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Original Research Article

Differential Expression of Claudin 3 and Claudin 4 in H. Pylori Infected Gastrointestinal Disease Patients

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Abstract:

Helicobacter pylori colonize epithelium lining of the gastrointestinal tract and disrupt the function of the epithelial barrier, leading to changes and inflammatory responses that rely on H. pylori virulence factors. This further leads to actin cytoskeleton reorganization in epithelial polarity, structural and functional changes leading transiting to mesenchymal characteristics. By following the changes from grade 1 to grade 4, our study examined epithelial mesenchymal transition in various grades of gastrointestinal diseases. Additionally, the function of claudin-3 and claudin-4 in different grades of gastrointestinal disease was investigated. In comparison to the control group, we observed reduced expression of claudin- 3 and claudin-4 in different grades of gastrointestinal diseases. Compared to grade 1 and grade 2 gastrointestinal diseases, there was a significant reduction in the expression of claudin-3 and claudin-4 in grade 3 and grade 4 gastrointestinal diseases. Tight junctions require claudin as a necessary component in order to preserve epithelial homeostasis and cell-to-cell integrity. Comprehending the roles of claudin-3 and claudin-4 will help to clarify the underlying molecular mechanisms causing gastrointestinal disorders as well as open new opportunities for exploring the creation of new treatments.

Keywords: H. pylori, Claidin 3 and Claudin 4, 1-4 grades Gastrointestinal Diseases, Gastric Adenocarcinoma, Peptic Ulcer Disease, Duodenal Ulcer, GERD Gastritis Epithelial - Mesenchymal Transition (EMT)

Introduction

H. pylori are one of the human infections that most effectively colonize the gastric lining epithelium of the stomach. (Wroblewski LE, et al 2010 and Hishida A, et al 2010) The cells of the gastrointestinal epithelium possess characteristics that enable them to act as a formidable barrier in innate mucosal immunity.

Further gastrointestinal epithelial cells provide cell barrier integrity, Cell turnover, autophagy, innate immune responses. (Alzahrani S et. al 2014) The stomach epithelium, which is firmly sealed by coordinated regulation of epithelial cell shape, polarity, and cell-to-cell and cell-to-matrix adhesions, constitutes an efficient initial barrier against pathogens in healthy persons. H. pylori breaks down the epithelial barrier function in connection with gastric mucus colonization to cause neoplastic alterations and inflammatory reactions that are dependent on H. pylori virulence factors [Wessler S, et al 2008]. Actin cytoskeleton reorganization leads to changes in epithelial polarity, structural and functional changes leading transiting to mesenchymal characteristics. The Epithelial - Mesenchymal Transition EMT phenotype requires complex morphogenetic programming, which can be observed in H. pylori-colonized cells [Yin Y, et al 2010]. This program can be triggered by alterations in gene expression, a loss of distinctive epithelial traits, and an increase in mesenchymal properties [Boyer B et al 2000].Cells develop a highly motile, mesenchymal shape during EMT and lose their polar, epithelial character. EMT is primarily characterized by the following processes: (i) disassembly of intercellular junctions; (ii) reorganization of the actin cytoskeleton from cell-matrix and cell-cell junctions into protrusive and invasive pseudopodial structures, such as actin stress fibers and actin-dependent protrusion of cell pseudopodia; and (iii) an increase in cell motility.

