H. Pylori bacterial gene cag A expression associated with gastric cancer development

Nazima Begum[1],Syed Mohammed Akbar Hassan[2], Avinash Bardia[3], Shaik Iqbal Ahmed[4],Puroshottam Padmanadhan[5], *, H. Surekha Rani [6] , Aleem Ahmed Khan[7*]

[1]Research Scholar, CLRD, Deccan College of Medical Sciences, Hyderabad, India
[2,5]Professor, Meenakshi Medical College and Research Centre, Kanchipuram, Tamil nadu
[3]Scientist, CLRD, Deccan College of Medical Sciences, Hyderabad, India
[4]Research Scholar, CLRD, Deccan College of Medical Sciences, Hyderabad, India
[6]Assistant Professor, Department of Genetics, Osmania University, Hyderabad, India
[7 *]Scientist, CLRD, Deccan College of Medical Sciences, Hyderabad, India

Corresponding author*
Dr. Aleem Ahmed Khan, PhD
Scientist,
Center for Liver Research and Diagnostics (CLRD),
Deccan College of Medical Sciences, Hyderabad, India.

***Abstract*: *Helicobacter pylori (H. pylori)* infects approximately half of the world's population with certain geographical variations. Being classified as a class I carcinogen and persisting mechanisms to resist gastric acidity, *H. pylori* has become a global public health issue. Yet, the precise molecular mechanism through which the *H. pylori* infection can cause a significant clinical result remains unknown. It was believed that a combination of factors like host genetic factors, environmental factors, and specific bacterial virulence genes are involved in *H. pylori* pathogenesis. Hence, the current study was aimed to identify the *H. pylori* pathogenesis at the molecular level by studying the expression of Cag A gene transcript in various gastrointestinal disease (GID) patients. by isolating RNA was isolated from tissue biopsies followed by cDNA construction and subjected to gene expression studies. Further, Cag A gene expression patterns were correlated with gastric cancer development and other inflammatory responses. The results obtained from this study revealed that the expression of the Cag A gene was significantly higher in GID group I patients than in GID group II patients. This correlative expression analysis of the Cag A gene transcript might be used as a predictive biomarker for diagnosing gastric cancer at an early stage.**

***Keywords*: *H. pylori*, Cag A, Gastric cancer, Gene expression, Predictive Biomarker.**

Introduction:

Gastric cancer (GC) remains the third leading cause of cancer deaths, worldwide. The most important risk factor for GC is the infection caused by Helicobacter pylori. Although there is a consistent decrease in its incidence, in 2015 imprecise 4.4 billion individuals that count to be more than half the world's population were infected with H. *pylori. (*1)

Helicobacter pylori were classified to be the class I carcinogen in the research on Cancer conducted by the World Health Organization in 1994. The estimated total of infection-attributable cancer is 1.9 million cases every year, which is 17.8% of the global cancer burden. The principal agent is *H. pylori*, the infection caused by H. Pylori can be accountable for approximately 63.4% of all stomach cancers or 5.5% of Global cancer.

The inter individual differences in risk of *H. pylori*-induced gastric diseases involve significant heterogeneity of both host genetics and *H. pylori* strain virulence factors. In the *H. pylori*-associated diseases pathogenic mechanisms, several strain-specific virulence factors were reported, such as *cag A* (cytotoxin-associated gene A), cag T, cag E, *vac A* (vacuolating cytotoxin A), and *hrg A*, genes. One of the main virulence factors is Cag A, which is associated with a higher risk of gastric cancer and peptic ulcer. Cag A protein can interact with intercellular proteins and activate signalling pathways through both tyrosine phosphorylation-dependent or independent mechanisms. (2)

About 70% of H. pylori strains have been found with the cag PAI gene. The cag A gene comprises 40 kb of chromosomal DNA with 32 open reading frames (ORFs), such as cag1 to cag 26, cag A to cag Z. The pathogenic mechanism of Cag A protein involves aberrant cellular signaling which may result in gastric cell malignancy (3,4). Previously it was observed that the expression of the cag A gene varies among H. pylori strains that might be due to several host interactions which opt for bacteria survival and its pathogenicity (5,6). Several in vivo studies have been reported the aberrant expression of virulent genes in the development of gastric cancers (7,8), however, there are no studies on the correlation of virulent gene expression and host gastric cancer development from the Indian population. The current study aimed to evaluate the *H. pylori* virulence gene cag A expression and correlate the expression pattern with gastric cancer development and other inflammatory responses.

