 ℃, 10′
Hp ureC-R	5′-AAG CTT ACT TTC TAA CAC TAA CGC-3′	1587-1563		
Hp cagA-F	5′-AAT ACA CCA ACG CCT CCA AG-3′	2593-2612	400	94 ℃, 4′, 39 cycle (94 ℃, 2′; 59 ℃, 2′; 72 ℃, 2′), 72 ℃, 10′
Hp cagA-R	5′-TTG TTG CCG CTT TTG CTC TC-3′	2992-2973		
β-globin-F	5′-GAA GAG CCA AGG ACA GGT AC-3′	195-176	268	94 ℃, 4′, 30 cycle (95 ℃, 30″; 55 ℃, 30″; 72 ℃, 1′), 72 ℃, 10′
β-globin-R	5′-CAA CTT CAT CCA CGT TCA ACC-3′	54-73		

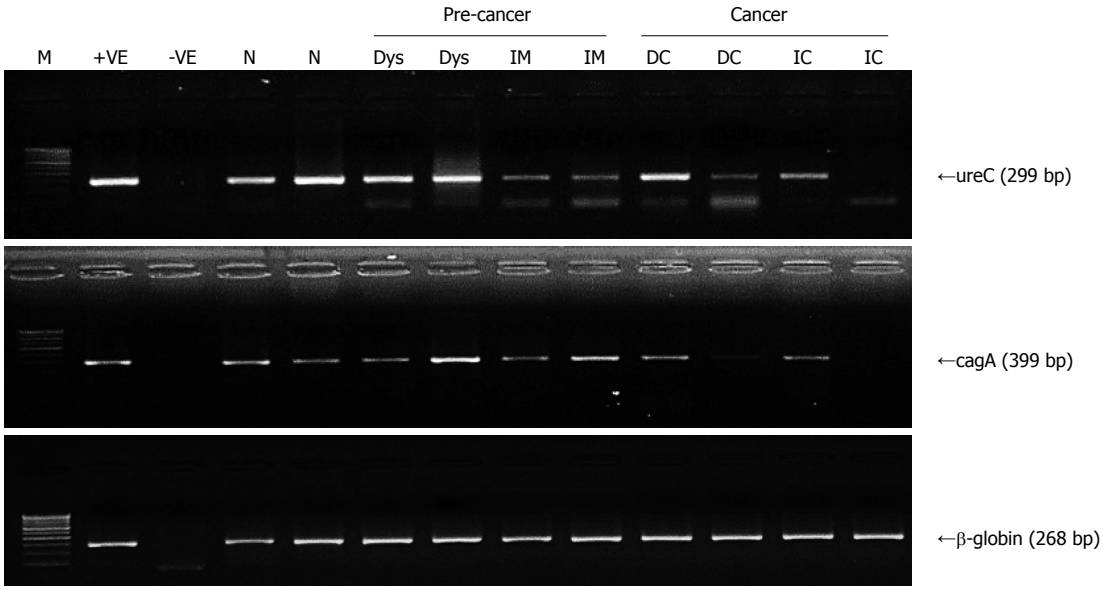

Figure 1 Detection of *Helicobacter pylori* infection by *ureC* and *cagA*-specific polymerase chain reactions in human gastric biopsies. Representative agarose gel photographs showing specific polymerase chain reaction amplification of *Helicobacter pylori ureC* and *cagA* gene segments in DNA isolated from different histopathological grades of gastric biopsies. N: Normal; Dys: Dysplasia; IM: Intestinal metaplasia; DC: Diffuse adenocarcinoma; IC: Intestinal adenocarcinoma; M: Marker 100 bp ladder.

Statistical analysis

The relationships between PCR analysis and clinico-epidemiological parameters were tested using χ^2 and Fischer's Exact Test. Two-sided *P* values were calculated and a *P* value of 0.05 or less was considered to be significant, while a *P* value of 0.001 or less was considered highly significant. Multivariate linear regression analysis was performed with age and gender stratification, and age gender bias was adjusted while doing the statistics.

