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ASSOCIATION OF *HELICOBACTER PYLORI* RESTRICTION ENDONUCLEASE-REPLACING GENE, *hrgA* WITH OVERT GASTROINTESTINAL DISEASES

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ABSTRACT - ***Background and Aim*** - *Helicobacter pylori* has been proven to be responsible for causing various gastrointestinal disorders including gastric adenocarcinoma. Several genes of pathogen (the genes of the *cag*-PAI, *vac*A, *ice*A, and *bab*A) either in combination or independently have been reported to significantly increase the risk of ulceration/gastric carcinoma, with the *cag*A gene having the strongest predictive value. Pursuit to identify new genes which could serve as a marker of overt disease progression, lead to the discovery of *hrg*A gene. ***Methods*** - Fifty-six indigenous strains of *H. pylori* from subjects with various gastric disorder were screened to assess the status of *hrg*A gene along with the *cag*A gene using simple polymerase chain reaction using specific oligonucleotide primers. Post-amplification, amplicons were subjected for sequencing to identify any strain specific variations in sequences from the *H. pylori* isolated from different disease manifestations. Histopathological analysis was done to ascertain any significant change in the histological scores of subjects infected with *cag*A+/*hrg*A+ and *cag*A-/*hrg*+ strains. ***Results*** - All the 56 (100%) subjects amplified with the oligonucleotide primers specific to *hrg*A gene, whereas 81.71% subjects showed the presence of *cag*A gene. Sequencing of the amplimers showed 99% homology. Histology of the *cag*A+/*hrg*A+ and *cag*A-/*hrg*+ subjects did not show any significant difference. ***Conclusion*** - *hrg*A gene of *Helicobacter pylori* is not a ideal surrogate marker for identifying individuals with higher risk of developing overt gastro-duodenal diseases such as neoplasia of the stomach.

HEADINGS – *Helicobacter pylori.* Stomach neoplasms. Adenocarcinoma. Bacterial proteins. Polymerase chain reaction.

INTRODUCTION

Adenocarcinoma of the stomach is one of the leading causes of cancer related deaths in the world[9]. Although the incidence of gastric cancer has declined much significantly in the West, it still remains a major type of neoplasm especially in the East Asian countries like Japan, China, and Korea[14]. Development of gastric cancer is believed to be a multi-factorial event and probably takes 3 to 4 decades to manifest. With the primary etiological agents being exposure to chemical carcinogens, *Helicobacter pylori* occupies a unique niche in the genesis of gastric cancer. In addition, epidemiological studies have indicated that infection with *Helicobacter pylori* is considered a major risk factor for gastric cancer[11], and the WHO/IARC[4] has classified this bacterium as a definite biological carcinogen in 1994. Though it is hypothesized that the development of cancer depends on a series of complex molecular interaction between the host and bacteria[4, 10, 11], the precise patho-mechanisms linking *H. pylori* infection and gastric carcinogenesis still remains an unsolved enigma.

The predisposition among *H. pylori* infected individuals to develop various gastro-duodenal diseases *viz* duodenal ulcer (DU), gastric ulcer, gastric carcinoma and MALT-lymphoma mainly depends upon the bacterial and host factors and in part on the topography of the gastric inflammation[5, 6, 10]. Studies have identified several strain specific factors that potentially are markers for the differential risk associated with *H. pylori* colonization[2, 15]; at present, the genes of the *cag* pathogenicity island (*cag*-PAI) and *vac*A, SADAKANE et al.[12]; MAEDA et al.[7]; TIWARI et al.[13] have been reported to possess strongest predictive values. However, most strains isolated from Indian sub-continent and other East Asian countries possess these determinants irrespective of their clinical outcome. Thus, identification of specific bacterial factors that can serve as surrogate marker for the progression to ulceration or to gastric cancer remains desirable.

ANDO et al.[1], in 2002, while working with restriction–modification (R-M) systems, discovered potential marker *Helicobacter pylori* restriction endonuclease-replacing gene (*hrg*A) that in conjunction with *cag*A identified individuals associated with gastric cancer. The same

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study reported that though *hrg*A was more prevalent among the Western countries than in Asian, its prevalence was more among gastric cancer patients in Asians compared to those with benign disease. The data also suggested that the *hrg*A occurred more among *cag*A+ve *H. pylori* strains than those lacking *cag*A. These results prompted us to evaluate the prevalence of this virulence determinant among the strains isolated from the South Indian population and evaluate the presence of *hrg*A as a surrogate marker of overt gastrointestinal disorders.

