Indian Journal of Science and Technology, Vol 9(2), DOI: 10.17485/ijst/2016/v9i2/78512 January 2016
ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

In Situ Nick End Labeling as a Molecular Immunopathological Indicator for the Severity of DNA Fragmentation and Gastroduodenal Tissue Damage among H. Pylori Cag A Positive Patients

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Abstract

Background and Objective: The aim is to determine the correlation between gastric Apoptotic Index (AI), inflammatory cells AI; H. pylori CagA; Gastric secretions; and other proposed factors that may induce apoptosis and subsequently induce different gastric lesions. **Methodology:** Gastroduodenal biopsies taken from 80 patients for histopathology and H. pylori diagnosis. Cag A and DNA fragmentation detected by in situ hybridization Serum samples used for evaluation of Serum pepsinogen I (PGI); II (PGII; Gastrin-17 (G-17) . **Findings:** Significant difference between gastric tissue (AI), inflammatory cells; inflammatory cells versus gastric tissue AI; CagA positivity among gastric disorders (p value = 0.022). Cag A correlated with gastric AI and inflammatory cells AI. Significant difference in Inflammatory Cells AI among Cag Apositive versus CagA negative cases (p value = 0.002). Significant difference in AI between gastric tissue and Inflammatory Cells among Cag A positive (p value = 0.000) with inverse correlation (p value = 0.014). Gastric AI and Inflammatory cells AI Inversely correlated with PGI/PGII level and CagA positivity. Marginal inverse correlation between gastric AI, Gastrin17 level and CagA positivity was reported (p value = 0.056). Inflammatory cells AI correlated with PMNs grade and CagA positivity (p = 0.044). Gastric cells AI correlated with PMNs grade (p = 0.001). Gastric AI and inflammatory cells AI correlated with age. Gastric AI and inflammatory cells AI in-versely correlated with gender. Significant difference in gastric AI; inflammatory cells AI and usage of PPIs; NSAID usage; smoking, drinking of tap water. Marginal inverse correlation between NSAID usage and gastric AI (p = 0.000), inflammatory cells AI (p = 0.001). **Conclusion:** Cag A correlated with AI among different gastro duodenal nonmalignant disorders. Inverse correlation between AI and PGI/PGII level; gastric AI and Gastrin17 level. AI correlated with PMNs grade; age and Cag A positivity. AI not correlated with PPIs, smoking, tap water. Inflammatory cells AI Inversely correlated with Gender.

Keywords: CagA, DNA Fragmentation, Gastric Disorders, H. pylori

1. Introduction

Gastroduodenal disorders associated with H. pylori infection are worldwide in occurrence. H. pylori infection responsible chronic (Type B) gastritis, which has been followed by atrophic gastritis, intestinal metaplasia in small group of patients while the majority are asymptomatic[1]. About 30% to 60% of duodenal ulcers and 70% of gastric ulcers are associated with H. pylori infection that can eventually lead to gastric adenoma and lymphoma[2,3]. However, the mechanisms leading from chronic active gastritis to other disease manifestations remain unclear. The usage of appropriate diagnostic method, and treatment strategies leading to clinical and histopathological improvement for patients[4].

Various bacterial and host factors are responsible for

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the development of disease after H. pylori infection. One of the most important bacterial factors was the Cytotoxin associated gene (Cag) bearing Pathogenicity Island (PAI)[5,6], which is associated with different pathological processes such as increase cell proliferation, increase motility, apoptosis and morphological change through different mechanisms[2]; increase gastric inflammation[5]. Increased gastric epithelial cytokines as a result of infection with Cag A positive H. pylori strains cause recruitment and activation of inflammatory and immune cells[7]. Cag A positive H. pylori strains activates transcription factors in gastric tissue ,such as nuclear factor KB[8] causing induction and increase in gastric cytokine production such as IL8 that cause recruitment and activation of neutrophils and macrophages that produce a large quantities of Reactive Oxygen (ROS) at the site of inflammation produced as a part of defense against this pathogen[8]. ROS also produced by H. pylori itself[9], proinflammatory cytokines. H. pylori associated oxidative stress cause increased expression of spermine oxidase[10], Which oxidizes polyamines that are abundant in epithelial cells to release hydrogen peroxide, suggesting another mechanism by which H. pylori induces oxidative stress[11].

Bacterial products and ROS, as well as reactive nitrogen species produce at the site of infection can leads to DNA damage which may extend to carcinogenesis duo to mutations or progress to initiates intracellular program of DNA fragmentation that generates a multitude of DNA Double-Strand Breaks (DSBs) with accessible 3'-hydroxyl (3'-OH) groups. This characteristic forms the basis for a well-established apoptosis detection method: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay[12].

The aim of the present study is to determine the correlation between gastric Apoptotic Index (AI) determined by *in situ* for DNA fragmentation, inflammatory cells AI; H. pylori CagA; Gastric secretions; and other proposed factors that may induce apoptosis and subsequently induce different gastric disorders.

2. Materials and Methods

2.1 Patients

In this cross sectional study, (80) patients, age range 16-80 years, mean (47.24±2.10) years, with clinical indications for upper gastrointestinal tract endoscopy during June 2013 to January 2015 were studied. Males represent 44 (55%) versus 36 (45%) females. This study conducted according to the principles of Helsinki declaration. Before endoscopy dully-filled consent, form obtained from all patients that agree to participate in the study. Approval of ethical review Committee of College of medicine –Diyala University-Iraq, taken prior to initiation of the work at gastroenterology department of Baqubah teaching hospital in Diyala province-Iraq. Any patient under antibiotics or colloidal bismuth compounds for past one month treatment; having a history of previous gastric surgery and recent or active gastrointestinal bleeding excluded from this study.