RESULTS

A total of 156 histologically-confirmed gastric tissues from 65 adenocarcinoma (median age: 55 years; range: 22-90 years), 30 pre-cancer (median age: 41.5 years; range: 13-78 years), and 61 symptomatic control patients (median age: 40 years; range: 7-85 years) were examined for *H. pylori* infection by *ureC* and *cagA* PCRs, which revealed the presence of generic and pathogenic *H. pylori* strains, respectively (Figure 1). Tissues were considered *H. pylori* positive if the DNA was positive for either of the two PCRs. Analysis of *H. pylori* positivity in the gastric tissue of the hospital visiting symptomatic control group revealed a *H. pylori* positivity of 61%, whereas only 34% of the control gastric tissues were *cagA*$^+$ (Table 3). On the other hand, the positivity of *H. pylori* infection was the highest in pre-cancer tissues (93%) compared to the control or cancer groups (Table 4). Moreover, the pre-cancer tissues, irrespective of histopathological grade, demonstrated a high prevalence of the *cagA*$^+$ *H. pylori* strain in this disease group (73%). Contrary to pre-cancer tissues, gastric tissues from cancer lesions (DC or IC) had a lower frequency of *H. pylori* infection (52%) as well as a *cagA* positivity (26%) that more closely resembled the symptomatic control group (Table 5).

In general, study samples had an overrepresenta-

Table 3 Analysis of *Helicobacter pylori* gene expression in normal gastric tissues with clinico-epidemiological parameters *n* (%)

Clinico-epidemiological characteristics	Total cases *n*	*ureC* positive	*P*	*cagA* positive	*P*	Hp +ve	*P*
Normal	61	33 (54.1)	-	21 (34.4)	-	37 (60.7)	-
Age (median 40 yr; range: 7-85 yr)							
< 40	29	13 (44.8)	0.167	8 (27.6)	0.284	15 (51.7)	0.174
⩾ 40	32	20 (62.5)		13 (40.6)		22 (68.8)	
Gender							
Male	42	18 (42.9)	0.009	14 (33.3)	0.789	22 (52.4)	0.049
Female	19	15 (78.9)		7 (36.8)		15 (78.9)	
Habits[1]							
Tobacco chewers	28	19 (67.9)	0.047	14 (50)	0.018	20 (71.4)	0.113
Tobacco non-chewers	33	14 (42.4)		7 (21.2)		17 (51.5)	
Tobacco smokers	22	13 (59.1)	0.557	11 (50)	0.055	15 (68.2)	0.366
Tobacco non-smokers	39	20 (51.3)		10 (25.6)		22 (56.4)	
Alcoholic	15	9 (60)	0.597	6 (40)	0.601	10 (66.7)	0.583
Non-alcoholic	46	24 (52.2)		15 (32.6)		27 (58.7)	
Multiple habits	22	14 (63.6)	0.262	10 (45.5)	0.173	15 (68.2)	0.366

[1]Tobacco chewing habits include betel quid, areca nut, and/or pan masala use; multiple habits include two or more of any tobacco and/or alcohol habit.

Table 4 Analysis of *Helicobacter pylori* gene expression in pre-cancer gastric tissues with clinico-epidemiological parameters *n* (%)

Clinico-epidemiological characteristics	Total cases *n*	*ureC* positive	*P*	*cagA* positive	*P*	Hp +ve	*P*
Pre-cancer	30	27 (90)	0.001[1]	22 (73.3)	< 0.001[1]	28 (93.3)	< 0.001[1]
Age (median: 41.5 yr; range: 13-78 yr)							
< 41.5	15	14 (93.3)	0.543	11 (73.3)	1.00	14 (93.3)	1.00
⩾ 41.5	15	13 (86.7)		11 (73.3)		14 (93.3)	
Gender							
Male	17	15 (88.2)	0.713	14 (82.4)	0.201	16 (94.1)	0.844
Female	13	12 (92.3)		8 (61.5)		12 (92.3)	
Types							
Dysplasia	12	12 (100)	0.136	9 (75)	0.866	12 (100)	0.503
Intestinal metaplasia	18	15 (83.3)		13 (72.2)		16 (88.9)	
Habits[2]							
Tobacco chewers	15	13 (86.7)	0.543	12 (80)	0.409	14 (93.3)	1.000
Tobacco non-chewers	15	14 (93.3)		10 (66.7)		14 (93.3)	
Tobacco smokers	11	11 (100)	0.165	10 (90.9)	0.098	11 (100)	0.265
Tobacco non-smokers	19	16 (84.2)		12 (63.2)		17 (89.5)	
Alcoholic	8	8 (100)	0.271	7 (87.5)	0.290	8 (100)	0.377
Non-alcoholic	22	19 (86.4)		15 (68.2)		20 (90.9)	
Multiple habits	9	9 (100)	0.232	8 (88.9)	0.207	9 (100)	0.338

[1]Normal *vs* pre-cancer; [2]Tobacco chewing habits include betel quid, areca nut, and/or pan masala use; multiple habits include two or more of any tobacco and/or alcohol habits.

tion of males in each disease group. Excluding gastric tissues from females in the symptomatic control group (who expressed significantly higher *H. pylori* positivity), there was no gender bias in *H. pylori* infection in pre-cancer or cancer tissue types. To understand the effect of confounding factors on the final outcome, age and gender stratification was performed and adjusted while doing the statistics. We also performed multivariate linear regression analysis, which again showed that these two factors did not play any significant role in the final outcome. Moreover, gastric tissue from these female patients showed no difference in the frequency of the pathogenic strain when compared to cagA positivity.