These processes typically happen simultaneously but separately from one another [Boyer B et al 2000]. Many alterations occur in tandem with the completion of EMT, including the breakdown of the basement membrane, the emergence of mesenchymal cells, the loss of cell polarity, the breakage of cell-cell junctions, and the remodelling of extracellular matrix (ECM). (Kalluri R, et al 2009 and Dongre A, et al 2019) The involvement of tight junctions (TJ) in gastritis associated with H. pylori has been thoroughly explored by numerous studies due to the disruption of epithelial cell-cell interaction in EMT. Claudin proteins are an essential component of tight junctions, which are large protein complexes made up of 40 distinct proteins. There are currently 27 members of the claudin family of transmembrane proteins, which is essential to a tight junction strand. In epithelial and endothelial cell sheets, claudin is essential for preserving cell-cell integrity, controlling paracellular permeability, and preserving cell polarity (Furuse M, et al 1998, Krause G, et al 2008 and Mineta K, et al 2011). Deregulation of claudin has been linked to a number of illnesses, from benign gastritis to deadly cancers. In human HCC827 lung cancer cells, the function of claudin-7 is studied because it regulates cell adhesion, migration, and invasion. (Kim DH, et al. 2019) Additionally, it has been shown that there are other Claudins with abnormal expression. These include Claudin-18, which is involved in gastric, pancreatic, and biliary cancers, and Claudin-1, which is involved in colorectal, neck, and squamous malignancies.(Babkair H, et al 2016 and Bhat AA, et al 2020) Furthermore, if claudin's potential barrier function is disrupted, it can lead to a number of intestinal diseases.(Barmeyer C, et al 2015)

A study that used immunostaining to show the expression of claudins 3, 4, and 5 in the human stomach found that corpus epithelial cells expressed these claudins at higher levels. In another study, immunostaining analysis of human tissues revealed that claudin 3 or 4 expression was either non-existent or very weak in the normal stomach mucosa.

While claudin 1 was expressed in approximately 50% of the normal stomach tissues next to the gastric tumors, claudin 3 was expressed in approximately 24% of the tissues, and claudin 4 was expressed in approximately 15% of the tissues [Zhu JL, et al 2013 and Cunningham SC, et al 2006] to as much as 40%–50% of the tissues [Ohtani S, et al 2009, Wang H, et al 2015 and Lee LY, et al 2008].The transmembrane protein claudin-4 is a crucial part of tight junctions. An increasing amount of experimental research has shown that claudin-4 is involved in the development of intestinal metaplasia, the epithelial to mesenchymal transition, and gastric cancer.

Furthermore, claudin-4 controls apoptosis, migration, invasion, and proliferation of cells. Claudin-4 is helpful in the classification of gastric cancer and may be a possible biomarker for the prognosis of patients with the disease. (Liu Wei et al, 2020).

Therefore, the present study used RT PCR, specifically targeting the claudin 3 and 4 gene, to examine the changes in the gastric epithelium and the consequent expression of claudin 3 and claudin 4 in numerous gastrointestinal illness categories.

Methodology

The present study was carried out during the course of three years, from 2019 to 2022 at Centre for Liver Research and Diagnosis laboratories (CLRD) and Department of Gastroenterology, Deccan college of Medical Sciences and Allied Hospitals, Hyderabad, India. Study as approved by Institutional Ethics Committee, Deccan College of Medical Sciences.

All patients aged between 16-60 yrs were recruited in study. All patients provided informed consent. Demographic information was collected by providing questionnaire about their occupation, dietary habits, smoking and alcohol consumption patterns, and place of origin. The clinical history was also documented, including the frequency and duration of heartburn, nausea, vomiting, and abdominal discomfort, as well as any prior medical history.

The study excluded subjects who were younger than 16 years old, receiving antibiotic medication, and who were taking H2 receptor blockers, proton pump inhibitors, or NSAIDs before their endoscopy. Other types of cancers (apart from gastric cancer), participants with HIV, HCV, or HBV infections, co-morbid illnesses, or active or latent tuberculosis infections were excluded from the study.

Study Population

The study was carried our of 250 subjects in total. Among the 250 participants, 240 had H. pylori infection, whereas 10 were used as the control group and did not have H. pylori infection. Out of the total 240 subjects who tested positive for H. pylori, 75 had gastric cancer, which were confirmed endoscopically; 40 had peptic ulcer disease; 45 had duodenal ulcer disease; 50 had gastroesophageal reflux disease (GERD); and 30 had gastritis.

