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Helicobacter pylori Virulent Genes in the Upregulation of *CCL20* and *β-actin* Expression and Progression towards Gastric Disorders

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ABSTRACT

Objectives: *Helicobacter pylori*, infection carryout a substantial role in the pathogenesis of gastric disorders. The pathogenic effect is shown to be associated with host bacterial interplay and *Vac A* and *Cag A* are recognized as imperative virulence determinants. A variety of carcinogenic pathways are triggered by *H. pylori* and *CCL20* and *β-actin* are responsible for cellular progress and upregulation of these genes leads to metaplasia, dysplasia and gastric adenocarcinoma. Therefore, it is of interest to investigate the association of *Cag A*, *Vac A* in the upregulation of *CCL20* and *β-actin* expression and progression towards gastric disorders.

Methods: Blood and gastric biopsy samples of chronic gastritis subjects (n=100) were collected to identify the incidence of *H. pylori* by ELISA and Rapid urease test. The samples positive for both RUT and ELISA were subjected to RT-PCR for expression studies.

Results: Among the study subjects, 36% and 58% showed positive result for RUT and ELISA of which 29% (p=0.0005) showed positive result for both RUT and ELISA. For expression studies, 48% and 90% showed higher significant (p=0.0005) expression levels of *Cag A* and *Vac A* m2. 10% (p=0.023) showed a significant expression levels of *Vac A* m1 region. Likewise 31% and 90% (p=0.0005) of patients showed higher significant expression levels of *CCL20* and *β-actin*.

Conclusion: Thus obtained results illustrated that *H. pylori* excites the production of *Cag A*, *Vac A*, *CCL20* and *β-actin* and expression of these factors signify a promising biomarker in the early diagnosis of the infection and in analyzing the progression towards gastric disorders. *J Microbiol Infect Dis 2019; 9(3):116-124.*

Keywords: *Helicobacter pylori, CCL20, β-actin, RT- PCR, gastric disorders*

INTRODUCTION

Helicobacter pylori, a microaerophilic gram-negative bacilli, inhabits in mucus lining of gastric epithelium of the stomach. Human beings are the bacterium's solitary host and majority of people harboring *H. pylori* are asymptomatic [1]. Even though in most infected people *H. pylori* do not cause illness, it is the leading driving force of chronic gastritis and infectivity with this organism is the chief etiological component in the pathogenesis of gastrointestinal disorders [2]. *H. pylori* infections primarily bring on chronic superficial gastritis, which can advances all the way through chronic atrophic gastritis, intestinal metaplasia, and dysplasia towards gastric carcinoma [3]. On the other hand, progression emerges in only few patients and it relies upon various factors, together with bacterial and host genetic makeup [4, 5]. *H. pylori* virulence factors, for instance cytotoxin-associated antigen A (*Cag A*), along with vacuolating cytotoxin (*Vac A*) do liable for majority of these events. One potential interpretation is that patients with severe gastroduodenal lesions are infected by virulent strains of *H. pylori*, while subjects with moderate

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gastritis without ulcer/cancer are infected by the organisms with low pathogenic potential [6].

H. pylori strains comprising *Cag A* are linked with enhanced threat in the evolution of atrophic gastritis and also gastric adenocarcinoma [7]. *H. pylori* pathogenesis firmly count on pathogenic determinants, such as *Vac A* that infuses the oncoprotein *Cag A* into the host cell. *Cag A* persuades transformation of cell morphology inside the host cell that is linked with improved cell migration. Therefore, *Cag A* interrupts normal cell and deliberates the carcinogenic potential which is demanding for the pathogenesis of *H. pylori* infection. Furthermore *Cag A* interrelates with *Vac A*, an added most important virulence factor for *H. pylori*, which bring about cellular vacuolization, and hinders cell proliferation and ulcer healing [8].