In a study, evidence indicates that the risk of gastric cancer or premalignant lesions is higher in persons infected with *cag A*-positive *H. pylori* strains than in persons infected with *cag A*-negative strains (9,10). The increased risk of gastric cancer observed with *cag A*-positive strains (which often contain the entire *cag* PAI) is attributed to the cellular effects of Cag A combined with an enhanced gastric mucosal inflammatory response (11). *Cag A* was described as the first bacterial oncogene but as with many other oncogenes in humans, the story became even more complex. The disease-associated factor Cag A is also associated with health,

and its lack can lead to disease. Although *cag A*$^{+}$*H. pylori* strains were preferentially associated with both ulcers and gastric cancer, we and others found that these same strains had an inverse (protective) relationship with premalignant and malignant conditions of the esophagus, specifically Barrett esophagus and esophageal adenocarcinoma (12). Hence, our study aims to analyze the correlation between cag A gene expression and gastric cancer development in various gastrointestinal disease (GID) patients infected with H. Pylori bacteria.

Materials and Methods

A total of 210 *H. pylori*-infected subjects (121 males and 89 females) in the age range between21 to 55 years who underwent for upper gastrointestinal endoscopy were included in the study. *H. pylori* diagnosis was confirmed by clinical diagnosis of gastrointestinal disease Clinical history such as age, gender, symptoms, previous medication, endoscopy and extra GIT diseases was taken from all subjects. Subject less than 22 Years and on antimicrobial therapy, treatment with H2 receptor blockers, proton pump inhibitors, and NSAID before endoscopy were excluded from the study. The endoscopic finding of each patient was classified into 6 categories: Gastric adenocarcinoma, Prepyloric ulcer, duodenal ulcer, peptic ulcer disease, GERD and NUD.

For each patient, two biopsy specimens were taken from the antrum and fundus using an endoscope. One small portion of tissue was placed in Brucella agar for gram stain, Urease test, culture, and another stored in 0.1 ml normal saline and stored in -80°C for DNA, RNA extraction,

Expression analysis of virulent gene cagA using Quantitative Real-Time PCR (qRT-PCR):

RNA was isolated from gastric biopsies using TRIZOL reagent (Thermo Fisher, USA). The Isolated RNA quality and quantity were evaluated by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA was subjected to cDNA conversion using a High-Capacity cDNA conversion kit (Thermo Fisher). The expression levels of virulent genes were analyzed by the SYBR Green method (Thermo Fisher, USA) All experiments were carried out in triplicate using ABI PRISM 7500 (Applied biosystems, USA). 16S gene was used as an internal control to normalize the expression levels. The data for gene expression was analyzed with the ∆∆Ct method. The primer sequences for the target genes are as follows. cagA-Forward: GATAACAGGCAAGCTTTTGA; cagA-Reverse: CTGCAAAAGATTGTTTGGCAGA. PCR conditions for amplifications are as follows.One cycle of 94°C for 2 min followed by 40 cycles of 30 s at 94°C, 30 s annealing at 56°C and 40 s at 68°C.

Confirmation for H. Pylori Culture and Gram stain:

Microscopy: The bacterial smear was prepared then stained with modified Gram's staining method and observed under the microscope at oil immersion lens (100x) of light microscope ***Gram's*** staining wasused to identify *H. pylori*colonies. (Figure:1)

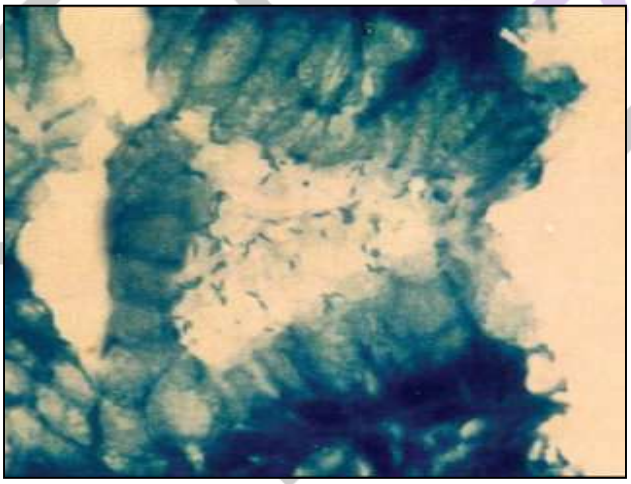

Figure 1: H. Pylori stained with modified Gram's

Urease test:

Rapid urease test: Rapid urease test is one of the invasive tests. It is based on the principle that abundant urease enzyme produced by *H. pylori* hydrolyses urea to ammonia. and carbon-di-oxide. The rise in the pH of the medium is detected by the phenol red indicator. The urea broth is usually pale yellow but it changes to pink color if *H. pylori* are present in the biopsy sample, as shown in Figure: 2 (Vandana Berry *et al.,* 2006).