2.2 Methods

After topical pharyngeal anesthesia for overnight fasted Patients, a sterile flexible endoscope was introduced for full investigation of Stomach and duodenum. Six biopsy samples from congested, inflamed or erosive lesions were picked via sterile biopsy forceps. Samples were placed in Serim® PylTek® Test Kit for detection of urease activity. Each PyloriTek strip has a built-in positive analyte control and a negative control, which run concurrently with the test specimen. The PyloriTek positive control automatically appears with every test within the normal 1-hour time. With competitive tests the positive control is run after waiting 24 hours then inserting a urease positive control material[13].

H. pylori Cag A gene expression detected by insitu hybridization procedure in 5µm thickness serial gastric mucosal sections fixed on positively charged slides using biotinylated long DNA probe for H. pylori/Cag A gene, Cat. No. IH-60061(HPY-6001-B) (Maxim biotech-USA) and the DNA Probe hybridization/Detection System – *In Situ* Kit-IH-60001 (Maxim biotech-USA), according to Maxim biotech instruction manual[14]. The examination and scoring were done under light microscope by pathologists at power X400 according to the scoring system[15].

Infiltrated lymphocytes score as following: 0: none, mild 10 cells/hpf, moderate10 cell/hpf to diffuse infiltration with dense chronic inflammatory cells, marked: Nearly whole gastric mucosa contains dense chronic inflammatory cells. Polymorphonuclear cells scored as following: None (Grade 0), Rare PMNs; 0-1 Intraepithelial (IE) PMN/hpf, Grade (3): 1-10 intraepithelial (IE) PMN/(hpf), grade(4): ≥10 IE PMN/hpf , grade (5): Pit abscess[8]

Gastric cells, apoptosis was detected using the DeadEnd™ Colorimetric TUNEL System[16], which is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells in situ at the single-cell level. The system can be used to assay apoptotic cell death in tissue sections by measuring nuclear DNA fragmentation, an important biochemical indicator of apoptosis in many cell types. Principally a biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, Diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown. To detect apoptotic cells in the gastric epithelium, using the Terminal Deoxynucleotide Nick-End Labelling (TUNEL) assay technique, the present study depends on[17] method in which at least 300 cells were counted in each tissue section and the number of Tunel positive apoptotic cells per 100 cells was expressed as apoptotic index in percent.

For serological assay blood was drawn from each patient during the visit to the endoscopy unit. Separated serum samples were stored at 27˚C until analyses .Serum Pepsinogen I (PGI) and II (PGII) and gastrin-17 (G-17) were assayed with ELISA using monoclonal antibodies to Pepsinogen I and II and gastrin-17 (BIOHIT Diagnostics, Biohit, Devon, UK). All procedures were carried out according to the manufacturer's instructions and results of pepsinogen I and II reported in µg/l and pmol/l for gastrin-17. The pepsinogen I: II ratio was calculated and reported in fraction[18].

2.3 Statistical Analysis

Frequency of variables express as percentage. PG I, II and G-17 values express as mean±standard deviation (Mean±SD). Pearson test for correlation was used for non-categorical data. Chi-test used to compare the PG I , PGII, and G17 according to CagA gene expression. The level of significance was 0.05 (two-tail) in all statistical testing; significant of correlations (Pearson, spearman) include also 0.01 (two-tail). Statistical analysis was performed using SPSS for windows TM version 17.0, and Microsoft EXCEL for windows 2010.

3. Results

As shown in Table 1, Figure 1, the frequency of gastric disorders according to endoscopy as following: gastric ulcers (18.75%), Gastropathy (15%), (11.25%); DU (8.75%); duodenitis and prepyloric ulcers (2.5%).

Table 1. Correlation between Gastric versus inflammatory cells apoptotic Index and gastric disorders

Parameters	Gastric Apoptotic Index		inflammatory Cells Apoptotic Index		χ2	P value	R	P value
	Cag A+	Cag A-	Cag A+	Cag A-				
Gastric ulcer	15 (18.75%) 0.6600±0.07493	0(0%)	15(18.75%) 0.2027±.06933	0(0%)				
DU	7(8.75%) 0.6540± .08849	5(6.25%) 0.48± 0.05477	7(8.75%) 0.2475± .06994	5(6.25%) 0.2960± .00548				
Gastropathy	12(15%) 0.6400± .05642	3(3.75%) 0.6833±0.7638	12(15%) 0.2667 ± .08797	3(3.75%) 0.2633 ± .07767	541.126	0.000	-0.192	0.089
Gastritis	9(11.25%) 0.7343 ± .02370	21(26.25%) 2.4495± 5.83588	9(11.25%) 0.3033± .09260	21(26.25%) 0.2486 ± .07812				
Duodenitis	2(2.5%) 0.6300± 0.00000	4(5%) 0.6175 ±0.06652	2(2.5%) 0.2200±0.000	4(5%) 0.2750 ± .05802				
prepyloric ulcer	2(2.5%) 0.8500±0.000	0(0%)	2(2.5%) 0.1500±0.000	0(0%)				
χ^2	122.604	35.879	123.287	27.584				
P value	0.000	0.118	0.000	0.152				
R	0.334	0.180	0.195 0.289*	-0.158				
P value	0.022	0.317	0.188 0.049*	0. 380				

*spearman correlation

Gastric cells apoptotic index among CagA positive gastric disorders was higher among prepyloric ulcers (0.8500±0.000), gastritis (0.7343±.02370), Gastric ulcer (0.6600±0.07493); DU (0.6540±.08849); gastropathy (0.6400±.05642), duodenitis (0.6300±0.000). Significant difference in (AI) among gastric disorders (p value = 0.000) (Figure 2). Significant correlation between Cag A and AI was determined (p value = 0.022). Neither significant difference nor correlation was determined in (AI) among CagA negative gastric disorders (p value = 0.118; 0.317). Inflammatory Cells apoptotic index among CagA positive gastric disorders was higher among Gastritis (0.3033±.09260), Gastropathy (0.2667±.08797); DU (0.2475±.06994); Duodenitis (0.2200±0.000); Gastric ulcer (0.2027±.06933), prepyloric ulcer (0.1500±0.000). Significant difference in (AI) among gastric disorders in inflammatory cells AI (p value = 0.000). Significant correlation between Cag A and inflammatory cells AI was determined (p value = 0.049). Significant difference in (AI) among inflammatory cells Versus gastric tissue AI regardless Cag A insitu positivity (p value = 0.000) without significant correlation between gastric and inflammatory cells AI (p value = 0.089) .

