

Pyrin Gene Polymorphisms and *H. pylori*-associated Dyspepsia: A Sri Lankan Study

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Abstract: Background/Aim: A considerable high number of dyspeptic patients were reported even with the decreasing prevalence of *H. pylori* in Sri Lanka. Several microbial, host, and environmental factors may associate with the disease outcome. Pyrin secreted by the white blood cells may modulate the inflammatory process by assembling inflammasome complexes in response to pathogen infection. This study focused on the role of pyrin gene polymorphism in gastric mucosal severity and *H. pylori* infection. Materials and Methods: Among the ninety dyspeptic patients three gastric biopsies were taken and the presence of *H. pylori*, yeast species and the gastric mucosal severity was determined. EDTA blood was used for DNA extraction and identification of pyrin gene polymorphism. 12 MEFV gene mutations were tested. Results: Most of the patients (61%) had mild chronic gastritis. Among them 11.1% specimens gave positive bands for NL1/LS2 PCR of yeast DNA. *H. pylori* was positive in 17 patients. No homozygous mutations were found in the MEFV gene. The most common three heterozygous mutations were E148Q (45%), P369S (5%), M680I (11.6%). No significant difference was observed between the presence of the gene polymorphism, gastric mucosal severity or the presence of *H. pylori* and yeast species in the study group. Conclusion: The absence of homozygous mutations in the MEFV gene suggests that it is not a main factor contributing to gastric mucosal severity. The presence of *H. pylori* and yeasts reinforce the concept that stomach is a non-sterile environment.

Keywords: *H. pylori*, Gut Microbiota, Pyrin

1. Introduction

The human stomach is considered a sterile organ. The unfavorable environment for microbial colonization is due to the inherent acidic environment, thick mucus layer, and gastric peristalsis [1]. In 1982, Warren and Barry Marshall discovered *Helicobacter pylori* from the stomach, and it was the first indication challenging the concept of “sterile

stomach” [2]. However, studies suggesting the presence of other microorganisms in the gastric mucosa have emerged recently further challenging this concept [3]. The microbial diversity of the gastric mucosa could be affected by microbial virulence factors, host genetic factors contributing to immunity, inflammation of the gastrointestinal mucosa, and environmental factors [4]. Recent studies have focused on the host immune-regulatory response against the gastric mucosal inflammation caused by *H. pylori*. There has been a

decreasing incidence of *H. pylori* in recent years reported in Sri Lanka [5]. However, there has been no considerable decline in patients seeking treatment for dyspepsia. Endoscopic investigations of these patients often reveal inflammatory changes in their gastric mucosa with varying severity despite the low *H. pylori* prevalence [6]. Thus, it is important to investigate other possible microbial etiologies contributing to gastric inflammation. Nevertheless, it is important to delineate transient flora from possible pathogens that could contribute to gastric pathology. Among the host genetic factors that could influence the gastric microbiota and inflammation are pyrin and pro-inflammatory cytokines [7]. Pyrin in the gastric mucosa modulates the inflammatory process by, assembling inflammasome complexes in response to pathogen infection [8]. IL-1 β is an important pro-inflammatory cytokine component of the inflammasome complex which contributes to pathology [9]. Mutations of the pyrin gene could result in nonfunctional pyrin and thus affect the amount of functional pyrin in the microenvironment. Thus, pyrin cannot perform its role in regulation of inflammatory responses and may lead to an irregular or prolonged inflammatory reaction which may damage the intestinal mucosal layer causing morbidity in patients [10]. Thus, pyrin may have a role in modulation of the inflammatory response resulting in the alteration of the microbial flora together which may contribute to gastric pathology.