An autonomous *H. pylori* loci associated with greater disease threat is vacuolating cytotoxin that encrypts the secreted toxin *Vac A*. The *Vac A* restrains T-cell responses towards *H. pylori* that possibly will commit to prolonged existence of infection. *Vac A* put forth multiple impacts on epithelial cell structure which bring about interruption in the function of gastric epithelial barrier and also intonation of inflammatory reaction. Further consequences of *Vac A* encompass interruption of late endosomal compartments that result in invitro vacuole formation [9]. *H. pylori* precise strain components are not perfect virulence agent, since majority of individuals inhabited with disease-linked strains persist asymptomatic and that has focused the demand to detect the host factors that control the pathological effects. Host mechanisms that are related to neoplastic conversion comprise improved cell turnover, injure to gastric mucosal layer by reactive oxygen metabolites and decline in the concentration of ascorbic acid in the gastric juice. In general it is established that for the breakthrough of oncogenesis, persistent inflammation persuaded through *H. pylori* is significant. A range of carcinogenic pathways are stimulated via *H. pylori* or else through its virulence components inside the host cell especially *Cag A*, *Vac A*, which deliberate the epithelial cell oncogenic potential and thus they are at present the goal of broad investigation [10].

H. pylori infection which increases the tumor invasiveness and metastasis is normally found to be a threat for the progression towards gastric cancer. *CCL20* is one of the chemokine that magnetizes immature dendritic cells along with memory T cells and may perform a significant function in gastric inflammation. The ability of *CCL20* to employ immature dendritic cells as well as T cells with these characteristics made to explore the expression of *CCL20* in *H. pylori*-induced chronic gastritis [11]. Earlier studies revealed that in swollen gastric tissue the expression of *CCL20* is elevated and *CCL20* may exhibit an important function in lymphocyte trafficking which is chemokine mediated in the event of gastric inflammation in *Helicobacter* infection [12].

β-actin, an essential component of the cytoskeleton and it is a housekeeping gene. It articulates in most of the eukaryotic cells, moreover implicated in organizing major housekeeping objectives. In addition it performs a significant function in regulation of transcription, chromatin remodeling, transport and processing of mRNA. In laboratory techniques to standardize protein and gene expressions *β-actin* is the frequently used endogenous reference controls as it is considered to have stable levels of expression in diverse physiological, cellular and experimental conditions. On the other hand, increasing data have revealed its differential expression in multiple cancers. In turn the knowledge about *β-actin* expression and dispersion in diverse cell types is not clear. Studies on *β-actin* expression in tissues will offer an enhanced comprehends of its function in carcinogenesis and it is used as a tumor biomarker because of its association with clinical pathological criteria. *β-actin* is also identified to be implicated in motility of cancer cells [13,14]. Dysregulation of oncogenic pathways with interruption of natural cell–cell communication results in the epithelial cell carcinogenic transformation. From the previous studies it is clear that gastric carcinomas involve *Helicobacter pylori* as primary carcinogen but the mechanisms by which *H. pylori* increases the risk of developing gastric cancer remain unclear. In the present study identification of host genetic determinant that inclined to the disease condition driven by *H. pylori*, provide insights to the carcinogenic mechanisms that

contribute to the understanding of the pathogenesis and early diagnosis of gastric cancer.

Hence, investigation on host–bacterial interplay significantly broadens our perceptive regarding the pathophysiology of persistent inflammation and carcinogenesis. Recognition of *H. pylori* assisted cellular responses can afford peculiar and fair insight in the molecular method of *H. pylori*-dependent pathogenesis in vivo and may direct to newer therapeutic approach. To explore its prospective, a study was conducted to interrogate the expression levels of *Cag A*, *Vac A*, *CCL20* and *β-actin* in subjects with chronic gastritis.

METHODS

The study was approved by the Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research (Deemed to be University) (year-2013). It is a prospective study and the study enrolled 100 chronic gastritis patients of both the sexes. Biopsy sample were collected through endoscopic procedures and simultaneously peripheral blood samples were also obtained from the Department of Gastroenterology at Sri Ramachandra Institute of Higher Education and Research (Deemed to be University). Subjects with chronic gastritis as well as positive for *H. pylori* infection were considered as case samples and the patients negative for *H. pylori* infection were considered as control samples, while subjects with co-morbid conditions besides chronic gastritis were excluded. A formalized informed consent form was acquired from the study subjects. The collected samples were employed for Rapid Urease Test (RUT) to identify the incidence of *H. pylori* and Enzyme-linked immunosorbent assay (ELISA) to determine the *H. pylori* IgG antibody and Real time PCR (RT-PCR) to study the *Cag A*, *Vac A*, *CCL20* and *β-actin* expression levels. RT PCR was carried out by SYBR Green method and the expression levels were analyzed by comparative CT method.