As shown in Table 2, regardless of the type of gastric disorder, Gastric AI (2.99±8.98) among Cag A positive versus (1.77±4.70) among Cag A negative with significant difference (p value = 0.001). Significant correlation between gastric tissue AI and insitu expression of CagA (p value = 0.005). Significant difference in Inflammatory Cells AI among Cag Apositive versus CagA negative cases (p value = 0.002). No Significant correlation between Inflammatory Cells AI and insitu expression of CagA (p value = 0.537). Significant difference in AI between gastric tissue and Inflammatory Cells among Cag A positive (p value = 0.000) with inverse correlation (p value = 0.014).

As shown in Table 3, significant difference in inflammatory cells AI among CagA negative, CagA positive cases , CagA positive versus CagA negative cases (p = 0.000) according to PMNs grade. Significant correlation between inflammatory cells AI, PMNs grade and CagA positivity (p = 0.044). Marginal correlation between inflammatory cells AI, PMNs grade (p = 0.057).

Significant difference in gastric cells AI among CagA negative, CagA positive cases, CagA positive versus CagA negative cases (p = 0.000) according to PMNs grade. Significant correlation between gastric cells AI and PMNs grade (p = 0.001).

Significant difference in inflammatory cells AI among CagA negative, CagA positive cases, CagA positive versus CagA negative cases (p = 0.010,0.001;0.019) according to Lymphocytes grade without significant correlation. Significant difference in gastric cells AI among CagA negative, CagA positive cases, CagA positive versus CagA negative cases (p = 0.000,0.015) according to PMNs grade without significant correlation.

As shown in Table 4, gastric AI among CagA positive

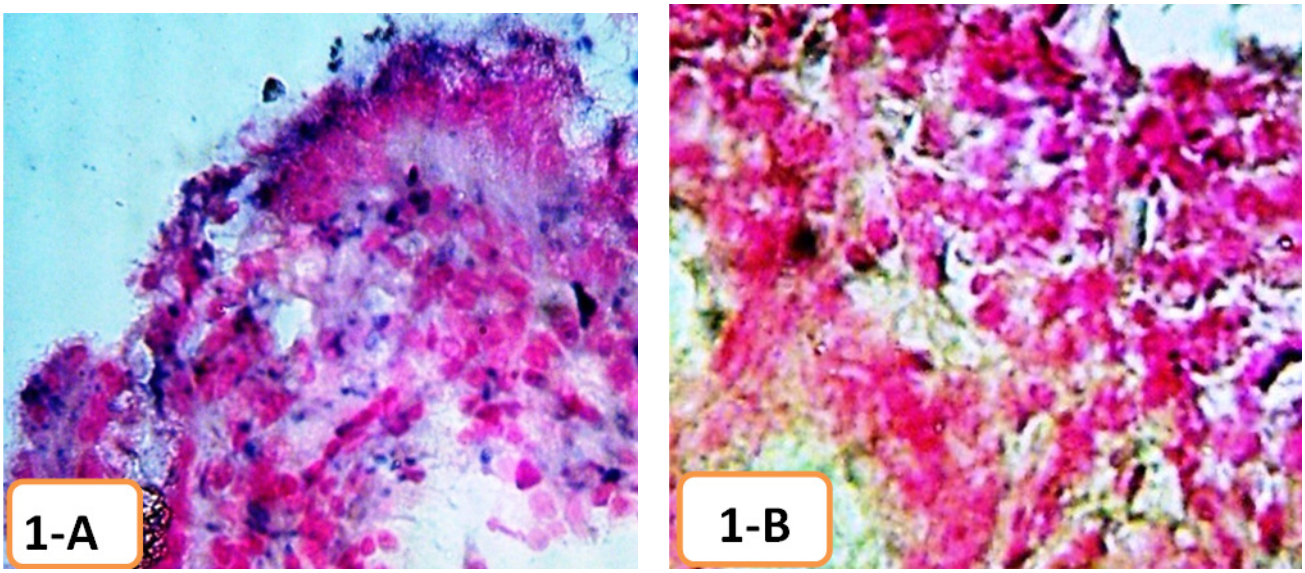

Figure 1. **(a)** In situ hybridization for CagA Positive H.pylori in gastric tissue section. Staining by BCIP/NBT (bluish purple) counterstained with nuclear fast red. Bar size = 50µm. **(b)** CagA negative H. pylori In situ hybridization in gastric tissue section for control group. Staining by BCIP /NBT (bluish purple), counterstained with nuclear fast red. Bar size = 50µm.