2. Materials and Methods

2.1. Study Design and Study Population

This was a comparative cross-sectional study carried out among 90 dyspeptic patients. Patients referred to endoscopy unit for routine endoscopy procedure with any combination of four dyspeptic symptoms (postprandial fullness, early satiety, epigastric pain, and epigastric burning) were selected for the study. The selected patients were over 18 years of age. The patients were not taking antibiotics during the past one month prior to endoscopy. Patients were excluded from the study if they were below 18 years of age, having duodenal ulcers or malignant diseases or on antibiotics. Informed written consent was obtained from participants enrolled in the study. A pre-tested interviewer-administered questionnaire was used for data collection on socio-demographic variables, risk factors, and selected clinical details.

2.2. Specimen Collection

Three antral biopsy specimens were taken from each

dyspeptic patient during endoscopy. One biopsy specimen was inserted to the in-house rapid urease test solution, one was placed in a 10% formalin saline solution for histological examination, the third biopsy was placed in a sterile eppendorf tube for DNA extraction. A 5 ml blood specimen was collected and 2ml was aliquoted into an EDTA tube for DNA extraction and 3 ml was aliquoted to a plain tube for obtaining serum. The blood and serum were separated into cryovials and kept at -80°C.

2.3. Histopathological Investigations

The biopsy sample was subjected to histopathological investigations for the presence of *H. pylori* and histopathology. Biopsies were transported in 10% formalin and dehydrated using an alcohol gradient. Clearing was done using the xylene and tissue was embedded in paraffin wax. Microtome sections were prepared and placed on autoclaved glass slides. Sections were stained separately by hematoxylin and eosin stain and Giemsa stain. Slides were examined by a histopathologist and grouped according to the updated Sydney classification system [11].

2.4. In-house Biopsy Urease Test

One antral biopsy was inserted into an in-house rapid urease test solution. The inoculated tubes were incubated at 37°C for 24 hours. Tubes were observed at different time intervals and a color change from yellow orange to pink red was recorded up to 24 hours [12].

2.5. DNA Extraction and PCR

DNA extraction was done using a commercially available DNA extraction kit (DNeasy Blood & Tissue Kit from QIAGEN). All the steps were performed according to the manufacturer guidelines. Extracted DNA from each biopsy sample was subject to PCR using previously published yeast primers [13] (Table 1). PCR was performed using Flexigene thermal cycler (version 31.04; Techne, Cole-Parmer, Staffordshire, UK). PCR mixture was prepared in a 50 μ l reaction mixture containing 10X PCR buffer (Sigma-Aldrich, St Louis, MO, USA), 0.2mM each of dATP, dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 0.2 mM of forward and reverse primer, 1.25U of Taq polymerase (Sigma-Aldrich) and 2 μ l (100 ng) of nucleic acid. Agarose gel electrophoresis of PCR products using a 1.5% agarose gel was done and products were visualized under a UV trans-illuminator (Quantum ST4; VilberLourmat, Marne-la-Vallee, France).

Table 1. The primer sequence used for the study.

| Target gene | Primer | Primer sequence 5' to 3' | Product size | PCR reaction condition |
|--------------------------------|--------------------------------|---|--------------|--|
| D1 region of 26S rDNA of yeast | NL1 (forward) LS2 (reverse) | GCC ATA TCA ATA AGC GGA GGA AAA G ATT CCC AAA CAA CTC GAC TC | ~ 250bp | 94°C for 1 min, 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, 72°C for 7 min (30 cycles) [13] |