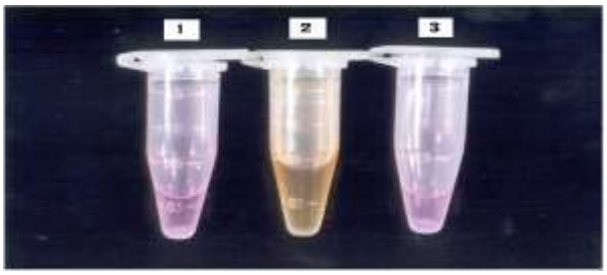

Figure 2: Tubes 1&3 showing the positive results, while tube 2 shows negative results

Confirmation of H. Pylori infection by amplifying 16S rRNA

The presence of *H. pylori* was confirmed by 16S rRNA amplification. The Polymerase Chain Reaction (PCR) was carried out using 16S rRNA primers and the DNA was isolated from the biopsy specimens of the subjects followed by amplification of 16S rRNA by using the following primer sequence. 5'–TAAGATCAGCCTATGCC – 3', R- 5-'TCCCACGCTTTAAGCGCAAT3'. The PCR conditions for the the16S rRNA gene amplification with both the primers were optimized and are as follows 95^0 C for 5 min (Initial denaturation step), 94^0 C for 30 sec 56^0 C for 30 secs, 40 cycles72^0 C for 30sec, 72^0 C for 5 min (Final extension step). The amplification was resulted a534 bp product which can be seen by using agarose gelelectrophoresis. (Figure: 4)

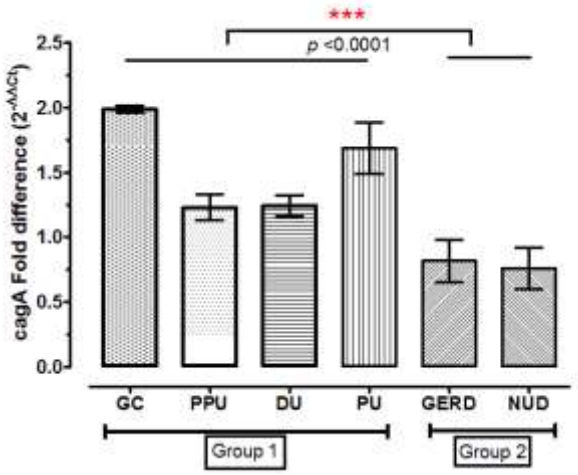

Figure 4: Expression of cag A gene in different gastrointestinal disease patients.

Statistical analysis Statistical analyses were carried out using Graph pad prism software. The data were given as mean ± standard deviation (SD) for all quantitative measurements. Unpaired t-test paired t-test and Pearson correlation coefficient tests were used to analyze the gene expression pattern. P-value at ≤ 0.05 was considered statistically significant.

Results:

Gram stain from homogenized biopsy shows gram-negative H. *pylori* curved rod bacteria whereas the culture of *H. pylori* is carried out in the laboratory in highly sterile conditions on selective media (Brucella agar media). Culturing upon streaking of the biopsy specimen on Brucella agar media transparent *H. pylori* colonies were grown as shown in the figure 3 after 72hrs of incubation at 37^0C anaerobic microaerophilic condition.

