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Molecular and Immunopathological Role of Gastric Versus lymphocytes Interleukin 8 Gene Expression in H.pylori Induced Fas-Fasl Apoptotic Pathway in Gastroduodenal Ulcer in Iraqi Patients

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Abstract
The main Objective was to determine the role of Cag A positive H.pylori in modulation of expression of FAS,FASL and IL8 mRNA in gastroduodenal tissue and infiltrated lymphocytes and its effects in development of gastroduodenal ulcer (GDU).Twenty one GDU patients and (10) control were chosen. Multiple gastric mucosal biopsies taken for rapid urease test and slides preparation from paraffin blocks. Biotinylated DNA probe for human FAS, FASL and IL8, genes used for detection of their expression in tissue lymphocytes and gastric epithelial cells using ISH technique. Biotinylated DNA probe for H.pylori Cag A gene used for detection of its expression in gastric epithelial cells. Insitu CagA was detected in (42.85%) of GDU cases. The toxin was not detected among control. There is a highly significant differences among CagA positive cases as well as Cag Anegative cases in FAS mRNA, FasL mRNA and IL8 mRNA expression between Gastroduodenal tissue(GDT) and Tissue infiltrating lymphocytes(TILs)($p < 0.05$).Significant difference in tissue FAS mRNA and tissue IL8 mRNA over expression ($p<0.05$) was detected. while no such difference was detected in tissue FASL mRNA between CagA positive and CagA negative cases was detected. Significant difference in TILs FasL and TILs IL8 mRNA expression between Cag A positive and Cag A negative GDU cases ($p<0.05$) .while no such difference in expression of FAS m RNA in TILs between Cag Apositive and CagA negative GDU cases .
Significant difference between control group and *H.pylori* Cag ^{positive} GDU cases (**P<0.05**) as well as *H.pylori* CagA^{negative} cases ($p< 0.05$) was detected.in normal control gastroduodenal tissue , Fas mRNA, FasL mRNA and IL8 mRNA expressed in less than 5% of infiltrated Lymphocytes which considered negative. Lymphocytes FAS mRNA expression has significant correlation with tissue FAS ($r= 0.435$, $p=0.049$). Tissue Fas mRNA expression has significant correlation with Lymphocytes FASL ($r= 0. 561$, $p=0.008$);Lymphocytes IL8 ($r= 0. 529$, $p=0.014$) and CagA ($r= 0.436$, $p=0.048$). Lymphocytes FASL mRNA expression has significant correlation with Lymphocytes IL8 ($r= 0.650$, $p=0.001$) .
In conclusions Cag A gene play a vital role in modulation of FAS , FASL and IL8 genes expressed in gastroduodenal ulcer tissue and infiltrated lymphocytes which reflect the severity of histopathological changes during the course of H.pylori infection.
Keywords: gastroduodenal ulcer, Fas, FasL, ,IL8 ,H.pylori ,Cag A ,Iraq

Introduction:

Incidence of peptic ulcer disease (PUD) ranges from 0.03% to 0.19% per year in different population settings [1]. Time trend studies show a decline in the incidence of PUD in the last decades. However, PUD remains a major cause of morbidity and, in a number of cases, is an underlying cause of mortality by subsequent complications, such as perforation or bleeding of the ulcer [2]. Peptic ulcer disease embraces both gastric and duodenal ulcers which have different pathogenesis: whereas duodenal ulcer disease is heavily acid-driven with immunopathogenetic components, the latter is likely to be the dominant aspect in gastric ulcer disease [3]. Therefore, it has been suggested that duodenal ulcer is an essentially Helicobacter pylori (H pylori)-related disease and gastric ulcer a mainly nonsteroidal anti-inflammatory drug (NSAID)-associated disease[4]. H pylori infection and NSAIDs have been identified as the main causes of PUD [4].

Though the prevalence of H.pylori infection appears to be decreasing in many parts of the world, H. pylori remains an important factor linked to the development of peptic ulcer disease, gastric malignancy and dyspeptic symptoms [5]. Majority of H. pylori infected persons remain asymptomatic. Approximately 10-15% of the infected persons develop associated illnesses, 1 to 10% developing peptic ulcer disease.