Five categories were created from each patient's endoscopic findings:

1. Gastric Adenocarcinoma,
2. Peptic Ulcer Disease,
3. Duodenal Ulcer,

4. GERD
5. Gastritis

Gastric biopsy collection and Processing

Total 250 recruited patients have undergone upper gastrointestinal endoscopic procedure and gastric biopsies were collected in phosphate buffered saline (PBS) using sterile forceps and all essential procedures to ensure aseptic collection of the sample.

RNA Isolation:

With the use of guanidium isothiocyanate (GITC, Catalog #: 50983, Sigma), total RNA was isolated from tissue samples. For further use, the entire freshly isolated RNA samples were transformed to cDNA.

Complementary DNA (cDNA) synthesis

Using a reverse transcriptase procedure, the extracted RNA was first converted into cDNA. To summarize, a conventional methodology was followed to convert cDNA: 10 minutes of incubation with Oligo dT at 65°C using a thermal cycler, followed by 2 minutes of snap cooling of the reaction. One microgram of RNA from each grade of sample was used for this purpose. Subsequently, a reaction mixture containing 1 unit of MMLV reverse transcriptase enzyme, 1X reaction buffer including DTT, and 10 mM dNTPs was added. The reaction mixture was then incubated at 42°C for 45 minutes and 72°C for 10 minutes.

Polymerase Chain Reaction (PCR)

240 H. pylori positive samples and 10 control (h. pylori negative) samples were subjected to PCR using CLDN-3 and CLDN-4 Oligonucleotide primers.

PCR Amplification

Claudin 3 and Claudin 4 RT-qPCR utilizing the SYBR-Green assay Using CLDN-3 (forward primer: 5′-ACCACCACCACCAACACC-3′, reverse primer: 5′-TGAGGT TTTACAGTCC ATGC-3′) and CLDN-4 (forward primer: 5′-GCGTG CAGATAATGACAAGG′, reverse primer: 5′-GGATTTGACGGCTCCTCTAC-3′) and GAPDH (forward primer: 5′-CAAGGTC ATCCATGA CAACTTTG-3′; reverser primer: 5’-GTCCACCACCCTGTTGCTGTAGCTGTAG-3′) primers, RT-qPCR was carried out using a Real-time PCR machine (ABI 7500, Applied Biosystems, USA). A PCR reaction was conducted in triplicate for each sample, individually for Claudin 3, Claudin 4, and GAPDH, using a total of 20 µL of reaction mixture. To ascertain the cycle threshold (Ct) values for GAPDH, Claudin 3, and Claudin 4, the following reaction conditions were established. The 40 cycles of the PCR cycle consisted of the following steps: (1) denaturation at 94°C for 2 minutes; (2) denaturation at 94°C for 30 seconds. (3) 30s of annealing at 56 °C, and 4 30s of extension at 72 °C. Additionally, a primer-dimer and amplicon melting curve was adjusted to diverge by a single step of 10 minutes.

Results

A total of 240 H.pylori positive subjects of various gastrointestinal diseases were graded 1-4. Grade-1, being less severe, 3 being highest and 4 being Gastric carcinoma. These subjects were analysed for gastric epithelial gap junction proteins (claudins).

Table 1:

Gastrointestinal Diseases	Grades
A. GERD B. Gastritis	Grade 1
C. Duodenal Ulcer	Grade 2
D. Peptic Ulcer Disease	Grade 3
E. Gastric Adenocarcinoma	Grade 4

Molecular Analysis of gap junction Proteins (claudins)

1. Relative expression analysis of claudin 3 in combined categories (grade 1-4) of gastrointestinal diseases.

Gene expression analysis of claudin-3 of different grades of gastrointestinal diseases revealed a 25% decrease in the expression of claudin-3 in all the stages of gastrointestinal diseases (combined together) when compared with the control group. (**fig A)**