Experimental procedure

We studied *H. pylori* strains from 56 dyspeptic patients (males: 32; females: 24; age range: 20-65 years) undergoing-gastroscopy in the Department of Gastroenterology, Deccan College of Medical Sciences and Allied Hospitals, Hyderabad, India, for the evaluation of upper gastrointestinal symptoms. The Institutional Ethical Committee (IEC) of the hospital approved the study protocol. A total of four gastric biopsy specimens (two from the antrum and two from the corpus) were collected from each patient after taking informed consent from the subjects to take part in the study. One antral biopsy collected in brucella broth supplemented with 2% fetal calf serum (FCS) was used for culturing *H. pylori*, one corpus biopsy for histological lesions and the remaining two biopsy one each from the antrum and corpus was collected in phosphate buffered saline for the DNA analysis.

Bacterial strains and growth conditions

The collected biopsy specimens were transported to the laboratory, the specimens were best processed within 1 hour, if delayed the specimen was preserved at 4^{0}C for 4–24 hours. The biopsy was inoculated onto the chocolate brucella agar medium (Difco Laboratories, Detroit, USA) supplemented with 7% sheep blood and antibiotics vancomycin (6 mg/mL), amphotericin-B (2 mg/mL) and polymixin-B (2500 units/mL). The plates were incubated in a microaerobic conditions at 37^{0}C for 3-5 days with 90%-100% humidity. Then the plates were examined after 72 hours for *H. pylori*.

The isolated colonies of *H. pylori* were again sub cultured in solid media. Rapid urease method (RUT) a touch cytology method was also done by inoculating few colonies into 250 µL urea broth comprising phenol red indicator. The presence of urease activity by a rapid change in color from yellow to pink indicated active *H. pylori*. The culture was considered to be positive for *H. pylori* by observing small, translucent, tiny colonies, which on staining gave Gram-negative staining.

DNA extraction and PCR amplification

Genomic DNA isolation from 36 pure *H. pylori* cultures and 56 biopsy samples was done using a standard protocol described previously[8]. The isolated DNA was then amplified for the presence of target genes viz., *cag*A, *hrg*A and *hpy*IIIR of *H. pylori* using specific oligonucleotide primers and PCR conditions listed in Tables 1 and 2. The products of amplification were subsequently electrophoresed in 1.5% agarose gel stained with ethidium bromide to visualize the presence of amplified genes. All the photographic registries were performed using a bio-rad gel documentation system.

TABLE1. List of oligonucleotide primers used during the study

Target gene(s)	Primer pairs	Sequence(5’- 3’)	Amplicon size (bp)	Reference
rRNA 165	16SrRNA-F	TAAGAGTACAGCCTATGTCC	534	13
	16SrRNA-R	TCCCACGCTTTAAGCGCAAT		
cagA	cagA-F	GATAACAGGCAAGCTTTTGA	499	This study
	cagA-R	CTGCAAAAGATTGTTTGGCA		
hrgA	hrgA-F	TCTCGTGAAAGAGAATTTCC	594	1
	hrgA-R	TAAGTGTGGGTATATCAATC		
hpyIIIR	hpyIIIR-F	CTCATTGCTGTGAGGGAT		
	hpyIIIR-R	TCTTGATAGGATCTTGCG	420	1

TABLE 2. Amplification conditions used for the PCR in the present study

Target gene	Amplification condition		
16S rRNA	Initial denaturation	95^{0}C for 5 min	
	Denaturation	94^{0}C for 30 sec	40 cycles
	Annealing	56^{0}C for 1 min	
	Extension	72^{0}C for 1 min	
	Final extension	72^{0}C for 7 min	
*hrg*A & *cag*A	Initial denaturation	95^{0}C for 5 min	
	Denaturation	94^{0}C for 30 sec	40 cycles
	Annealing	52^{0}C for 1 min	
	Extension	72^{0}C for 1 min	
	Final extension	72^{0}C for 7 min	
*hpy*IIIR	Initial denaturation	95^{0}C for 5 min	
	Denaturation	94^{0}C for 45 sec	40 cycles
	Annealing	47^{0}C for 1 min	
	Extension	72^{0}C for 1 min	
	Final extension	72^{0}C for 7 min	