There are several lines of evidence implicating H. Pylori in the development of gastric and duodenal ulcers. 1. H. Pylori is found in most patients who have peptic ulcers in absence of NSAID use.2. Presence of H. Pylori is a risk factor for the development of ulcer.3. Eradication of H. Pylori significantly reduces the recurrence of gastric and duodenal ulcers.4. Treatment of H. Pylori infection leads to more rapid and reliable ulcer healing than does treatment with anti-secretory therapy alone[5].

Several mechanisms are proposed to describe the pathogenicity of H. pylori: change in expression of host genes, infection-induced cell proliferation, loss of polarity and elongation of cell, cell-cell junctions

degradation, decrease in acid secretion and inflammation [6]. Cytotoxin associated gene pathogenicity island (cag PAI) with a size of 40 Kbp contains 27 genes encoding for T4SS pilus which is responsible for pathogenicity and inflammation. When bacteria colonize the stomach, inflammation induces G cells of the antrum. G cells secrete hormone gastrin, which travels to parietal cells of the fundus via blood stream[7] . Gastrin stimulates secretion of the acid from the parietal cells and also increases the number of parietal cells. Increased load of acid damages epithelial cells of the duodenum resulting in ulcers [8].

The main Objective was to determine the role of Cag A positive H.pylori in modulation of expression of FAS,FASL and IL8 mRNA in gastroduodenal tissue and infiltrated lymphocytes and its effects in development of gastroduodenal ulcer.

Materials and methods:

Patients: The subjects of the present study were selected from Patients attending the gastroenterology unit of Al-kadymia teaching hospital in Baghdad. This study was conducted according to the principles of Helsinki declaration. Dully-filled consent form was obtained from all patients participating in the study before endoscopy. Approval of ethical review committee of medicine college –Al-Nahrain University-Iraq, was taken prior to initiation of the work. At first the aim of study was explained for all participants and after obtaining their oral consent they have been studied. The subjects were diagnosed as having atrophic gastritis by upper gastrointestinal endoscopy from May 2005 to December 2006.Twenty one patients suffering from gastroduodenal ulcer were chosen. Ten apparently healthy volunteer were enrolled as control.

Exclusion criteria used in the study:

Patients who had undergone or were currently undergoing H.pylori eradication therapy; Patients who had received antiulcer treatment in the last three months, and still receiving proton-pump inhibitors (PPI) or H 2 receptor blockers; Patients who had received nonsteroidal anti-inflammatory drugs (NSAIDs) in the two months before the examination; The result of rapid urease test was negative informed consent was not signed before endoscopy. Data were collected through direct interview with the patient, and by seeking his/her hospital record as well as previous medical reports.

Sample collection: Patients were fasted for at least eight hours before endoscopic examination. Endoscopic examination was performed under local pharyngeal anesthesia. Using fiber optic endoscope, multiple mucosal biopsy specimens were taken via sterile standard biopsy forceps from the stomach (inflamed area of gastric mucosa). One biopsy specimens were used for rapid urease test (RUT) for detection of H.pylori in tissue sample. Rest biopsy specimens were fixed with 10% buffered formalin for preparation of paraffin embedded tissue blocks for histological examination using haematoxylin-eosin. Biopsy specimens obtained from the antrum were used to evaluate the grades of Polymorphonuclear leukocytes and lymphocytes [9] . The presence of H.pylori in tissue section was established by typical appearance on scanning along the mucosal surface and the individual gastric pits.

DNA Probes and DNA Probe hybridization/Detection System : Biotinylated long DNA probe for human Fas Gene, Cat. No.: IH-60047(fas-6001-B). (Maxim biotech-USA); Biotinylated long DNA probe for human FasL gene, Cat. No. IH-60048(fas-6010-B) (Maxim biotech-USA). Biotinylated long DNA probe for human IL8 Gene, Cat. No.: IH-60047(IL8-6001-B) , Biotinylated long DNA probe for H.pylori/ Cag A Gene, Cat. No.: IH-60061(HPY-6001-B) (Maxim biotech-USA). The DNA Probe hybridization/Detection System – In Situ Kit (Maxim biotech-USA).

Biopsy urease test (BUT): The biopsies were placed directly into the rapid urea medium at the time of endoscopy. Positive results indicated by development of a pink-red or red-violet color[10] .