2.6. ELISA Assays for Human IL-1 β and TNF α in Serum

Human IL-1 β and TNF α concentrations in human serum were determined using an ELISA assay (Human IL-1 β - Mabtech, AB Sweden and TNF α - TNF- α ELISA development kit) as stated in the kit manual. While coating the ELISA plate two blank wells were kept empty. Remaining cells was coated with respective antibody diluted with PBS (2 μ g/ml in Phosphate Buffered Saline) and plate was incubated at 4°–8°C overnight. The serum specimens and the plate were kept on the bench to bring them to room temperature (20°–25°C) and the coated plate was washed with PBS. Blocking solution (PBS with 0.05% Tween 20 with 0.1% BSA) was added to each well (except blanks) and kept for one hour at room temperature for incubation. The plate was then washed with washing buffer (PBS with 0.05% Tween) followed by addition of standards and patient serum in duplicate. After a 2-hour incubation, plates were washed well, and secondary antibody was added to each well. Plates were incubated for 1 hour at room temperature. The plate was washed and Streptavidin-HRP solution was added. Then plate was kept at room temperature for another 1 hour for incubation. After washing, TMB substrate was added and permitted to incubate for 45 minutes. The reaction was stopped by addition of stop solution and plate was read in an ELISA plate reader at an absorbance of 450 nm. Absorbance values obtained for the standards were plotted using Graph pad prism software (version 7.04) to prepare the standard curve. Serum cytokine expression was determined by interpolation of the standard curve.

2.7. Detection of MEFV Gene Polymorphism

MEFV gene mutations were identified using a commercially available FMF strip assay kit based on DNA isolation, polymerase chain reaction (PCR), and reverse-hybridization. All steps were carried out according to the manufacturer instructions provided by the kit. EDTA blood specimen was used to extract the DNA and PCR amplification was done using biotinylated primers. Allele-specific oligonucleotide probes are immobilized into the test strips as an array of parallel lines. Amplified PCR products were hybridized into the test strip and bound biotinylated sequences were distinguished using streptavidin-alkaline phosphatase and color substrates. The assay covered 12 mutations in the MEFV gene: E148Q, P369S, F479L, M680I (G/C), M680I (G/A), I692del, M694V, M694I, K695R, V726A, A744S, R761H. The genotype was identified using the enclosed collector TM sheet provided by the kit. For each position out of 12 lines a colored line taken as a positive result. Control line should always stain positive for interpretation.

Ethics:

Ethical approval for the study was obtained from the Ethics Review Committee University of Sri Jayewardenepura (14/19) and the Colombo South Teaching Hospital, Kalubowila.

3. Results

3.1. Demographic Data

The current study consisted of 90 patients with dyspeptic symptoms aged between 18-84 years with a median age of 53 years. Forty-six were male and 44 were female. According to the family dimension analysis 58 of them had three to five members in their family, 27 had less than three family members while 5 of them had more than five family members in their family. The patients complained of abdominal pain (95.5%), belching (30%), abdominal rumbling (10%), nausea (63.3%), and emesis (13.3%).

On analysis of the medication taken by the patients at the time of presentation 58 (63%) the patients were taking antacids while 20 (22%) were not on any medication, 10 (11%) were taking NSAIDs and 3 (3.3%) patients were taking other medications such as nutritional supplements, vitamins, and hormones.

3.2. Laboratory Diagnosis of *H. pylori* Infection by Histology and In-house Biopsy Urease (IBUT) Test

Five patients out of 90 were positive for *Helicobacter pylori* by histology and 13 patients were positive for *H. pylori* by the IBUT test. Out of the 13 IBUT positive specimens one developed the color change within 10 minutes of incubation while 4 in 1-4 hours, 3 in 4-6 hours, and 5 in 10-12 hours. Histology was considered as the gold standard method for the diagnosis of *H. pylori* infection. However, when histopathological severity was reported according to modified Sydney classification system, 55 (61% patients were diagnosed with mild chronic gastritis, 25 (28%) had active chronic gastritis with moderate severity and 9 (10%) patients had a normal gastric pathology. Among the study population 18.8% were positive for *Helicobacter pylori* either by IBUT or histopathology.

3.3. Endoscopic Examination Findings

Endoscopic findings suggest that majority of the patients had mild antral gastritis (54.4%), while 15.5% had severe antral gastritis, 14.4% had moderate antral gastritis. Pan gastritis was seen in 10% of patients while 5.5% had normal gastric pathology.

3.4. Molecular Biology Investigations for Determination of Yeast DNA in Biopsy Specimens

Out of 90 specimens 10 specimens gave positive bands for NL1/LS2 PCR for amplification of yeast DNA, suggesting the presence of yeasts in the gastric mucosa.