Identification of *H. pylori* infection in gastric biopsy sample by RUT

The RUT detects the existence of urease activity in the gastric biopsy samples for the determination of *H. pylori* infection. In brief, the hydration reagent was placed on top of the substrate pad, allowed to fully absorb and biopsy sample was positioned directly onto the reaction pad. Then the substrate pad was fold over onto the specimen and placed into the reaction chamber or pouch for an hour. The formation of the deep blue or purple color on the specimen within 60 minutes signifies that the specimen is infected with *H. pylori* (positive). Whereas, formation of a faint gray haze or pale blue on the specimen at 60 minutes is observed as a negative result [15]. The results were confirmed by running positive control along with test samples.

Identification of *H. pylori* IgG antibody in serum sample by ELISA

The identification of IgG antibodies across *H. pylori* antigens was based on the use of sandwich-variant of solid-phase enzyme immunoassay. About 10 µl of positive, negative control (received along with the kit) and patient serum sample was added to the wells which bind to the antigen on the surface of the wells, the resulting complex is detected using conjugate- mouse monoclonal antibodies to human IgG with horseradish peroxidase (HRP). As a result, a "sandwich", bound to the plastic, containing peroxidase is formed and then incubated with tetramethylbenzidine substrate (TMB). Reaction of a substrate with the enzyme produces a colored product, thus indicating a positive reaction. The intensity of the color is directly proportional to the content of specific IgG antibodies to *H. pylori* antigen. Optical density was measured at a wavelength of 420 nm [16-18].

Positivity index (PI %) of each sample was calculated using following formula

Positivity Index = Optical density of test sample / Cut off value

Where the cut off value = Average of negative + 0.3

RT PCR analysis for the expression of *H. pylori Cag A, Vac A, CCL20* and *β-actin*

Single step RNA isolation method with TRIzol reagent was employed to isolate the high quality RNA from tissue samples [19]. The gastric biopsy sample was homogenized with 1 ml of TRIzol reagent to which 200 µl of chloroform

was added, incubated at room temperature (25±1 ^{0}C) for 10 minutes and centrifuged at 10,000 rotations per minute (rpm) for 10 minutes. The upper aqueous phase was collected and to that 500 µl of 100% ice cold ethanol was added to precipitate RNA and pelletized at the bottom after centrifugation. Discard the supernatant and re-suspend the pellet in 20 µl of sterile Diethyl pyrocarbonate (DEPC) water and it was stored at -80 ^{0}C until further use. Agarose gel electrophoresis was employed to determine the quality of the RNA; the quantification was achieved by Nanodrop. The quantity of the RNA on an average was found to be between1.8-2.0.

The qRT PCR analysis was accomplished by two step process; the isolated mRNA was transformed into cDNA using high capacity cDNA conversion kit followed by expression analysis. The primers for *Cag A*, *Vac A*, *CCL20* and β- actin gene were acquired from the NCBI database. Gene sequence was downloaded in FASTA format and gene specific primers were designed. The following primers were used for *Cag A*, 5'- GATAACAGGCAAGCTTTTGAGG -3 (forward primer) 5 - CTGCAAAAGATTGTTTGCGAGA-3 (reverse primer) and 5 - GGTCAAAATGCGGTCATGG -3 (forward primer) 5 - CCATTGGTACCTGTAGAAAC - 3 (reverse primer) for *Vac A* m1 region and for *Vac A* m2 region 5 - GGAGCCCCAGGAAACATTG -3 (forward primer) 5'- CATAACTAGCGCCTTGCAC -3 (reverse primer) and for *CCL20* 5 - GCGCAAATCCAAAACAG -3' (forward primer) 5 - GTCCAGTGAGGCACAA -3 (reverse primer)were used and 5'- AGAAAATCTGGCACCACACC -3 (forward primer) 5 - CCATCTCTTGCTCGAAGTCC-3 (reverse primer) for *β-actin*. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an exogenous control. Subsequently, the synthesized cDNA was employed for expression analysis using RT PCR by SYBR Green method. RT-PCR was performed in a 20 µl reaction volume which contains 2 µl of template (cDNA) (50ng/µl), 10 µl of SYBR Green mix (2x), 1 µl of forward and reverse primers (5 pmol/ µl), and 7 µl of DEPC water. Two sets of reactions were prepared for each sample, one with the desired primers and the other with the exogenous control GAPDH. PCR was performed for 10 min at 95˚C, pursued by 40 cycles at 95˚C for 15 sec, at 50˚C for 30 sec, 60˚C for 30 sec and final extension for 1 min at 60˚C [20,21]. The comparative threshold method (CT method) was adopted to quantify the expression level of the above said genes. In this method the RT PCR Ct values (cycle threshold) of the test sample was compared with a control sample (negative for *H. pylori*); Ct values of the sample of interest and the control were normalized to an appropriate endogenous housekeeping gene.