Insitu hybridization procedure: Human (Fas,FasL&IL8) genes as well as Cag A gene expression for H.pylori were detected in 4-6 µm thickness serial gastric mucosal tissue sections fixed on positively charged slides. According to Maxim biotech instruction manual [11]. Two pathologists, independently at powerX40 according to the scoring system, did the examination and scoring under light microscope and discordances were solved by consensus.

For the evaluation of Fas, FasL, IL8 expression, a semi quantitative evaluation system was used to record the number of positive cells. Expression patterns were graded according to [9].Negative result (grade 0) estimated if less than 5% of the cells were positively stained in contrast Positive result estimated if more than 5% of cells were positively stained. The positively stained cell arranged from low expression (grade 1) if (5-25%) of cells give positive signals; intermediate expression(grade 2) if (26-50%) of cells give positive signals, High expression(grade 3) if more than 50% of estimated cells give positive signals. The presence of CagA positive H.pylori was detected by direct visualization of bacteria with a characteristic blue-black color in gastric epithelial surface which reflect colonization with this pathogen [12].

Statistical analysis

Data analysis was performed using T-test to find out the significance of differences between two groups that

composed from continuous variables. Pearson test for correlation was used for non-categorical data. The level of significance was 0.05(two-tail) in all statistical testing; significant of correlations include also 0.01 (two-tail) .The level of confidence limits was 0.095.Statistical analysis was performed using SPSS for windows TM version 14.0, and Microsoft EXCEL for windows 2007.

Results:

a) Demography:

In the present case control hospital base study, 21 patients were proved to have GDU by endoscopic examination were enrolled .The mean age of Patients was (51.14±3.86) years with a range of (25-80) years as seen in table(1). Among GDU patients, the more frequent (28.57%) ,age group was(28-37)years in, then 5/21(23.8%) for(48-57)years and (68-77) years. The least incidence of GDU was recorded among the age group (18-27)years ,(38-47)years, (58-67)years and (78-88)years .Males represents (52.4%) versus (47.6%) for females. Ten apparently healthy volunteers (5 males &5 females) were included as a control group .The mean age was (38+3.44) years, ranged (23-58) years. Fifty percent of control group were included at the age range (38-47) years.

b) Insitu Detection of Cag A cytotoxin in GDU cases:

As shown in table (2) *Insitu* CagA Cytotoxin was detected in 9(42.85%) out of 21 case of GDU as shown in figure (1-A).

c) Tissue versus lymphocytes insitu expression of FAS,FASL,IL8 mRNA In Cag A positive/Cag A negative GDU Patients:

Positive expression of tissue FAS mRNA was detected in 9/21 case of CagA positive GDU (42.85%). Tissue expression of Fas mRNA was more than 50% in 8/21 (38.09%) of CagA positive GDU cases. Among CagA negative tissue expression of Fas mRNA was more than 50% in 6/21 (28.57%). Among CagA positive GDU cases tissue expression of Fas mRNA was intermediate in 1/ 21 (4.76%) case compared with that of CagA negative cases in which intermediate expression was detected in 4/21 (19.04%) . Low expression of tissue FAS was detected only in 1/21 (4.76%) of CagA negative cases. Expression of lymphocyte Fas mRNA was more than 50% in 1/21 (4.76%) of CagApositive GDU cases compared with (9.52%) of CagA negative GDU cases. Intermediate expression of lymphocyte Fas mRNA was detected in 1 /21(4.76%) of CagApositive GDU cases compared with 7/21 (33.33%) of CagA negative cases. Low expression of lymphocyte Fas mRNA was detected in 1/21 (4.76%) of CagA positive GDU cases compared with 4 /21 (19.04%) of CagA negative cases. Up to (70%)of normal control cases tissue Fas mRNA expression was negative and only in (30%) of cases low level of expression was detected on the other hand no detectable level of Fas mRNA expression was found regarding to lymphocytes. Details of tissue Fas mRNA expression and scoring system was explained in table (3). Figure (1-B) shows the typical in situ staining for tissue Fas mRNA.