TABLE 3. Comparison of the cagA and hrgA status in *H. pylori* positive subjects

S. No	Clinical status (n)	*cag*A status (%)	*hrg*A status (%)
1	Prepyloric ulcer (14)	14 (100%)	14 (100%)
2	Duodenal ulcer (15)	11 (73.3%)	15 (100%)
3	Gastric adenocarcinoma (14)	14 (100%)	14 (100%)
4	NUD (8)	6 (75%)	8 (100%)
	Total (51)	45(88.23%)	51 (100%)

Sequencing of *hrgA* gene of *H. pylori*

Following amplification using *hrgA*F and *hrgA*R primers, the amplimers were sequenced directly after purification with the QIAquick gel extraction kit (Qiagen) using the big dye terminator v3.1 cycle sequencing kit and injected to an ABI 3730 *xl* Genetic Analyzer (Applied Biosystems, Germany).

Histpathological analysis

Histopathological analysis was mainly performed to detect the presence of H. *pylori* and also to ascertain the presence

of significant preneoplastic and neoplastic lesions. Briefly, two sections (4 µM) were cut from each block: one section was stained with a modified Giemsa stain, and the other section was stained with hematoxylin-eosin (H-E) to assess the presence of intestinal metaplasia and dysplasia. A single pathologist (Z. A.) who was blinded to the patient's clinical conditions evaluated all the histologic sections. Grading of the histological lesions was done according to the updated Sydney system of classification[3].

Statistical analysis

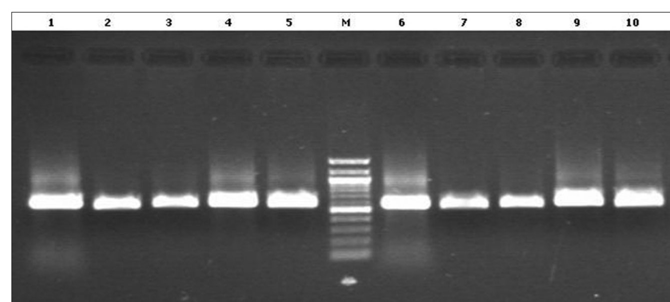
The data obtained was evaluated using Student Chi (χ^2) square test and probability values less than 5% ($P<0.05$) was considered statistically significant.

RESULTS

Out of 56 patients enrolled for the study, we categorized the patients according to their disease status (prepyloric ulcer-14, DU-18, non-ulcer dyspepsia-10, and gastric adenocarcinoma-14). *H. pylori* positivity was found in 36 (64.28%) by culture, 44 (78.57%) by RUT, 28 (50%) by histopathology, and 51 (91.07%) by biopsy DNA amplification.

Status of *hrg*A, *w*A and hpyIIIR gene locus

Of the 56 genomic DNA isolated (36 cultures and 51 biopsy samples), *hrg*A gene amplification was seen in all the samples screened giving a product size of 594bp. Besides this, the *cag*A gene was found in all (100%) the patients with pre-pyloric ulcers and gastric adenocarcinoma but only in 11 (61%) with DU and 6 (60%) with non ulcer dyspepsia (NUD), respectively. However, among the remaining 11 subjects with DU and NUD that did not amplify for the *cag*A gene, *hrg*A amplified giving the expected product (Figure 1 and Table 3). The prevalence of *hrg*A was then compared



Lanes 1 - 10 *H. pylori* DNA from various patients with different gastric diseases
Lane M - 100bp Molecular weight DNA marker (New England Biolabs)

FIGURE 1. Gel picture showing amplification of *hrgA* gene of *Helicobacter pylori* from various subjects

with the strain's *cag*A status, and clinical manifestations and also amplification for assessing the status of *hpy*IIIR gene locus PCR were performed in all the reported strains and was found to be negative except for the ATCC strain 26695. (Figure 2) All the 56 strains were found to lack the *hpy*IIIR locus and positive for *hrg*A gene. Samples were amplified