Table 2. Correlation between Gastric versus inflammatory cells apoptotic index and insitu H.pylori CagA

Parameters	Gastric AI	Inflammatory Cells AI	χ2	P value	R	P value
CagA+	2.99±8.98	0.25±0.092	275.098	0.000	-0.283 -0.356*	0.054 0.014*
CagA-	1.77± 4.70	0.26±.06948	211.200	0.000	0.153	0.394
χ2	38.192	31.963				
P value	0.001	0.002				
R	0.081 0.309*	-0.07				
P value	0.476 0.005*	0.537				

*spearman correlation

Table 3. Correlation between Gastric versus Inflammatory cells apoptotic index and gastric disorders

INSITU		PMNs grade			Total	χ2	P value	R	P value
		2	3	4					
CagA negative	Cellular AI(0.233± 0.77)	2(2.5%)	24(30%)	7(8.75%)	33 (41.25%	53.821	0.000	0.176	0.328
CagA positive	Cellular AI(0.2458± 0.101)	4(5%)	9(11.25%)	34(42.5%)	47(58.75%)	63.526	0.000	0.296	0.044
χ2		85.148							
P value		0.000							
R		0.214							
P value		0.057			Total	χ2	P value	R	P value
CagA negative	Gastric AI(2.5350 ± 6.13761)	2(2.5%)	24(30%)	7(8.75%)	33 (41.25%	51.212	0.000	-0.067	0.711
CagA positive	Gastric AI(3.6933 ± 10.48932)	4(5%)	9(11.25%)	34(42.5%)	47(58.75%)	80.791	0.000	0.150	0.313
χ2		128.709							
P value		0.000							
R		0.123 0.354*							
P value		0.277 0.001*							
Insitu		Lymphocytes grade							
		1	2	3	Total	χ2	P value	R	P value
CagA negative	Cellular AI (0.233± 0.77)	2(2.5%)	17(21.25%)	14(17.5%)	33 (41.25%	29.104	0.010	-.361	0.039
CagA positive	Cellular AI (0.2458± 0.101)	0(0%)	19(23.75%)	28(35%)	47(58.75%)	32.744	0.001	.020	0.895
χ2		43.109							
P value		0.019							
R		-0.136							
P value		0.228			Total	χ2	P value	R	P value
CagA negative	Gastric AI (2.5350 ± 6.13761)	2(2.5%)	17(21.25%)	14(17.5%)	33 (41.25%	54.333	0.000	-.580	0.000
CagA positive	Gastric AI (3.6933 ± 0.48932)	0(0%)	19(23.75%)	28(35%)	47(58.75%)	23.561	0.015	.215	0.147
χ2		125.071							
P value		0.000							
R		-0.009							
P value		0.934							

*spearman correlation

cases was higher in patients with normal PGI (30-160µg/L), (0.6669±0.07149). Gastric AI among CagA negative cases was higher in patients with above normal PGI (160 µg/L), (0.5960±0.13426). According to PGI levels, significant difference in gastric AI was detected among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000). No significant correlation between gastric AI and PGI levels regardless of CagA positivity (p value = 0.141;0.191;0.248).

Gastric AI was higher in patients with normal PGII (3-15 µg/L), (0.6773±.04606) among CagA positive cases versus (0.6300±0.09442) among CagA negative cases. Significant difference in gastric AI was detected among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000) according to PGII level .inverse correlation between AI and PGII among Cag A negative cases (p value = 0.029).

Gastric AI among CagA positive cases was higher in patients with under normal PGI/PGII (3 µg/L), (0.6700±0.06661) while AI was higher (0.6165±0.12389) among CagA negative cases with normal PGI/PGII. Significant differences in gastric AI reported among CagA positive; CagA negative; CagA positive versus CagA negative cases (p value = 0.000) according to PGI/PGII level. Inverse correlation between gastric AI, PGI/PGII level and CagA positivity was reported (p value = 0.039).

Gastric AI among CagA positive cases was higher in patients with normal Gastrin17 (1-7 pmol/ml), (0.6600±0.10642). Gastric AI among CagA negative cases was higher in patients with above normal Gastrin17 7pmol/ml, (0.6600±0.10832). According to Gastrin17 levels, significant difference in gastric AI was detected among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000). Marginal inverse correlation between gastric AI, Gastrin17 level was reported (p value = 0.056).

As shown in Table 5, inflammatory cells AI among CagA positive cases was higher in patients with normal PGI (30-160µg/L), (0.2535±.08270). Inflammatory cells AI among CagA negative cases was higher in patients with above normal PGI (160 µg/L), (0.3150±0.2415). According to PGI levels, significant difference in inflammatory cells AI was detected among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000). No significant correlation between inflammatory cells AI and PGI levels regardless of CagA positivity (p value = 0.743;0.74;0.706).

Inflammatory cells AI was higher in patients with above normal PGII (15 µg/L), (0.2694±0.09411) among CagA positive cases versus (0.2644±.07148) among CagA negative cases. Significant difference in inflammatory cells AI was detected among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000) according to PGII level. No significant correlation between inflammatory cells AI; PGII and Cag A .

Inflammatory cells AI among CagA positive cases was higher in patients with under normal PGI/PGII (3 µg/L), (0.3106±0.09601) versus (0.2906±.03714) among CagA negative cases. Significant differences in gastric AI reported among CagA positive; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000) according to PGI/PGII level. Inverse correlation between Inflammatory cells AI, PGI/PGII level and CagA positivity was reported (p value = 0.016).