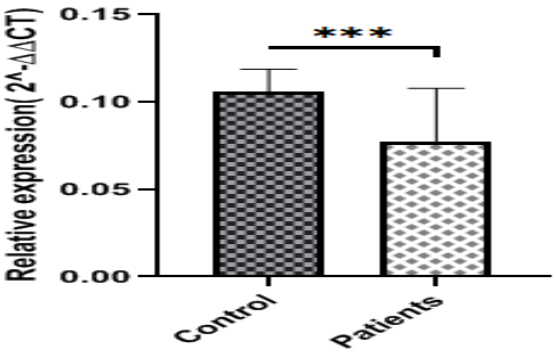

Figure A:

Relative fold change in claudin-3 expression in all the grades of gastrointestinal diseases (combined together) compared with the control group. N = 10 for the control group and 240 for the patients group. Significant differences ($p<0.0001$, Mann-Whitney test) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

2. Relative fold change in claudin-3 expression in different grades of gastrointestinal diseases (grade 1, 2, 3 and 4) when compared with control.

When the claudin-3 expression was compared across different grades with the control group it showcased that in grade 3 and grade 4, claudin-3 expression is 60% and 40% of the control group respectively. Though slight variations in claudin-3 expression were evident in grade 1 and grade 2 when compared with the control group, the effects were not statistically significant (fig B).

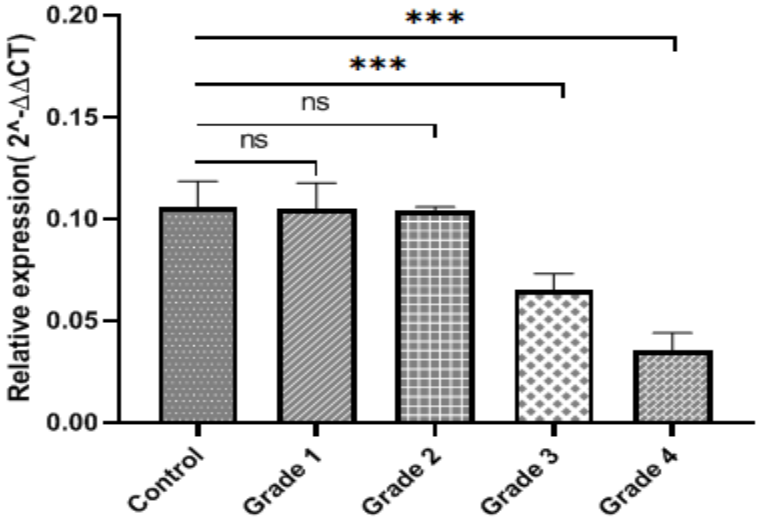

Figure B

N = 10 for each group. Significant differences ($p<0.0001$, One way ANOVA) between the groups is indicated by a (***). Non significance is indicated by the abbreviation'ns'. Values are mean +/- SD.

3. Relative expression analysis of claudin 4 in combined categories (grade 1-4) of gastrointestinal diseases.

In a similar manner, examination of claudin-4 expression profiles of different grades of Gastrointestinal diseases revealed 24% decreased expression of claudin 4 in all the stages of Gastrointestinal diseases (combined together) when compared with the control group(Fig C).

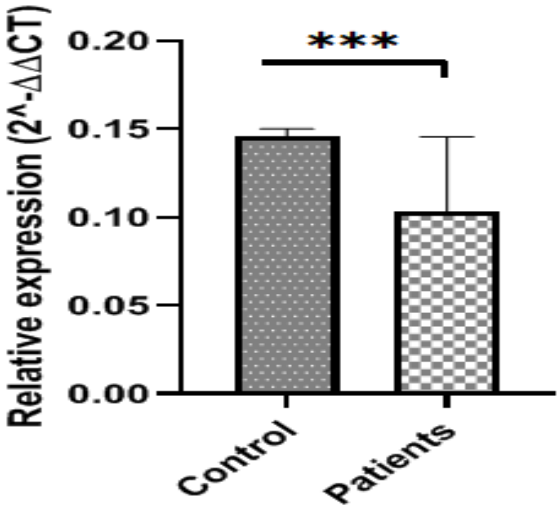

Figure C

(C) Relative fold change in claudin-4 expression in all the types of gastrointestinal diseases (combined together) compared with the control group. N = 10 for the control group and 240 for the patients group. Significant differences ($p<0.0001$, Mann-Whitney test) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

4. Relative fold change in claudin-4 expression in different grades of Gastrointestinal diseases (grade 1, 2, 3 and 4) when compared with control.