3.5. Expression of IL-1 β and TNF- α

Human serum IL-1 β and TNF- α expression was analyzed among 90 dyspeptic patients in this population. IL-1 β mean concentration was 2.78 pg/ml and TNF- α mean concentration was 6.36 pg/ml. The mean IL-1 β concentration of *H. pylori* positive group was 2.3 pg/ml and *H. pylori* negative group

had a mean IL-1 β concentration of 3.0 pg/ml. The mean TNF- α concentration of *H. pylori* positive group and *H. pylori* negative group were 5.86 pg/ml and 6.48 pg/ml respectively. There was no statistical difference observed between cytokine expression and the presence of *H. pylori*.

Mean IL-1 β concentration of yeast PCR positive (10) and yeast PCR negative (80) groups were 3.0 pg/ml and 2.7 pg/ml respectively. The mean concentration of serum TNF- α levels of the yeast PCR positive group was 3.3 pg/ml and the yeast PCR negative group was 6.7 pg/ml. No statistical significance was observed between serum cytokine concentrations and the presence of yeast species ($P>0.05$)

Human serum IL-1 β and TNF α cytokine concentrations were measured, and mean concentrations were analyzed along with the histopathological severity of the gastric mucosa (Table 2). Mean IL-1 β concentrations were slightly increased with the severity of the gastric mucosa. Mean IL-1 β concentration of patients with active chronic gastritis with moderate severity had 2.9 pg/ml, Mild chronic antral gastritis

had 2.85 pg/ml and patients with normal gastritis had 2.17 pg/ml. There was no statistically significant difference between IL-1 β concentrations and gastric mucosal severity of these dyspeptic patients.

Mean concentrations of human IL-1 β and TNF α cytokines were analyzed with endoscopic findings (Table 3). According to the endoscopic evaluation, a group of patients with severe antral gastritis had the highest mean concentrations of both IL-1 β (3.7 pg/ml) and TNF α (8.8 pg/ml). The mean IL-1 β concentrations observed for pan gastritis, moderate antral gastritis, mild antral gastritis, and normal gastric pathology were 3.02 pg/ml, 2.2 pg/ml, 2.59 pg/ml, and 2.91 pg/ml respectively. The mean concentration of TNF α of patients with pan gastritis was 6.4 pg/ml, moderate antral gastritis was 5.7 pg/ml, mild antral gastritis was 5.9 pg/ml and patients with normal gastric pathology had 4.9 pg/ml. The expression of IL-1 β and TNF α did not have a significant statistical association with endoscopic findings.

Table 2. Mean concentrations of IL-1 β and TNF α according to the histopathological severity.

| Histopathological findings | Mean IL-1 β concentration | Mean TNF α concentration |
|---|---------------------------------|---------------------------------|
| Normal | 2.17 pg/ml | 7.17 pg/ml |
| Mild chronic antral gastritis | 2.85 pg/ml | 6.83 pg/ml |
| Active chronic gastritis with moderate severity | 2.9 pg/ml | 5.11 pg/ml |

Table 3. Mean IL-1 β and TNF α concentration along with the endoscopic findings.

| Endoscopic findings | Mean IL-1 β concentration | Mean TNF α concentration |
|---------------------------|---------------------------------|---------------------------------|
| Normal | 2.91 pg/ml | 4.9 pg/ml |
| Mild antral gastritis | 2.59 pg/ml | 5.9 pg/ml |
| Moderate antral gastritis | 2.2 pg/ml | 5.7 pg/ml |
| Severe antral gastritis | 3.7 pg/ml | 8.8 pg/ml |
| Pan gastritis | 3.02 pg/ml | 6.4 pg/ml |

3.6. MEFV Gene Polymorphism

Twelve MEFV gene mutations were tested in 60 patients. None of the individuals were positive for homozygous mutations. The most common three heterozygous mutations within the population were E148Q (45%), P369S (5%), M680I (11.6%). No significant difference was observed

between presence of *H. pylori*, yeasts species and the gene polymorphism (E148Q, P369S, M680I) in this population. MEFV gene mutations were compared with endoscopic findings and histopathological severity of gastric mucosa (Tables 4 and 5). In both categories patients with mild antral gastritis had the highest number of patients with any mutations of E148Q, P369S or M680I.