Statistical analysis

The collected data were examined with IBM.SPSS statistics software 23.0 Version. To illustrate data descriptive statistics frequency analysis, percentage analysis was employed for categorical variables and for continuous variables the mean and standard deviation were employed. Chi-Square test was used to find the significance in categorical data; similarly the Fisher's Exact was used if the expected cell frequency is less than 5 in 2 × 2 tables. The probability value ≤0.05 is considered as significant in the above statistical tool.

RESULTS

Demographic details of the study subjects

Gastric biopsy samples and blood samples (n=100) were collected from the study subjects. Among the study subjects, 86% were males and the remaining 14% were females. The age ranges between 23 to 63 years with a mean of 42.5 ± 11.9 years.

Identification of *H. pylori* infection in gastric biopsy sample by RUT

RUT identifies the existence of urease activity in the gastric biopsy samples for the determination of *H. pylori* infection. The formation of the deep blue or purple color on the specimen signifies the *H. pylori* infection. Out of 100 subjects, 36% of patients showed positive for infection with *H. pylori* and rest of the patients showed a negative result.

Identification of *H. pylori* IgG antibody in serum sample by ELISA

Presence of *H. pylori* in the serum sample was measured by sandwich ELISA. Reaction of a

substrate with the enzyme produces a colored product, (Figure 1) thus indicating a positive reaction. The intensity of the color is directly proportional to the content of specific IgG antibodies to *H. pylori* antigen. Among the study subjects, 58% showed positive result signifying the incidence of *H. pylori* IgG antibody.

Identification of *H. pylori* infection in gastric biopsy and blood samples

About 29% (p=0.0005) of samples showed a positive result both by RUT and ELISA, signifying the existence of *H. pylori* infection.

RT PCR analysis for the expression of *H. pylori Cag A*, *Vac A*, *CCL20* and *β-actin*

The samples which showed positive result for RUT and ELISA were subjected to RT PCR to study the expression levels of *Cag A*, *Vac A* and host genetic factors (*CCL20* and *β-actin*). The 2-ΔΔCT value of *H. pylori* virulent genes and host genetic factors was calculated and the expression levels are given in Figures 2 and 3.

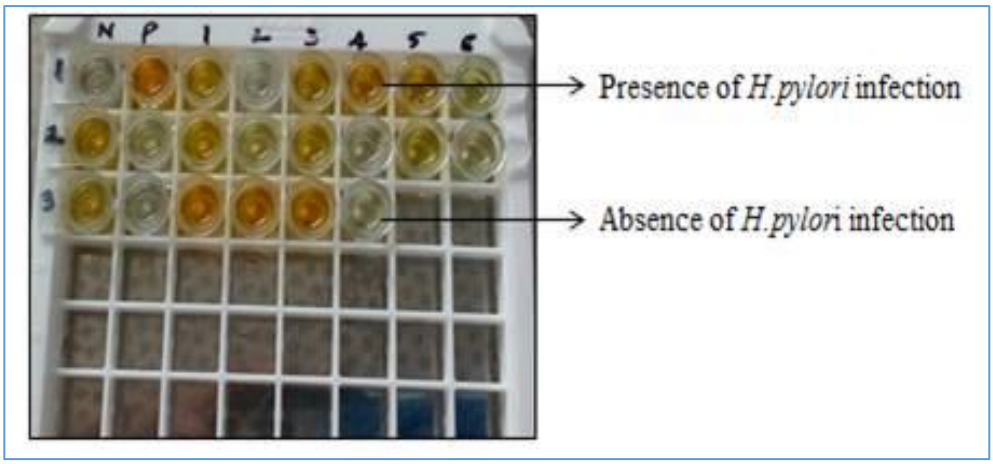

Figure 1. ELISA test showing the presence and absence of *H. pylori* infection in serum sample.