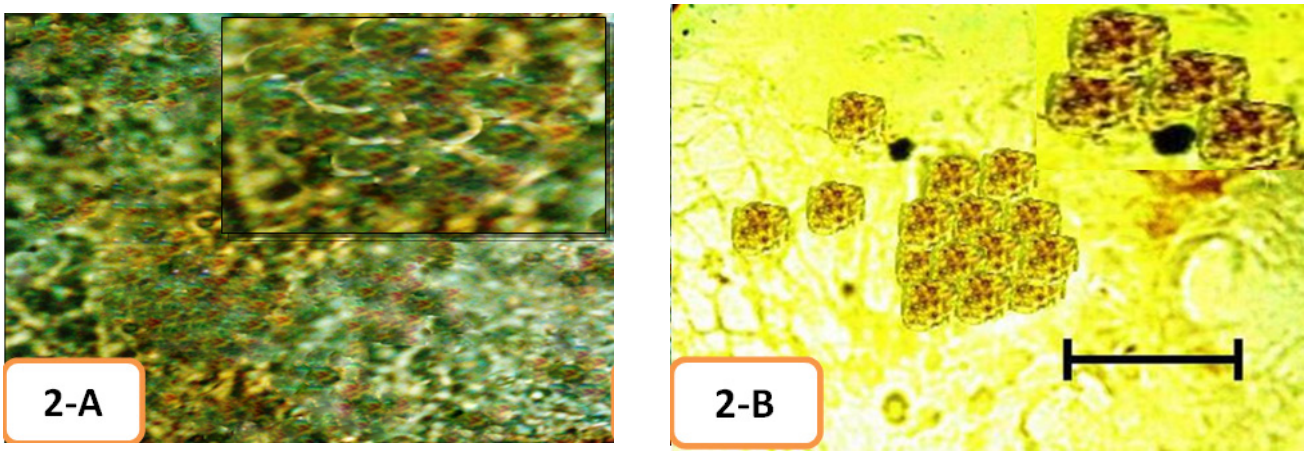

Figure 2. **(a)** During the early stages of apoptosis, enlarged nuclei with chromatin condensation surrounded by a clear halo due to cytoplasmic shrinkage could be noted. ***(b)*** Gastric tissue: Intracellular chromatin fragments (micronuclei) were demonstrated in some apoptotic cells (apoptotic bodies) Bar size = 50µm.

Table 4. Correlation between Gastric cells Apoptotic Index and gastric secretions

Parameters		Gastric cells Apoptotic Index (2.4869 ± 7.49993)		χ2	P value	R	P value
		CagA+	CagA-				
PGI	<30 µg/L	6(7.5%) 0.6550± 0 .07450	0(0%)	625.609	0.000	0.131	0.248
	30-160µg/L	26(32.5%) 0.6669 ± 0.07149	23(28.75%) 0.5565± 0.15221				
	>160 µg/L	15(18.75%) 0.6287± 0.15815	10(12.5%) 0.5960± 0.13426				
	χ2	318.979	199.277				
	P value	0.000	0.000				
	R	0.218	-0.234				
	P value	0.141	0.191				
PGII	<3 µg/L	0(0%)	0(0%)	642.424	0.000	-0.015	0.896
	3-15 µg/L	11 (13.75%) 0.6773 ± .04606	8(10%) 0.6300± 0.09442				
	>15 µg/L	36(45%) 0.6458 ± 0.11833	25(31.25%) 0.5488 ± 0.15552				
	χ2	360.895	225.333				
	P value	0.000	0.000				
	R	-0.021	-0.119 -0.380*				
	P value	0.891	0.510 0.029*				
PGI/ PGII	<3 µg/L	17(21.25%) 0.6700 ± 0.06661	16(20%) .5175 ± 0.15416	1059.846	0.000	-.039 .231*	.730 .039 *
	3-20 µg/L	30(37.5%) 0.6437± 0.12333	17(21.25%) 0.6165± 0.12389				
	>20 µg/L	0(0%)	0(0%)				
	χ2	485.915	287.277				
	P value	0.000	0.000				
	R	0.000	-.137				
	P value	0.999	0.446				
Gastrin17	< 1pmol/ml	0(0%)	0(0%)	377.145	0.000	-.033 -.215*	.769 .056*
	1-7 pmol/ml	44(55%) 0.6600 ± .10642	29 (36.25%) 0.5559 ± 0.14754				
	>7pmol/ml	3(3.75%) 0.5667± .05774	4 (5%) 0.6600 ±0.10832				
	χ2	244.323	165.393				
	P value	0.000	0.000				
	R	-.082	-.046				
	P value	0.583	0.801				

*spearman correlation

Inflammatory cells AI among CagA positive cases was higher in patients with normal Gastrin17 (1-7 pmol/ml), (0.2530±0.09367). Inflammatory cells AI among CagA negative cases was higher in patients with above normal Gastrin17 7pmol/ml, (0.2929±.06726). According to Gastrin17 levels, significant difference in Inflammatory cells AI was detected among CagA positive ; CagA negative cases; CagA positive versus CagA negative cases (p value = 0.000). No significant correlation between Inflammatory cells AI, Gastrin17 level and CagA positivity was reported (p value = 0.091; 0.172; 0.258).

As shown in Table 6, the mean age of patients was

47.24±2.104 (years). Significant difference (p = 0.000) and correlation between gastric AI (p = 0.002), inflammatory cells AI (p = 0.008) according to age. Males represent (55%) versus (45%) females. Significant difference (p = 0.000) and inverse correlation with gastric AI (p = 0.044), inflammatory cells AI (p = 0.01) and gender. In current study Proton pump inhibitors (PPI) used by (11.3%) of patients. Significant difference (p = 0.000) in gastric AI; inflammatory cells AI and usage of PPIs. No significant correlation between PPI usage and AI. In current study NSAID used by (22.5%) of patients. Significant difference in gastric AI (p = 0.000), inflammatory cells AI (p = 0.001) according to NSAID usage. Marginal inverse correlation between gastric AI and NSAID using (p = 0.057). In current study only (1.3%) of patients were drinkers. Neither significant difference nor correlation in gastric AI, inflammatory cells AI and alcohol drinking. In current study smokers represent (31.3%) of patients. Significant

Table 5. Correlation between inflammatory cells apoptotic index and gastric secretions