H. pylori infection in general and *cagA* positivity in particular was strongly associated with the habit of chewing tobacco irrespective of the disease group, whereas no association between *H. pylori* infection and tobacco smoking, alcohol consumption, or a combination of the two could be ascertained (Tables 3-5).

DISCUSSION

The present study was conducted to perform a comparative analysis of *H. pylori* infection, and to examine the potential contribution of carcinogenic *cagA*$^+$ strains in gastric cancer progression and their association with tobacco use and alcohol abuse that may play a permissive role in carcinogenic transformation. Our results demonstrate that the frequency of *H. pylori* infection was highest in precancerous gastric lesions and was associated with the habit of chewing tobacco. Pre-cancer lesions, irrespective of histopathological grade, had a relatively

Table 5 Analysis of *Helicobacter pylori* gene expression in adenocarcinoma gastric tissues with clinico-epidemiological parameters *n* (%)

Clinico-epidemiological Characteristics	Total cases *n*	*ureC* positive	*P*	*cagA* positive	*P*	Hp +ve	*P*
Adenocarcinoma	65	33 (50.8)	< 0.001[1] 0.708[2]	17 (26.2)	< 0.001[1] 0.312[2]	34 (52.3)	< 0.001[1] 0.345[2]
Age (median 55 yr; range: 22-90 yr)							
< 55	31	14 (45.2)	0.388	10 (32.3)	0.285	15 (48.4)	0.546
⩾ 55	34	19 (55.9)		7 (20.6)		19 (55.9)	
Gender							
Male	42	22 (52.4)	0.725	10 (23.8)	0.561	23 (54.8)	0.592
Female	23	11 (47.8)		7 (30.4)		11 (47.8)	
Types							
Diffuse	44	24 (54.5)	0.378	13 (29.5)	0.368	25 (56.8)	0.292
Intestinal	21	9 (42.9)		4 (19)		9 (42.9)	
Habits[3]							
Tobacco chewers	24	19 (79.2)	< 0.001	12 (50)	0.001	19 (79.2)	0.001
Tobacco non-chewers	41	14 (34.1)		5 (12.2)		15 (36.6)	
Tobacco smokers	20	10 (50)	0.934	8 (40)	0.090	11 (55)	0.772
Tobacco non-smokers	45	23 (51.1)		9 (20)		23 (51.1)	
Alcoholic	16	9 (56.2)	0.614	4 (25)	0.904	9 (56.3)	0.716
Non-alcoholic	49	24 (49)		13 (26.5)		25 (51)	
Multiple habits	16	9 (56.2)	0.614	6 (37.5)	0.234	9 (56.3)	0.716

[1]Pre-cancer *vs* adenocarcinoma; [2]Normal *vs* adenocarcinoma; [3]Tobacco chewing habits include betel quid, areca nut, and/or pan masala use; multiple habits include two or more of any tobacco and/or alcohol habits.

high proportion of carcinogenic *cagA*$^+$ strains. However, frequency of *H. pylori* infection or *cagA* positivity was detected at low levels in tumor tissue of adenocarcinoma lesions that more closely resembled the symptomatic control group.