Tissue versus lymphocytes insitu expression of FAS mRNA in CagApositive / CagA negative GDU Patients was explained in table (6) in which the mean ± SE of CagApositive tissue FAS was (69+ 3.4238) compared with (54 ±7.3978) in case of CagA negative Cases. Mean ± SE of Lymphocyte FAS mRNA expression among CagApositive GDU cases was (35.6667 ± 28.25) compared with that of lymphocyte FAS mRNA expression among CagA negative cases. Based on T-test ,there is a highly significant differences among CagA positive cases as well as Cag Anegative cases in FAS mRNA expression between GDT and TILs ($p < 0.05$). Also there is a significant difference between CagApositive and CagA negative cases regarding tissue FAS mRNA over expression ($p<0.05$) While no such difference was found in the expression of FAS m RNA in TILs between CagApositive and CagA negative GDU cases .

Tissue versus lymphocytes insitu expression of FASL mRNA In Cag Apositive/Cag Anegative GDU Patients:

As shown in table (4), Positive expression of tissue FASL mRNA was detected in 9/21case of Cag A positive GDU (42.85%). Tissue expression of FasL mRNA was Low in 8/21 (38.09%) of Cag A positive cases compared with that of CagAnegative cases in which 6 out of 21 (28.57%) patient GDT gave low in situ expression of FASL. Among Cag Apositive cases, only 1 out of 21 cases (4.76%) do not show any expression of FASL in GDT while 2/21 (9.52%) patient give that result of expression. Figures (1-C) show the typical in situ staining for tissue FasL mRNA.

Among CagA negative GDU cases tissue expression of FasL mRNA was intermediate in 3/21 (14.28%) case compared with that of CagA positive cases in which intermediate expression was not detected in GDTs. Expression of lymphocyte FasL mRNA was more than 50% in 6/21 (28.57%) of CagA positive GDU cases compared with 8 /21 (38.09%) of CagA negative GDU cases. Intermediate expression of lymphocyte FasL mRNA was detected in 3 /21 (14.28%) of CagA positive GDU cases compared with 2/21 (9.52%) of CagAnegative cases. Low expression of lymphocyte Fas mRNA was not detected among CagA positive GDU cases compared with 1/21 (4.76%) of CagA negative cases. Up to (70%)of normal control cases tissue FasL

mRNA expression was negative and only in (30%) of cases low level of expression was detected on the other hand no detectable level of FasL mRNA expression was found regarding to lymphocytes.

Tissue versus lymphocytes insitu expression of FASL mRNA in Cag Apositive / Cag A negative GDU Patients was explained in table (6) in which the mean± SE of Cag Apositive tissue FASL was (11.11± 2.16) compared with (16.25± 2.99) in case of Cag Anegative Cases. Mean±SE of Lymphocyte FASL mRNA expression among Cag Apositive GDU cases was (74.77± 7.43) compared with (46.66 ±5.66) of lymphocyte FASL mRNA expression among Cag Anegative cases.

Based on T-test, significant differences in FASL mRNA expression between GDT and TILs among Cag A positive cases as well as Cag A negative cases ($p<0.05$). There was no significant difference between Cag A positive and Cag Anegative Cases regarding to the tissue FASL mRNA over expression ($p>0.05$).While there was highly significant difference in TILs FasL expression between Cag A positive and Cag A negative GDU cases ($p<0.05$) as shown in table (6).

Tissue versus lymphocytes insitu expression of IL8 mRNA in Cag Apositive/Cag Anegative GDU Patients.

Positive expression of tissue IL8 mRNA was detected in 9/21case of CagApositive GDU (42.85%). Tissue expression of IL8 mRNA was more than 50% in 2 /21 (9.52%) both in CagA positive and CagA negative GDU. Among CagA positive and Cag A negative GDU cases, Intermediate tissue expression of IL8 mRNA was (19.04%) as shown in table(5). Figure (1-D) show the typical in situ staining for tissue IL8 mRNA.

Tissue expression of IL8 mRNA was Low in 3/21 (14.28%) of CagApositive patients and in 4/21(19.04%) of CagAnegative cases. Expression of lymphocyte IL8 mRNA was more than 50% in 7/21 (33.33%) of CagApositive GDU cases compared with 6 /21 (28.57%) of CagA negative GDU cases. Intermediate expression of lymphocyte IL8 mRNA was detected in 2/21 9.52%) of CagApositive GDU cases compared with 5/21 (23.80%) of CagA negative cases .Low expression of lymphocyte IL8 mRNA was not detected among CagApositive GDU cases compared with 1/21 (4.76%) of CagA negative cases. Up to (70%)of normal control cases tissue IL8 mRNA expression was negative and only in (30%) of cases low level of expression was detected on the other hand no detectable level of IL8 mRNA expression was found regarding to lymphocytes.