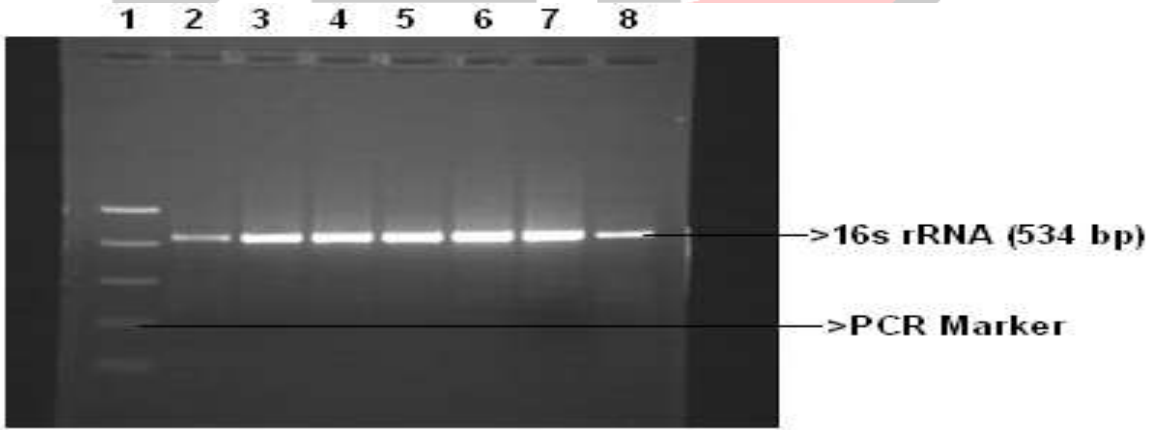

Figure 4: Gel imaging showing the amplified product of 16s rRNA gene.

Lane 1: DNA ladder (100bp)

Lane 2-8: Amplified 16s rRna gene product (534 bp)

Urease enzyme produced by *H. pylori* hydrolysis urea to ammonia. The consequent rise in the Ph of the medium is detected by phenol red indicator, which turns orange-red color to (low pH) pink color solution in high pH condition.

The 16srRNA amplification was carried out in the initial stages by using the DNA isolated from the biopsy samples of the patient. 16srRNA was the most conserved region to depict the presence of any organism. Also because of the higher sensitivity and specificity of 16srRNA primers to detect *H. pylori* in comparison with other primers specific to *H. pylori*, we havecarried out this 16srRNA amplification. All the 210 subjects were found to be *H. pylori*-positive based on the 16s rRNA amplification of product size 534 bp.

The expression of the cag A gene was significantly elevated in group 1 patients (gastric cancer patients, PPU, DU, and PU) compared to Group 2 patients (GERD and NUD patients). Nearly 2-fold difference was observed in GC and PU patients whereas 1.2-fold difference in PPU and DU patients. The difference between the two groups was statistically significant (p= 0.0001).

DISCUSSION

Helicobacter pylorus is a human gastric pathogen that causes chronic gastric inflammation. The infection typically persists for life long and responsible for several clinical manifestations such as gastric cancer, peptic ulcer diseases, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Upon establishment of infection, digestive acid/enzyme-producing gastric mucosa undergoes several metaplastic and dysplastic transformations which leads to gastric adenocarcinoma.

Several bacterial virulence factors are involved in gastric carcinogenesis along with host factors and gastric environment (13). Among, cag A, cag E, cag T, hrg A, and vac A S1 are the important bacterial virulent factors that influence pathogenicity and malignant cell development. Virulent factors such as cag A, cag E, and cag T are located in Cytotoxin-Associated Gene Pathogenicity Island (cag PAI) which is present in 70% of H. pylori strains. Cag PAI not only encodes bacterial virulent genes and also encodes the bacterial type IV secretion system (T4SS), which is essential to deliver Cag A into gastric epithelial cells. Upon delivery, cag A influences several components of gastric epithelial cells thereby induce disease development. In our study, the expression of cag A, cag E, and cag T was significantly elevated in gastric cancer, DU, and PPU patients. This might be due to the oncogenic and pathogenic activity of cag PAI. Previously, it was shown that cag A expression was significantly increased the gastric cancer risk (14,15). Moreover, the presence of cag PAI has been associated with the development of clinical manifestations such as peptic ulcer diseases and chronic gastritis. (16). Also, cag A responsible for malignant cell progression by influencing several signaling pathways. Cag A induces the anti-apoptotic protein MCL1 (myeloid cell leukemia sequence-1) expression levels by activating pro-survival MEK-ERK signaling (17) and suppressing p53 activity (18). It can be postulated that the presence of cag PAI might be involved in gastric cancer development. In conclusion, our study suggests that the expression of bacterial virulent genes might be involved in gastric cancer development and other gastric inflammatory responses. Hence, the cag A gene might be used as a predictive biomarker for gastric cancer development in *H. pylori*-infected patients.

Conflict of interest: None

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