Parameters		inflammatory Cells Apoptotic Index 0.2534 ± 0 .08342		χ2	P value	r	P value
		CagA+	CagA-				
PGI	<30 µg/L	6 (7.5%) 0.2400±.15218	0(0%)	596.664	0.000	0.043	0.706
	30-160µg/L	26 (32.5%) .2535± .08270	23(28.75%) 0.2365± .06952				
	>160 µg/L	15(18.75%) .2433±.08633	10(12.5%) 0.3150 ± 0.2415				
	χ2	337.430	162.938				
	P value	.000	0.000				
	R	-0.049	0.315				
	P value	0.743	0.074				
PGII	<3 µg/L	0(0%)	0(0%)	562.157	0.000	0.156	0.168
	3-15 µg/L	11 (13.75%) 0.1800±.03899	8(10%) 0.2475± 0.06563				
	>15 µg/L	36(45%) 0.2694± 0.09411	25(31.25%) 0.2644± .07148				
	χ2	334.973	159.734				
	P value	0.000	0.000				
	R	00.261	0.261				
	P value	0.166	0.142				
PGI/PGII	<3 µg/L	17 (21.25%) 0.3106± 0.09601	16(20%) 0.2906± .03714	867.817	0.000	-0.074 - -0.268*	0.516 0.016*
	3-20 µg/L	30(37.5 %) 0.2133± 0.07009	17 (21.25%) 0.2318 ± .08110				
	>20 µg/L	0(0%)	0(0%)				
	χ2	461.644	222.338				
	P value	0.000	0.000				
	R	-0.220 -.364*	0.143				
	P value	0.138 0.012*	0.426				
Gastrin17	< 1pmol/ml	0(0%)	0(0%)	233.017	0.006	0.128	0.258
	1-7 pmol/ml	44 (55%) 0.2530± 0.09367	26 (32.5%)0.2515± 0.06868				
	>7pmol/ml	3(3.75%) 0.1833± 0.02887	7 (8.75%) 0.2929 ± .06726				
	χ2	170.365	124.740				
	P value	0.000	0.000				
	R	-0.249	0.244				
	P value	0.091	0.172				

*spearman correlation

difference in gastric AI (p = 0.006), inflammatory cells AI (p = 0.006) according to smoking habit, without correlation between AI and smoking . Tap water used by (90%) of patients. Significant difference in gastric AI (p = 0.001), inflammatory cells AI (p = 0.006) according to drinking of tap water, without correlation between AI and tap water .

Table 6. Correlation between proposed risk factors, inflammatory cells and gastric apoptotic Index

Parameters	Statistics	Gastric AI	Cellular AI
Age 47.24±2.104 (years)	χ^2	803.891	530.555
	P VALUE	0.000	0.000
	R	0.344	-0.294
	P VALUE	0.002	0.008
Gender Male: 44(55%) Female: 36(45%)	χ^2	41.457	33.922
	P VALUE	0.000	0.001
	R	-0.039 -0.226*	-0.285
	P VALUE	0.730 0.044*	0.01
Proton pump inhibitors Positive: 9(11.3%) Negative: 71(88.8%)	χ^2	62.640	39.937
	P VALUE	0.000	0.000
	R	-0.085	-0.029
	P VALUE	0.454	0.800
NSAID Positive: 18(22.5%) Negative: 62(77.5%)	χ^2	40.456	33.915
	P VALUE	0.000	0.001
	R	-0.136 -.213*	-0.054
	P VALUE	0.231 0.057*	0.632
ALCOHOL Drinker : 1(1.3%) Nondrinker:79 (98.8%)	χ^2	4.388	19.241
	P VALUE	0.996	0.116
	R	-0.027	0. 131
	P VALUE	0.812	0.246
SMOKING: Smoker:25(31.3%) Nonsmoker:55(68.8%)	χ^2	72.116	64.370
	P VALUE	0.006	0.006
	R	-0.152	0.059
	P VALUE	0.180	0.603
WATER Tap water:72(90%) Others :8(10%)	χ^2	39.444	29.265
	P VALUE	0.001	0.006
	R	-0.081	-0.149
	P VALUE	0.474	0.186

*spearman correlation

4. Discussion

In the present study apoptotic events were detected using terminal deoxynucleotidyl Transferase-Mediated UTP nick End-Labeling (TUNEL) technique in non-cancerous gastric disorders. The apoptotic events were detected in gastroduodenal mucosa epithelial cells; gastric glands and lamina propria.

In current study, gastric cells apoptotic index among CagA positive disorders was higher among prepyloric ulcers (0.8500±0.000), gastritis (0.7343±.02370), Gastric ulcer (0.6600±0.07493); DU (0.6540±.08849) ; gastropathy (0.6400±.05642), duodenitis (0.6300±0.000) which come in line with others[19,20].

Among gastric disorders, In current study, Significant difference (p value = 0.000) reported in gastric tissue as well as in inflammatory cells AI among gastric disorders infected with CagA positive H. pylori. Significant difference in inflammatory cells versus gastric tissue AI (p value = 0.000) regardless CagA positivity without significant correlation between gastric and inflammatory cells AI (p value = 0.089) was reported. These results come in line with other studies[17] who elucidated that number of apoptotic cells that were detected in (83%) antral biopsy specimens of chronic gastritis, in surface epithelium, antral pyloric glands and lamina propria using Tunel detection system. Colonization of H. pylori CagA positive strain play obvious role in apoptotic process as appear from current study presence of significant correlation between CagA positive H.pylori infection and gastric AI (p value = 0.022); inflammatory cells AI (p value = 0.049). These results of causal relationship between CagA positive H. pylori and AI come in line with[21] stated that apoptotic cells were located throughout the depth of the gastric glands and increased in to (16.8%) in patients colonized with of CagA positive strain . Other study agree with current work[22] found that gastric antral ulcers AI (82.24±18.9) and duodenal ulcer AI (81.5±22.1) TUNEL-positive cells which is higher than non-infected persons. Apoptosis usually associated with loss of mucosal integrity, increased permeability and a breakdown in the cytoprotective mechanisms that guard the epithelium against damage via luminal acid and pepsin, increase of cell associated death receptors as well as expression of intrinsic mitochondrial pathway molecules[23] and increase in reactive oxygen species due

to inflammatory cells infiltration mainly neutrophils and macrophages and mitochondrial activity, leading to DNA fragmentation process[24].