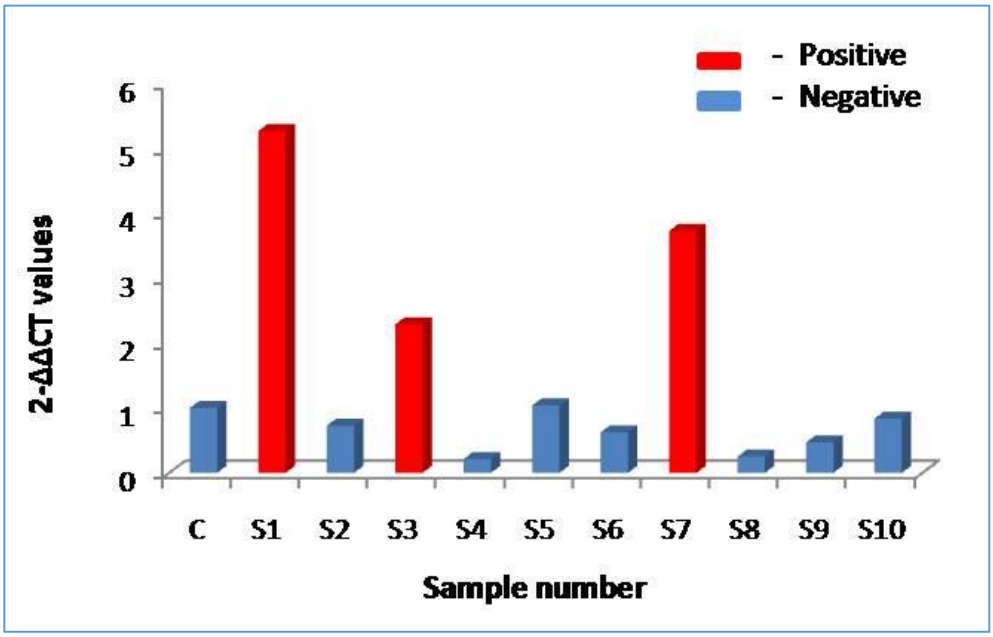

Figure 2. The 2-ΔΔCT values of *CCL20* expression from the gastric biopsy sample of the study subjects.

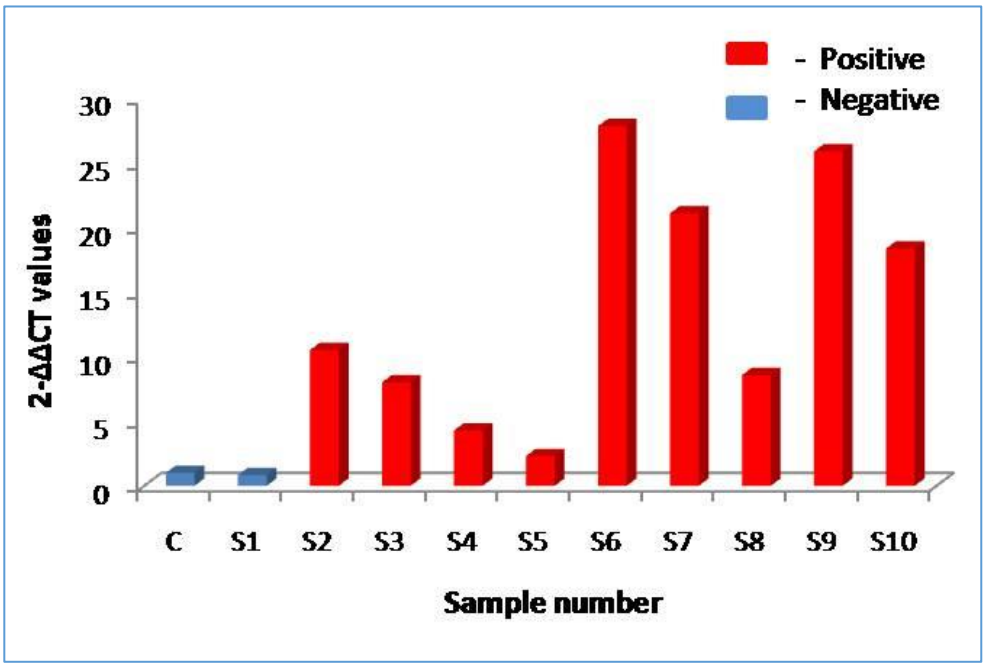

Figure 3. The 2-ΔΔCT values of *β-actin* expression from the gastric biopsy sample of the study subjects