Table 4. MEFV gene polymorphism and histopathological severity.

| Histopathology | No mutations | Any mutations |
|---|--------------|---------------|
| Normal | 2 | 6 |
| Mild chronic antral gastritis | 19 | 18 |
| Active chronic gastritis with moderate severity | 5 | 10 |

Table 5. MEFV gene polymorphism and endoscopic findings.

| Endoscopic findings | No mutations | Any mutations |
|---------------------------|--------------|---------------|
| Normal | 0 | 3 |
| Mild antral gastritis | 15 | 22 |
| Moderate antral gastritis | 6 | 3 |
| Severe antral gastritis | 2 | 4 |
| Pan gastritis | 3 | 2 |

Human IL-1 β and TNF α cytokines expression were analyzed with MEFV gene polymorphism. Patients with any of mutations within E148Q, P369S, or M680I genes had a mean IL-1 β concentration of 2.74 pg/ml and TNF α concentration of

5.1 pg/ml. Patients without any mutations in E148Q, P369S or M680I genes the mean cytokine concentrations were observed for IL-1 β and TNF α were 3.48 pg/ml and 5.0 pg/ml respectively. There was no statistically significant association

observed in MEFV gene polymorphism and cytokines expression among these patients.

Among patients with yeasts PCR positive (10) by NL1/LS2 PCR, 6 of them were identified with active chronic

gastritis with moderate severity while 4 presented with mild chronic gastritis (Table 6). There was a statistical significance observed in yeast positivity among these three groups ($P=0.059$).

Table 6. Yeast positivity and histopathological severity.

| Histopathology | Yeast positive | Yeast Negative |
|---|----------------|----------------|
| Normal | 0 | 8 |
| Mild chronic antral gastritis | 4 | 52 |
| Active chronic gastritis with moderate severity | 6 | 20 |

Among the *H. pylori*-positive patients (17), 9 patients were diagnosed with active chronic gastritis with moderate severity while 7 patients had mild chronic gastritis and only one patient had normal gastric pathology. There was a statistical significance between *H. pylori* positivity among these three groups ($P=0.05$).

4. Discussion

The human stomach was considered as an unfavorable environment for microbes due to its acidic conditions and other antimicrobial factors. The detection of *H. pylori* and other microorganisms provided ample evidence for the concept of a non-sterile stomach [14]. Research has been directed to understand the mechanisms by which these organisms adapt to and survive in a hostile environment. Cutting edge molecular investigations have proved to be the turning point in understanding the microbiota of previously thought to be a sterile site in the human body. Despite the potential importance of a gastric microbial community in human health and disease, few data on the complexity of this biota have been available especially in the stomach.

This is the first study conducted in Sri Lanka to report the presence yeast in the gastric mucosa. Based on recent literature *Candida albicans* is also known to be responsible for chronic gastritis and peptic ulcer disease [15]. *Candida albicans* poses an array of virulent factors. As a result, the organism has great flexibility and adaptability to different host environments. *Candida* species are oral commensal in 20-40% of healthy people [16]. Further studies have found oral *Candida* prevalence is significantly high in the dyspeptic group compared to the healthy group [17]. *C. albicans* can grow well in low pH even at pH 2. Oral *Candida* colonizers might be the origin of these gastric mucosal strains; however, this strain relationship needs to be further investigated.

Despite the low proportion of *H. pylori* in the local community dyspeptic symptoms are a frequent complaint. Therefore, the possible role of other etiological agents and host genetic factors may contribute to this gastric pathology leading to dyspepsia. It is possible that in this population the presence of yeast may be accountable for the gastric pathology and justify further investigation.