This reaction perhaps independent or not only associated with Cag A toxin, other virulence factors HP-NAP, Urease, Vac have vital role. Regardless the types of gastric disorder, significant correlation between gastric tissue AI and insitu expression of CagA (p value = 0.005) which disagree with others stated that no significant difference in AI among H. pylori cag A positive and negative cases[25] due to statistically small sample size or due to P53 mutation[26]. Significant difference in Inflammatory Cells AI among Cag Apositive versus CagA negative cases (p value = 0.002), which come in agreement with others stated AI significantly decrease after successful eradication therapy for H. pylori[19], also other stated that increase in Fas, FasL expression in CagA positive chronic gastritis cases with obvious correlation between gastric, inflammatory cells apoptosis and CagA positivity[6].

In current study, inverse correlation between, gastric AI, inflammatory cells AI and CagA positivity (p value = 0.014) was reported. This can be attributed to several factors mainly the number of infiltrating PMNs, lymphocytes is relatively low compared with gastroduodenal epithelial cells; the degree of inflammatory response and the protective role of histamine which is a gastric mucosal constituent that protect lymphocytes from the free radicals that could be produced via monocyte oxidase in response to proinflammatory peptide of H. pylori like HP(2-20)[27]. All these differences give as a bright image about the role of CagA in apoptosis of gastroduodenal epithelial cells whether its occur in superficial epithelia or glandular epithelia as well as it reflect the possibility of other contributors in the apoptosis. One of these possible contributors is the genetic back ground of infected individuals, density of H. pylori in infected tissue[24], as well as the heterogeneity of infective strains, and the possibility of co-infection with CagA positive and CagA negative strain must be kept in mind.

In current study, gastric tissue AI; inflammatory cells AI affected by the grade of PMNs and H. pylori CagA positivity. Meanwhile no significant correlation between inflammatory cells AI, gastric tissue AI and lymphocytes grade even significant differences appear in AI. The direct connection between H. pylori and AI confirmed in various studies, indicating that eradication of this pathogen associated with significant reduction in gastric and inflammatory cells AI[19,28].

The majority of patients in current study have chronic active gastric inflammation after short period of neutrophilic inflammation with little disruption of gastric acid secretion. As a result of infection, gastric tissue produce IL8 and other chemokines also proinflammatory cytokines to instigate recruitment of macrophages, PMN, mast cells, lymphocytes to infected tissue. Recruited PMNs secrete more inflammatory mediators that amplify the primary signal and mediates directly the influx of more PMN to the gastric mucosa. H. pylori soluble proteins act as neutrophils chemoattractants. Tissue invading PMNs, induce oxidative burst leads to damage of cellular membranes via lipid peroxidation and this effect extended to mitochondrial membrane. Beside H. pylori stimulates overproduction of mitochondrial ROS (O^-) beyond the ability of mitochondrial superoxide dismutase and glutathione peroxidase which cause detrimental effects on mitochondrial membrane and initiation of intrinsic apoptotic pathway and leading finally to gastric mucosal damage[24,29]. However, ROS cannot eradicate H. pylori. The role of oxidative stress on gastric mucosa is multi-functional. For neutrophils, it is the result of excessive defense reactions of the body against H. pylori intrusion, and for H. pylori, it is a convenient tool for invading the human gastric mucosa[24,29].

In current study this explanation is true as PMNs grades significantly associated with H. pylori and mainly with CagA secretion which correlated directly with AI of gastric as well as PMNs AI which come in line with[19,21]. Meanwhile in current study, no significant correlation between inflammatory cells AI, gastric tissue AI and lymphocytes grade even significant differences appear in AI. This may be due to several factors mainly the number of infiltrating lymphocytes is relatively low compared with that of gastroduodenal epithelial cells due to chronic active inflammatory response beside the protective role of histamine which is gastric mucosal constituent that protect lymphocytes from the free radicals that could be produced via monocyte oxidase in response to proinflammatory peptide of H. pylori like HP(2-20)[27,30].

In current study, Among CagA positive cases gastric and Inflammatory cells AI and was higher in patients with normal PGI (0.6669±0.07149), (0.2535±.08270). Meanwhile among CagA negative cases gastric and inflammatory cells AI was higher in patients with PGI (160 µg/L), (0.5960± 0.13426), (0.3150±0.2415). No significant correlation between gastric AI; inflammatory cells AI and PGI levels regardless of CagA positivity.

Which indicate that main effector on PGI was the density of H. pylori regardless possessing of CagA. H. pylori distributed gradually to be pangastric, stimulating intracellular nitric oxide and calcium production leading to the assembly of the superoxide-forming NADPH oxidase on the neutrophil plasma membrane finally and sever inflammatory response that subsequently induce PGs disturbance[31,32]. This result come in line with others stated that gastric tissue apoptosis depends on the activity of inflammatory cells mainly PMNs (gastritis activity) and grade of H. pylori colonization[33] others link the severity of gastric AI with degree of chronic inflammatory infiltrates[34], other indicating that CagA strain induce more sever DNA fragmentation compared with CagA negative which also have the ability to Do via its virulence factors other than CagA[35].

Gastric AI (0.6773±0.04606); inflammatory cells AI (0.2694±0.09411) were higher in patients with normal PGII (3-15 µg/L), among CagA positive cases versus (0.6300±0.09442); (0.2644±.07148) among CagA negative cases. Inverse correlation between gastric AI and PGII among Cag A negative cases (p value = 0.029). Meanwhile no significant correlation between inflammatory cells AI; PGII and Cag A.