The RT PCR analysis for the expression of *H. pylori* virulent genes reveals that among the study subjects 48% and 90% showed higher significant (p=0.0005) expression levels of *Cag A* and *Vac A* m2. Whereas, 10% (p=0.023) showed a significant expression levels of *Vac A* m1 region. While 31% and 90% (p=0.0005) of patients showed higher significant expression levels of *CCL20* and *β-actin* respectively. An association analysis was performed between the *H. pylori* virulent genes and host gene expression levels, in which the association analysis between *Cag A*, *Vac A* m2 and *CCL20*, *β-actin* showed a higher significant expression levels (p= 0.0005), that signifies a strong association towards disease progression and *Vac A* m1 did not show any significant result (p=1.000) which indicates a weak association towards gastric disorders.

DISCUSSION

H. pylori are identified as the governing organism of human gastric environment and its existence leads to constant inflammatory response and alter host cell signaling pathways. Therefore, in the pathogenesis of gastric disorders, the chronic infection caused by *H. pylori* is an eminent risk determinant for chronic gastritis and peptic ulcer [22].

Virulence factors articulated by most of the *H. pylori* strains have emerged to alter host cell signaling pathways. Long-standing bearer of the organism extensively enhances the threat of establishing site-precise diseases [23]. In accordance with the clinical importance of *H. pylori* a study was performed to determine the role of virulent genes (*Cag A* and *Vac A*) in the upregulation of *CCL20* and *β-actin* expression and progression towards gastric disorders in subjects with chronic gastritis. Out of 100 samples, 36% and 58% of patients showed positive result for RUT and ELISA respectively, signifying the existence of infection with *H. pylori* and 29% showed positive for both the test and were subjected to RT-PCR for expression studies. It was determined that, *Cag A*, *Vac A* m2, *CCL20* and *β-actin* showed higher significant expression levels whereas, *Vac A* m1 showed a significant expression levels respectively. An association analysis was performed between the *H. pylori* virulent genes and host gene expression levels, which signifies a strong / moderate or weak association towards the progression of gastric disorders.

Among the individuals infected with *H. pylori*, the variations in gastric disease progression may be linked to variable host characteristics, disparity among *H. pylori* strains, precise interactions

between host and microbial determinants and environmental influences. The antibodies to the *Cag A* protein is found in the sera of 100% of subjects infected with this bacterium who have peptic ulcer however merely 60 to 62% of patients who have gastritis [24-26]. As a result, the existence of *Cag A* is linked to infection with *H. pylori* to bring about peptic ulceration and it is a significant method to find out the *Cag A* status of *H. pylori* in biopsy samples and are likely to be imperative.

The most essential virulence determinants formed by *H. pylori* is *Vac A*. Various studies revealed that *H. pylori* strains that encompass definite *Vac A* types such as s1, m1 are linked with an elevated risk of gastric intestinal disorders than s2 or m2 *Vac A* types. In patients who are tested positive for *Vac A* but negative for *H. pylori* the threat for gastric cancer was greater when correlated with individuals who were tested positive for both. Studies on *H. pylori* have linked vacuolating cytotoxin with particular signal sequence (m1 and m2) allele of the underlying *Vac A* gene to gastritis. Many recent studies have confirmed clinical and pathological disparity among strains holding diverse *Vac A* polymorphisms [27]. Studies on biological behavior and clinical organizations related to dissimilar *Vac A* genotypes are essential for a profound knowledge of their role in disorders induced by *H. pylori* and for the evolution of new strategy to counteract their outcomes. In the present study, the *Vac A* allele m2 region of *H. pylori* was found to be strongly linked with chronic gastritis than allele m1. Therefore these virulence factors of *H. pylori* can be employed as indicator to determine the risk of budding serious gastrointestinal disorders.