Our PCR data demonstrated the presence of *H. pylori* infection in each disease group (a significant proportion of which was *cagA*$^+$), although its prevalence varied in different types of gastric tissues. Our study showed the presence of *H. pylori* infection in 61% of histologically normal, but symptomatic, individuals. Studies carried out using serological or microscopic tests reported a variable presence of *H. pylori* infection in the gastric mucosa that ranged from 15%-92% in different adult populations worldwide[8]. An earlier study on *H. pylori* revealed a high prevalence of this pathogen, which could reach as high as 80%[20,29]. However, the prevalence of *H. pylori* shows major variations in ulcers, gastritis, and dyspepsia in India[20,21,29,30]. Despite high *H. pylori* prevalence, its importance in the progression of malignant gastric lesions is still debated. The high prevalence of *H. pylori* infection but correspondingly low incidence of gastric cancer in Asian countries like India is still a major paradox[18]. Accumulating evidence indicates a major variation in *H. pylori* prevalence that ranged from 45%-87.5%[20,21,31]. Moreover, it is important to note that prevalence of *H. pylori* infection in asymptomatic individuals increases with age; 56%-63% in the adult population[31]. The association between carcinogenic *H. pylori* and progression of gastric carcinoma may have been previously under- or overestimated due to the poor accuracy of serologic *H. pylori* markers, lack of discrimination of infecting genotypes, and the inclusion of previously resolved exposure with concurrent infection. Studies showed that PCR is a more accurate and sensitive technique than conventional methods like histopathology, rapid urease tests, and serological assays in detecting *H. pylori* infection[32]. Among the different primers of *H. pylori* genes, *ureC* and *cagA* gene primers are most frequently used in different studies[25,26], and represent the identification of generic and pathogenic *H. pylori* strains, respectively.

Our PCR-based analysis revealed the presence of a lower percentage of the pathogenic *cagA*$^+$ strain in overall *H. pylori* infection in symptomatic individuals. However, other investigators have demonstrated a higher percentage of *cagA* positivity that ranged up to 90% in duodenal ulcers and gastritis in different geographical location[33]. Further investigation of *H. pylori* infection in pre-cancer gastric tissues revealed a high frequency of *H. pylori* infections in intestinal metaplasia (89%) and dysplasia (100%); interestingly, the majority of these infections were *cagA*$^+$. Information regarding the prevalence of pathogenic *cagA*$^+$ *H. pylori* in pre-cancer lesions in India is grossly inadequate. Prevalence of *H. pylori* has been described in some reports that addressed pre-cancer lesions in isolation[34,35]. However, a comparative analysis of *H. pylori* infection within pre-cancer and adenocarcinoma groups in a single population have not yet been made, which, in purview of the variability in the frequency of *H. pylori* and its virulent strain, is essential in order to understand the pathogenic role of *H. pylori* in different stages of gastric carcinogenesis. Nevertheless, data from other geographical regions demonstrate that the prevalence of *H. pylori* ranges from 34%-71% in different studies[36-38], whereas prevalence of the *H. pylori cagA* strain showed a frequency that ranged from 40%-52% in other studies[39,40]. In contrast, we observed a high frequency of *cagA* in pre-cancer lesions. *CagA* is a bacterial oncoprotein that functionally mimics the mammalian Gab family of adaptor proteins and is re-

sponsible for gastric carcinogenesis[41]. *H. pylori* infection is significantly associated with intestinal metaplasia (72%) and dysplasia (75%) of pre-cancerous lesions, while in adenocarcinoma *H. pylori-cagA* infection was significantly higher in the diffuse type than the intestinal type of adenocarcinoma. It is unknown as to why there are high overall *H. pylori* or *cagA*$^+$ strains in pre-cancer lesions but not in cancer lesions in our clinical specimens, as well as the reason for the low incidence of gastric cancer. In view of observations by both us and others, it is likely that the Indian population is highly prone to carcinogenic *H. pylori* infection. However, due to the prevailing medical practice of indiscriminate and widespread overuse of antibiotics in the treatment of gastric and other ailments[42], carcinogenic progression of *H. pylori* induced lesions may be prevented.

In contrast to pre-cancer lesions, the prevalence of *H. pylori* was characteristically lower in cancer tissues (52%); more closely resembling the symptomatic control group. Globally, prevalence of *H. pylori* infection ranges from 27%-92% in advanced gastric carcinoma (predominantly intestinal type)[18]. On the other hand, *H. pylori* infection in gastric cancer lesions in studies from different regions of India demonstrates similar *H. pylori* positivity (43%-86%)[35,43,44], which was closer to the frequency detected in the present investigation. Interestingly, we found a low frequency of *cagA*$^+$ strains in gastric cancer tissues, whereas a study from another group from India showed a high proportion of *cagA* positivity (up to 100%), of which 86% cases were found to possess *H. pylori* with intact *cagA*-PAI[45]. It is likely that *cagA*$^+$ *H. pylori* in cancer lesions are a remnant of a more carcinogenic infection that could have been involved in tumorigenic transformation.