When the claudin-4 expression was compared across different grades with the control group, it was manifested that in grade 3 and grade 4 claudin-4 expressions was 65% and 35% of the control group respectively. Despite changes in claudin-4 expression in grade 1 and grade 2 when compared with the control group, the results lack statistical significance (Fig D).

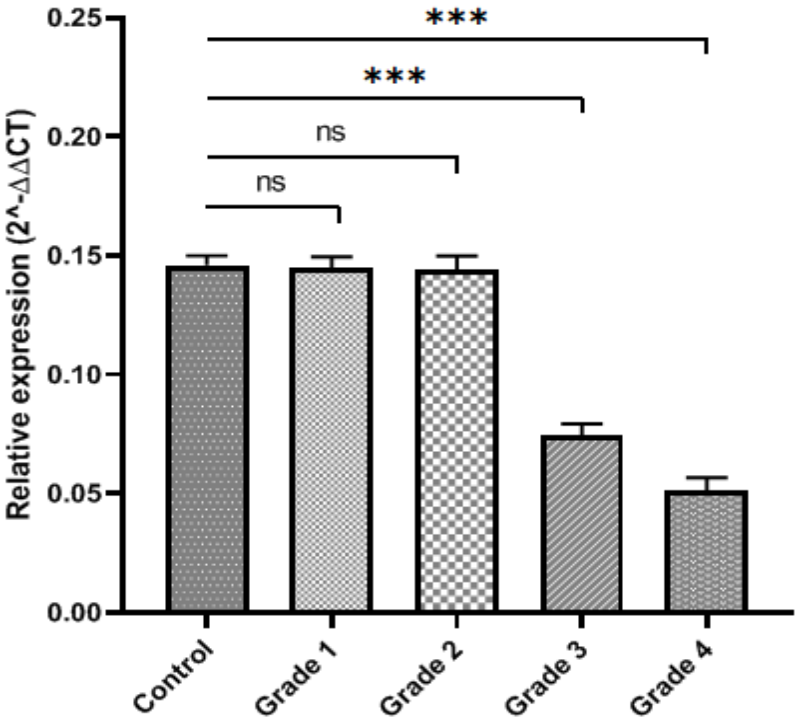

Figure D

Relative fold change in claudin-4 expression in different grades of gastrointestinal diseases (grade 1, 2, 3 and 4) when compared with control. N = 10 for each group. Significant differences ($p<0.0001$, One way ANOVA) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.....

Discussion

Data generated on differential expression of gap junctional proteins shown significant difference when compared to non-infected subjects. Gastrointestinal diseases are classified into several types, based on the severity and the pathology of the disease.

Changes associated with different grades, include alteration in cellular morphology and differences in gene expression of several proteins. Among the myriad of proteins that are altered in gastrointestinal diseases, claudin (claudin 3 and 4 - gap junction proteins) role in pathogenesis. Severity of pathogenesis generally involves a series of steps: (a) Firstly, epithelial cell-cell contacts (tight junctions, adherens junctions, desmosomes and gap junctions, and hemi-desmosomes) are disrupted. (b) Secondly, cells lose their polarity through degradation of Crumbs, partitioning defective (PAR) and Scribble (SCRIB) polarity complexes. (c) Thirdly, expression of epithelial genes is turned off with concomitant activation of mesenchymal/stromal cell genes. (d) Finally, actin architecture is reorganized by forming lamellipodia, filopodia and invadopodia that brings invasiveness and motility to the cells. (Lamouille S, et al. 2014)

Gastric epithelium represents effective first line barriers against pathogens, which is tightly sealed by coordinated regulation of epithelial cell shape, polarity, cell-to-cell and cell-to-matrix adhesions. Concomitantly with colonization of the gastric mucus, H. pylori dismantles the epithelial barrier function to induce inflammatory responses and neoplastic changes dependent on H. pylori virulence factors (Wessler S, et al. 2008).