β-actin, a housekeeping gene, is expressed nearly in every eukaryotic cell and is implicated in managing basic housekeeping functions and it diversely expressed in multiple cancers. The over expression of *β-actin*, bring about modification of various signaling pathways. On the whole in gastritis, improved *β-actin* expression has been described, which modifies the cellular growth, distribution and proliferation. Knowledge on *β-actin* expression in biopsy sample will offer a enhanced comprehend of its function in carcinogenesis. Thus, it could possibly signify a potential biological marker in early identification and prognosis of gastric carcinoma. A study by Xu J et al, illustrated that *β-actin* level was extensively higher in gastric cancer tissues and the correlation between *β-actin* expression and the neighboring lymph node metastasis was statistically important, which explained that over expression of *β-actin* might be partly responsible to the local lymph node metastasis of gastric cancer. Thus *β-actin* was found to be the most conventional and dynamic protein and it is over expressed in gastric cancer tissues and may perhaps perform a vital role in metastasis of gastric cancer [28]. Similarly Shafqat A Khan et al, investigated the association of cell type precise *β-actin* expression in gastric carcinoma by means of clinicopathological specifications by RT-PCR and western blotting. Their study result revealed a significant over expression, equally at protein and mRNA level. The above studies imply the potentials of *β-actin* as a cancer biomarker and proposed as a promising target for chemotherapy [13]. In the current study expression of *β-actin* is upregulated in 90%of subjects with *H. pylori* persuaded gastritis.

A precise chemokine receptor for *CCL20* is *CCR6* which is particularly articulated on few memory T cells and dendritic cells. *CCL20* has proved to preferably attract memory T cells, and perform a significant task in gastric inflammation. In gastric mucosa, infection with *H. pylori* is correlated with an inflammatory reaction leading to persistent gastritis, peptic ulcers and gastric carcinoma. At the site of infection with *H. pylori* there is an improved T-cell infiltration. In the present study 31% of samples showed over expression of *CCL20* and rest of the samples showed normal expression when compared to the control sample. Previous studies confirmed that in irritated gastritis tissue the *CCL20* expression is elevated. The *CCR6*/*CCL20* plays a vital part in the gut-associated lymphoid tissue homeostasis and also in the organization of mucosal immunity and increased expression of *CCL20* has been noticed in gastrointestinal inflammation. *CCL20* acts as a ligand for chemokine receptor *CCR6* and Yi-Ying Wu et al examined the function of *CCR6* and *CCL20*, in promoting an inflammatory reaction in the gastric epithelial cells during infection with *H. pylori*. The study result indicated that there was drastically improved *CCR6* expression in the gastric mucosa. Their results proposed that the

interplay between *CCR6* and *CCL20* might perform a critical function during gastric inflammation [12]. The over expression of *CCL20* were certainly linked with the *Helicobacter pylori* colonization density. A study by Akira Yoshida et al to detect the protein and mRNA levels of *CCL20* by RT-PCR and ELISA, revealed that the expression of *CCL20* at protein and mRNA levels were considerably high in subjects positive for *H. pylori* and significantly declined following complete eradication. The correlation between *CCL20* concentrations and the extent of chronic gastritis demonstrated that the *CCL20* interplay may possibly be entailed in the evolution of gastritis induced by *H. pylori* [29].

In summary, the obtained study result proposed that *Cag A*, *Vac A*, *CCL20* and *β-actin* might play significant role in categorizing the disease severity and knowledge on *H. pylori* virulence factors provide the clarification on disease pathogenesis. Moreover *CCL20*, *β-actin* and its associated signaling pathways perform substantial role in retaining the functions of gastric mucosa and dysregulation of these determinants contributes to gastric cancer initiation as well as progression. The limitations in the present study indicates that altered expression of host genetic factors is not found at a higher percentage as compared to cancerous tissues but comparatively found at a rational rate in chronic gastritis as it may be one of the reasons for its cause or may develop in time.

Conclusion

Infection with *H. pylori* excites the production of *Cag A*, *Vac A*, *CCL20* and *β-actin* and expression of these factors is upregulated in the gastric epithelium of subjects with gastritis induced by *H. pylori* and may signify a promising biomarker in the early identification of infection with *H. pylori* and in analyzing the progression towards gastric disorders.

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