This is probably related to the effect of H. pylori density and severity of inflammation on PGII that secreted from cardiac, fundic, antral mucosa as well as proximal duodenal mucosa. This is not affected by absence or presence of CagA. Gastric and inflammatory cells AI and PGII level affected by age, gender and type of gastric mucosal lesion[36,37] beside the level of proinflammatory cytokine TNFα,γ interferon in gastric tissue induce Fas receptor expression in gastric tissue and infiltrated PMNs oxidative burst as well as lymphocytes which triggers the apoptotic signals and DNA fragmentation in tissue and inflammatory cells[38,39]. Although in current study, AI of inflammatory cells in current study was less than AI of gastric tissue.

Gastric AI (0.6700±0.06661); Inflammatory cells AI (0.3106±0.09601) among CagA positive cases was higher in patients with under normal PGI/PGII (3 µg/L), while gastric AI was higher (0.6165±0.12389) among CagA negative cases with normal PGI/PGII. Low level of serum PGI/PGII ratio indicate the presence of atrophic changes in gastric mucosa which in turn explain the increase of AI in gastric tissue and Inflammatory cells which is appear to be affected by the presence of CagA although without significant correlation with gastric AI. The presence of Inverse correlation between gastric, inflammatory cells AI, PGI/PGII level in current study reflect the role of H. pylori density in mucosal damage due to inflammatory reaction and cytotoxic effects of urease[7,36]. Beside the possibility of protective role of PPI intake by (11.3%) of patients[40] which enhance proliferation and regeneration of damaged gastric mucosal cells.

In current study, H. pylori infection cause elevation of gastrin secretion in (8.75%) regardless CagA status, but gastric (0.6600±0.10642); Inflammatory cells (0.2530±0.09367) AI was higher in patients infected with CagA positive H. pylori with normal Gastrin17 (1-7 pmol/ml), which give the possibility that patients might be under PPIs treatment that modulate the serum level of gastrin[40]. Increased AI among patients with normal gastrin level reflects the effects of gastrin in growth and regeneration of damaged mucosal cells due to ROS, proinflammatory cytokines and cytotoxic activity of CagA and other H. pylori virulence factors[7].

Gastric (0.6600±0.10832); Inflammatory cells (0.2929±0.06726) AI among CagA negative cases was higher in patients with above normal Gastrin17 7pmol/ml, which come in line with[41] and indicate the presence of chronic atrophic changes among gastritis cases[42]. Marginal inverse correlation between gastric AI, Gastrin17 level reported (pvalue = 0.056) which explain the role of normal gastrin in mucosal epithelial cells homeostasis[41].

Correlation between patients age and H. pylori infection is controversial. In current study, the mean age of patients was 47.24±2.104 (years). Significant correlation between gastric AI (p = 0.002), inflammatory cells AI (p = 0.008) and age, this come in accordance with[6,7], indicating that distribution of H. pylori infection is pangastric in the younger patients. In current work, Males represent (55%) versus (45%) females. Significant invers correlation between gastric AI (p = 0.044), inflammatory cells AI (p = 0.01) and gender. This result proved by experimental exposure of mice to H. felis or H. pylori that induce sever inflammatory response and gastric epithelial cell apoptosis was more sever in females than males[43,44]

In current study Proton pump inhibitors (PPI) used by (11.3%) of patients. No significant correlation between PPI usage and AI. This due to minority of our patients under PPI. Several studies reveal a more direct effect of PPIs on the integrity of the gastric mucosa which comprises strong antiapoptotic, anti-inflammatory, and free radical

scavenging activities[40]. In current study NSAID used by (22.5%) of patients. Marginal inverse correlation between gastric AI and NSAID using (p = 0.057). This appear reasonable gastric mucosal damage in H. pylori infected patients already having NSAID resulting in decrease in pH, imbalance between apoptosis and proliferation, reduction in mucosal blood flow and recruitment of polymorph nucleates in distinct compartments that accelerate inflammatory reaction depending on H. pylori density, dose and duration of NSAID usage[45]. Although alcohol considered as a risk factor for gastroduodenal diseases due to increase free radicals[24], in current study only (1.3%) of patients were drinkers. For this reason no correlation between alcohol drinking and gastric AI, inflammatory cells AI reported .

Cigarette smoking has tremendous adverse effects on the stomach maybe through the change in apoptosis in the stomach due to mucosal irritation and induction of inflammatory reaction and increase free radicals. In current study smokers represent (31.3%) of patients. Although, Significant difference in gastric AI (p = 0.006), inflammatory cells AI (p = 0.006) according to smoking habit, No significant correlation between AI and smoking. This differences in AI due to the effect of nicotine, the major constituent of cigarettes, that modulate the mucosal gastroprotective mechanism, alteration of apoptosis/ proliferation balance and delay in healing of gastric and duodenal ulcers[46].

Tap water used by (90%) of patients. Significant difference in gastric AI (p = 0.001), inflammatory cells AI (p = 0.006) according to drinking of tap water, without correlation between AI and tap water. Presence of such differences does not abolish the possibility of water as source of H. pylori transmission without losing of virulence factors responsible for attachment and colonization as well as gastroduodenal pathology[47].

5. Conclusion

Cag A correlated with gastric AI and inflammatory cells AI among different gastroduodenal nonmalignant disorders. Gastric AI Inversely correlated with PGII among Cag A negative cases; AI Inversely correlated with PGI/PGII level; gastric AI Inversely correlated with Gastrin 17 level. AI correlated with PMNs grade; age and CagA positivity. AI was not correlated with PPIs, smoking and tap water. Inflammatory cells AI Inversely correlated with Gender.

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