Though the sample size in our study showed a male gender bias in gastric pre-cancer and cancer lesions, *H. pylori* infection was not age or gender specific. Incidentally, symptomatic females showed significantly higher *H. pylori* infection than males, though they did not differ in *cagA* positivity. Contrary to this observation, gastric cancer incidence in India is reported to be lower in Indian females[12]. The reasons behind such discrepant observations are not clear. The high prevalence of *H. pylori* infection may be due to poor household hygiene in rural areas and addiction to tobacco, particularly chewing tobacco, in females which is quite frequent in northern India[11,46]. According to a population-based epidemiological study, women significantly ($P < 0.00001$) preferred smokeless/chewable tobacco compared to smoking[11]. Moreover, it has been generally observed that females from rural areas usually do not come to referral hospitals until they have significant symptoms, whereas males report even if symptoms are milder. It is quite likely that this phenomenon resulted in the higher percentage of females who came for endoscopy having *H. pylori* infection than males in the control and pre-cancerous groups, whereas in cancer cases, where both genders had equally severe symptoms, such a bias did not exist.

Analysis of *H. pylori* infection with habits of tobacco and alcohol abuse in different disease groups revealed an association of tobacco chewing with *H. pylori* infection, whereas its association with tobacco smoking or alcoholism could not be established in our study. Tobacco smoking and alcoholism have been indicated as risk factors for gastric cancer[47,48], and the association of tobacco use and *H. pylori* infection is equally frequent[49,50]. However, the mechanism by which these factors cooperate during gastric carcinogenesis is not clear. Tobacco chewing is a highly prevalent practice in the low socioeconomic strata population in India[11,46]. Our study demonstrates that habit of tobacco chewing in general, and specifically in combination with contaminated hands or unhygienically-prepared tobacco in such forms as khaini, local snuff, and lime-tobacco, could be a major factor promoting the transmission of *H. pylori*. Interestingly, in a study on a Yemeni population, a similar habit of chewing qat was found to be associated with high *H. pylori* infection[51], which supports our findings regarding the association of chewing tobacco with infection of *H. pylori*.

Although the onset of *H. pylori* infection starts in early childhood, our study group comprised subjects older than 17 years (except two cases of 7 and 13 year-olds who did not use tobacco in any form). Therefore, *H. pylori* positive subjects in the present study might have received prior exposure to *H. pylori* infection in early childhood by tobacco use or other means. A significant association between tobacco chewing and *H. pylori* in the cancer group, but not in the control or pre-cancer groups, further shows that *H. pylori* and tobacco may act as a co-carcinogen. It is likely that the toxic effect of tobacco may further promote *cagA*$^+$ *H. pylori*-mediated carcinogenic transformation in gastric epithelial cells over a long time period. A large cross-sectional epidemiological study in school children demonstrated that 11.2% of school-going children were addicted to tobacco in some form. Interestingly, 2.5% of the study subjects were "exclusive tobacco chewers" and the mean age of initiation of these habits in school children was found to be around 12.4 years[52]. Moreover, the likelihood of concurrent *H. pylori* infection due to tobacco being contaminated by being rubbed on the palm prior to use cannot be ruled out.

Overall, our study provides a comprehensive account of generic and *cagA*$^+$ *H. pylori* infection in different gastric pre-cancer and cancer lesions, and demonstrates a high incidence of *H. pylori* infection and carcinogenic *cagA*$^+$ strains in pre-cancer lesions during gastric carcinogenesis that may be acquired orally due to the habit of chewing tobacco or other similar abusive products.

COMMENTS

Background

With nearly one million new cases estimated in 2008, gastric cancer accounts for 7.8% of the total cancer burden and is the second leading cause of cancer deaths reported worldwide (738000/year). The distribution of gastric cancer is particularly high in Asia, Latin America, and some areas of Europe and Africa. De-

spite some of the high risk populations being in Asian countries such as Japan, Korea, and China, other Asian countries like India present relatively low rates of gastric cancer.

Research frontiers

The present study was conducted to perform a comparative analysis of *Helicobacter pylori* (*H. pylori*) infection and potential contribution of carcinogenic *cagA*$^+$ strains in gastric cancer progression, as well as to examine their association with tobacco use and alcohol abuse that may play a permissive role in carcinogenic transformation.

Innovations and breakthroughs

The results demonstrate the frequency of *H. pylori* infection was highest in pre-cancerous gastric lesions and was associated with habit of tobacco chewing. A high incidence of *H. pylori* infection and carcinogenic *cagA* positive strains in pre-cancer lesions during gastric carcinogenesis may be associated with the habit of chewing tobacco.

Peer review

This manuscript is a well-written article with some clinico-epidemiological significance in the Indian population.

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