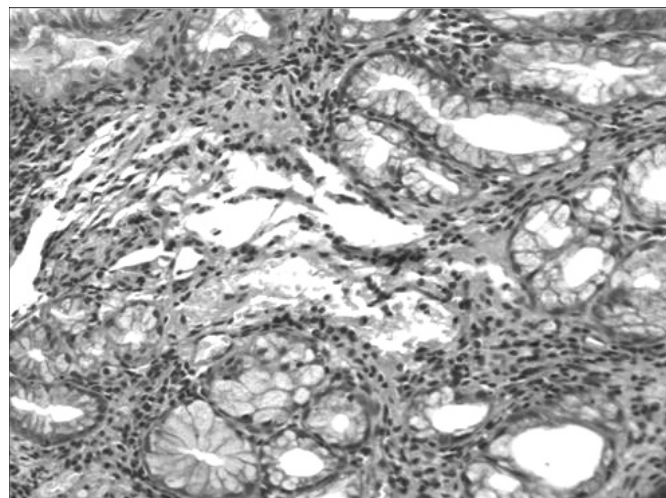


FIGURE 2. Corpus biopsy showing focal intestinal metaplasia with mild dysplasia

twice to confirm the presence of *hrg*A gene and absence of *hpy*IIIR locus. No mixed genotypes of *hpy*IIIR/*hrg*A were found in this study.

Histological assessment

Analysis of the sections from each biopsy from various patients showed chronic gastritis grade II among 10 (66.67%) subjects with DU, 8 (57.14%) with pre-pyloric ulcer, 3 (37.5%) with NUD, whereas chronic grade III gastritis with atrophic gastritis was seen in 2 (13.33%) with DU, 5 (35.71%) with pre-pyloric ulcers. H-E staining showed mild intestinal metaplasia (IM) in one (6.67%) subject with DU and one (7.14%) with pre-pyloric ulcer. Among 14 gastric carcinoma subjects, high grade dysplasia was seen in 8 (57.14%) and low grade dysplasia in 3 (21.42%) subjects, the remaining 3 (21.42%) subjects showed foci of chronic atrophic gastritis with moderate IM (Figures 3 and 4).

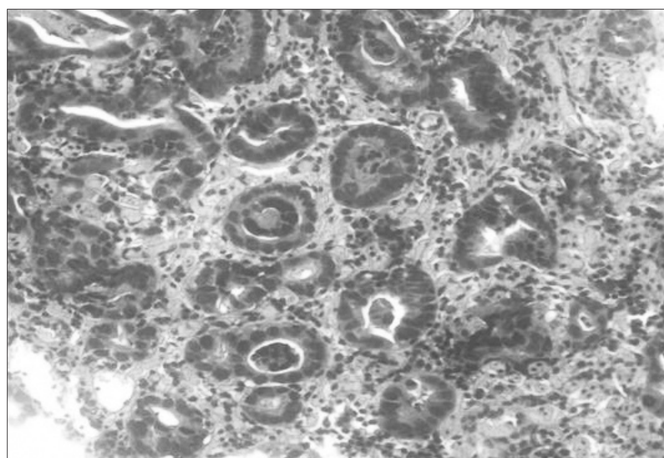
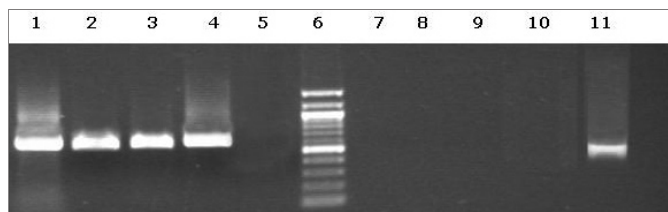


FIGURE 3. Invasion of stroma, dysplasia associated with invasive intestinal type cancer



Lane 1-4 representing hrgA gene amplification (596bp) of H. pylori strains
Lane 5 & 11 indicates control (ATCC 26695 strain) used for hrgA amplification & hpyIIIR locus amplification.
Lane 6 100bp Molecular marker (New England Biolabs)
Lane 7-10 represents hpyIIIR amplification in hrgA amplified strains.

FIGURE 4. Gel photograph showing amplification of the hgrA and hpyIIIR Status in *H. pylori* strains

Sequence analysis

Sequences obtained were compared using BLAST^n with the NCBI database. Sequencing analysis showed the presence of *hrg*A gene of *H. pylori* with a homology of 99%.

DISCUSSION

The present study evaluated the presence of *Helicobacter pylori* restriction endonuclease-replacing gene (*hrg*A) among subjects with various gastric disorders. The results of this study showed the presence of *hrg*A gene in all the 51 (100%) *H. pylori* strains isolated from each subjects with different clinical presentations.