Few studies have investigated the association of pyrin gene polymorphism and gastric pathological changes. A study conducted in Turkey has revealed that most of the MEFV gene mutated patients presented with dyspeptic symptoms and identified M694V mutation (valine for methionine at

position 694 in MEFV gene) as the most common in this population [18].

In the present study there were only heterozygous mutations which suggest that MEFV gene mutation may not be an important determinant contributing to the gastric pathology. Thus, the absence of a significant association between *H. pylori* and yeast species positivity and the presence of heterologous gene polymorphism (E148Q, P369S, M6801) is acceptable. In this study, most of the patients with or without any mutations (E148Q, P369S, M6801) had mild chronic gastritis. These findings suggest that the presence of any heterogeneous mutations (E148Q, P369S, M6801) had no association with the gastric pathology. Another study by Agin et al., 2018 has concluded similarly that MEFV gene polymorphism was not strongly associated with histopathological severity with the evidence showing 50% had antral gastritis while 43% of patients had normal gastric pathology in this study [18].

An association between yeast positivity and histopathological severity of the gastric mucosa was observed ($P=0.059$). The majority ($n=6$) of yeast PCR positive patients had active chronic gastritis with moderate severity suggesting that yeast species may have a role in gastric pathology in these patients. This finding is further supported by Pereira, V et al., 2018 who reported that *Candida* spp. were found in the gastric mucosa in 8.9% of dyspeptic patients. *C. albicans* can actively alter the pH of its environment and induce its switch to the hyphal form⁽⁶⁾. The change in pH is caused by the release of ammonia from the cells produced during the breakdown of amino acids. Therefore, the low pH assumed to be not a barrier to survival and pathogenic action of fungal species [19].

TNF- α is a pro-inflammatory cytokine associated with the immune response and is produced by macrophages, neutrophils, NK cells, B cells, and T cells. TNF- α protein mediates the immune response by binding to TNF- α receptors (TNFRs) and activating NF- κ B signaling, resulting in T-cell proliferation and pro-inflammatory responses. According to the recent study in 2018, between *H. pylori*-positive and negative groups in Sri Lanka; showed that there was no significant difference in serum TNF- α expression among these two groups [18]. Similarly, the current study identified that there was no statistically significant difference between *H. pylori* positivity or the yeast positivity with serum pro-inflammatory cytokines (IL-1 β , TNF α).

The IL-1 β has been broadly reported to play an important role in *H. pylori* infection and associated gastric pathology,

but little is known about the mechanisms triggering its expression and release from immune cells upon infection [20]. IL-1 β and TNF α have a role in contributing to gastric pathology and gastric inflammation. Contradictory to the expectation, the mean IL-1 β and TNF α expressions were low in this dyspeptic population despite the presence of gastric mucosal inflammation. In this study group expression of IL-1 β did not increase with the severity of gastric mucosa. However, when compared to previous studies the overall TNF- α and Interleukin 1 β expression was low. This observation may be due to the PPI medication (Eg: Omeprazole) taken by many patients (64%) such as Lansoprazole, Omeprazole, etc. has strong acid suppression effect and anti-inflammatory effect. PPI can inhibit the activation of NF-kB and significantly reduce the production of IL-1 β and TNF- α [21].

5. Conclusion

Study demonstrated the presence of heterozygous mutations in E148Q, P369S, M680I and absence of homozygous mutations in the study population. Thus, the MEFV gene mutations does not play a role as a main factor in contributing to gastric mucosal severity. The presence of *H. pylori* and yeasts were observed in these patients. These findings suggest the stomach is not a sterile environment. Further identification should be done to identify the yeasts species. The expression of serum cytokines IL-1 β and TNF α were low in this study population which may be attributed to PPI medications. There was no direct association found between serum pro-inflammatory cytokines and the presence of *H. pylori* and yeast species.

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