This might be facilitated by a rearrangement of the actin cytoskeleton as a central mechanism in those processes. Earlier studies has Supported this findings that H. pylori induces the formation of protrusions and massive stress fibers in cultured gastric epithelial cells accompanied by the loss of epithelial morphology and cell-to-cell adhesions leading to a mitogenic- invasive scattering phenotype in vitro (Wessler S, et al. 2008 and Schneider S, et al 2008) further leading to Epithelial-Mesenchymal Transition (EMT).

Claudins are very important gap junctional proteins which play an important role in providing intercellular communication. Caludin 3 and claudin 4 are important gap junctional protein in gastric epithelium. Our generated data of claudin 3 and 4 signifies that as the disease progresses from grade 1 to grade 4, expression of claudin 3 and claudin 4 decreases.

Similar data reported by Pan XY, et al, that down-regulation of claudin-3 and claudin-4 in severe form of gastrointestinal diseases suggesting pathogenic role of claudins in of gastrointestinal diseases. In contrast, Studies have reported localization of claudin-2 and claudin-3 is identical in various diseases and concluded that claudin-2 and claudin-3 do not contribute to the pathogenesis of gastrointestinal diseases. (Hoerscher A, et al. 2020.)

However, these studies are carried out in small sample size. In order to address this limitation we took large sample size (total of 240 h. pylori positive subjects with different grades of gastrointestinal diseases). Moreover till date studies on the role of claudin-3 and claudin-4 on the Indian population have not been evaluated. We investigated the role of claudin-3 and claudin-4 in Indian subjects who are suffering from gastrointestinal diseases by collecting their tissue biopsies. In our study the role of claudin-3 and claudin-4 in different grades of gastrointestinal diseases was investigated by examining their expression profile (m-RNA) using RT-PCR. The expression of claudin-3 and claudin-4 in grades 3 and grade 4 was significantly lower when compared with control. On the other hand, changes in expression of claudin-3 and claudin-4 in grade 1 and grade 2 when compared with control were few and insignificant (Figure 3 and figure 4). Claudin, being an integral component of tight junctions, are indispensable for cell-cell contact/integrity and epithelial cell homeostasis. Down regulation of claudin-3 and claudin-4 disrupts the tight junctions and liberates the cells from the basement membrane. The reduction or absence of claudin-3 and claudin-4 expression appears critical in the invasive phenotype of epithelial cells. This signifies the importance of preservation of tight junctions (TJs) function to avert the damage to epithelial lining.

In our study, expression of Claudins 3 and claudin 4 have shown similar trend 25% and 24% respectively in the severe form of GI diseases (Combined grades of gastrointestinal diseases).

The results of our study also demonstrate that, when claudin-3 expression was examined between grades and the control group, it was 60% and 40% of the control group in grades 3 and 4, respectively. Even though there were minor differences in claudin-3 expression between grades 1 and 2 and the control group, the differences were not statistically significant. Similarly, when the

expression of claudin-4 was examined across grades and the control group, it was observed that the expression of claudin-4 in grades 3 and 4 was, respectively, 65% and 35% of the control group. Although there were alterations in the expression of claudin-4 in grades 1 and 2 in comparison to the control group, the findings did not reach statistical significance. Our study also corroborates with the findings published by several investigators

In all the above three observations, ignoring the extent of statistical significance, we observed gradual transition in the characteristics (morphological changes, changes in percentage of cells expressing mesenchymal markers, and claudin-3 and claudin-4 expression changes) from grade 1 to grade 4 GI diseases when compared with control.

Tracking these progressive changes from grade 1 to grade 4 gastrointestinal diseases might assist us in forecasting the severity of disease, taking preventive measures, and developing biomarkers for prognosis. This study clearly demonstrates infection of H.pylori brings gastric epithelial changes at the level of gap junctional protein complex.

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