Even though number of bacterial virulence determinants such as the genes of the *cag*-pathogenicity island, *vac*A, *ice*A, and *bab*A, have been extensively studied in the past (in association with) the clinical status in various geographical regions. No significant association could be established with the presence of any of these genes with respect to the clinical outcome, the main reason being the highly plastic genome of *H. pylori*[6, 12, 15], therefore in a continued pursuit to identify novel bacterial markers of *H. pylori*, which could serve as a surrogate marker of disease progression, ANDO et al.[1] reported the presence of *hrg*A to be high among patients with gastric cancer of the Asian population than those with non-cancerous or benign form of disease. However the present study could not find any such specific association of this gene with any of the disease conditions and found that the *hrg*A gene was present in all the subjects included in the study irrespective of their disease status. Further this study also found that the prevalence was not dependent on the presence of the *cag*A gene as it was evenly distributed among the patients with *cag*A positive and *cag*A negative *H. pylori* strains.

As evident from the results, we could not find any significant difference in the histological pattern among the subjects infected with *cag*A+ve/*hrg*A+ve *H. pylori* and those with *cag*A-ve/*hrg*A+ve. These findings suggest that development of overt disease such as ulceration or gastric carcinoma cannot be predicted based on the presence or absence of a one or two bacterial virulence factors. Rather development of severe form of disease is a result of complex molecular interactions between the host and the bacterial factors over a period of time that causes significant damage to the host. In addition, the site of the stomach where *H. pylori* colonizes also has an important bearing on the predisposition to develop pre-pyloric ulcer, DU and gastric cancer. Further, the environmental factors also play major role in disease manifestation besides the host and bacterial factors. This could have been one of the main reasons in ANDO et al.[1] study that could not establish a correlation between *hrg*A gene and gastric cancer patients of the Western countries whereas the same was possible among the Asian gastric cancer patients.

The results obtained in the present study are in contrast to those obtained by ANDO et al.[1] thereby suggesting that, though presence of *hrg*A had a higher predictive value for gastric carcinoma, our study however, could not hint at any such correlations, as *hrg*A gene was found to be present unequivocally in all the strains screened from various disease pathologies. The exploration for reasons responsible for these varied results warrants further investigations on large cohort population from different geographic areas. Besides this, an in depth molecular profile of the R-M systems and especially the functional role of *hrg*A gene would further be helpful to delineate the direct/indirect role played by this gene in gastric carcinogenesis.

In conclusion, *hrg*A gene of *H. pylori* may not be used as a ideal surrogate marker for identifying individuals at higher risk of overt form of gastro-intestinal disorders among the South Indian population.

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RESUMO – ***Racional e Objetivos*** – O *Helicobacter pylori* tem sido incriminado como causador de vários distúrbios digestivos, incluindo o adenocarcinoma gástrico. Diversos genes patogênicos (os genes do cag-PAI, vacA, iceA e babA), em combinação ou independentes, têm sido reportados como fatores de aumento de risco para ulceração/carcinoma gástrico, tendo o gene cagA forte valor preditivo. A procura da identificação de novos genes que possam vir a ser marcadores da progressão da doença levaram à descoberta do gene hrgA. ***Métodos*** – Cinqüenta e seis amostras de *H. pylori* provenientes de pacientes com diversas afecções gástricas foram examinadas para caracterizar a presença do hrgA juntamente ao cagA, usando iniciadores específicos da reação de cadeia da polimerase. Após amplificação, os produtos amplificados pela PCR foram seqüenciados para a identificação de variações específicas nas seqüências do *H. pylori* isolado de diferentes doenças gastroduodenais. A análise histopatológica foi feita para assegurar qualquer mudança significativa nos escores dos indivíduos infectados com cagA+hrgA+ e cagA-/hrgA+. ***Resultados*** – Todas as 56 amostras (100%) foram amplificadas com iniciadores específicos para o hrgA, enquanto que 81,71% mostraram a presença do cagA. O seqüenciamento do produto amplificado pela PCR mostrou 99% de homologia. A histologia entre os grupos cagA+/hrgA+ e cagA-/hrgA+ não mostrou nenhuma diferença significante. ***Conclusão*** – O gene hrgA do *H. pylori* não é o marcador ideal para identificar indivíduos com alto risco de desenvolvimento de doenças gastrointestinais como a neoplasia de estômago.

DESCRITORES – *Helicobacter pylori.* Neoplasias gástricas. Adenocarcinoma. Proteínas de bactérias. Reação em cadeia da polimerase.

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