Tissue versus lymphocytes insitu expression of IL8 mRNA in Cag Apositive / Cag Anegative GDU Patients was explained in table (6) in which the mean ± SE of CagA positive tissue IL8 was (51.44± 9.59) compared with (25.50±5.17) in case of Cag A negative Cases. Mean ± SE of Lymphocyte IL8 mRNA expression among Cag Apositive GDU cases was (80.33± 6.48) compared with (46.75 ±5.49) of Lymphocyte IL8 mRNA expression among Cag A negative cases. Based on T-test, there is a highly significant differences in IL8 mRNA expression between GDT and TILs ($p<0.05$) among Cag Apositive cases as well as among Cag A negative cases as shown in table (6). There was a significant difference in tissue IL8 mRNA as well as TILs IL8 mRNA over expression between Cag A positive and CagAnegative Cases ($p<0.05$).

d) Comparative expression of tissue versus cell FAS ,FASL&IL8 in GDU & Normal Cases:

By making simple comparative analysis for evaluation of Tissue Fas , FasL and IL8 mRNA expression in GDU and control group ,there is significant difference between control group and *H.pylori* Cag ^{positive} GDU cases (**P<0.05**) as well as for *H.pylori* CagA^{negative} cases ($p< 0.05$) as shown in table (6).in normal control gastroduodenal tissue , Fas mRNA, FasL mRNA and IL8 mRNA expressed in less than 5% of infiltrated Lymphocytes that considered negative.

Correlations among tissue and cellular markers of GDU cases:

Lymphocytes FAS mRNA expression has significant correlation with tissue FAS (Pearson correlation coefficient= 0.435 , p=0.049). Tissue Fas mRNA expression has significant correlation with Lymphocytes FASL (Pearson correlation coefficient= 0. 561 , p=0.008);Lymphocytes IL8 (Pearson correlation coefficient= 0. 529 , p=0.014);CagA (Pearson correlation coefficient= 0.436, p=0.048). Lymphocytes FASL mRNA expression has significant correlation with Lymphocytes IL8 (Pearson correlation coefficient= 0.650, p=0.001) as shown in table (8).

Table (1): Description of age and Gender for patients and control groups

Clinical presentation			
	Parameters	GDU	Control
		No. of patients	No. of patients
		21	10
Age(Years)	Minimum	25	23
	Maximum	80	58
	Range	55	35
	Mean± SE	51.14±3.86	38±3.44
	Age group	No.(%)	No.(%)
	18-27	1 (4.67%)	3(30%)
	28-37	6 (28.57%)	1(10%)
	38-47	2 (9.52%)	5(50%)
	48-57	5 (23.8%)	0(0%)
	58-67	1 (4.67%)	1 (10%)
	68-77	5 (23.8%)	0 (0%)
	78-88	1 (4.67%)	0 (0%)
	Total	21 (100%)	36(100%)
Gender	Male	11(52.4%)	5(50%)
	Female	10(47.6%)	5(50%)
	Total	21(100%)	10(100%)

Table (2): Frequency distribution of Insitu CagA Cytotoxin among GDU cases

Clinical presentation	Insitu Cag A detection		
	Positive	Negative	Total
GDU	9(42.85%)	12(57.14%)	21
Control	0(0%)	10(100%)	10

Table (3): Tissue versus lymphocytes insitu expression of FAS mRNA in Cag $A^{positive}$/Cag $A^{negative}$ GDU Patients

Cag A In situ	Marker CD95(APO1/FAS)	Negative	Positive			Total No.
		Grade	Grade			
			Low	Intermediate	High	
		< 5%	5-25%	26-50%	>50%	
Cag $A^{positive}$	Tissue FAS	0 (0%)	0(0%)	1 (4.76%)	8(38.09%)	9(42.85%)
	Lymphocyte FAS	0(0%)	1 (4.76%)	7(33.33%)	1 (4.76%)	
Cag $A^{negative}$	Tissue FAS	1(4.76%)	1 (4.76%)	4(19.04%)	6(28.57%)	12 (57.14%)
	Lymphocyte FAS	2(9.52%)	4(19.04%)	4(19.04%)	2(9.52%)	
Normal Control	Tissue FAS	7(70%)	3(30%)	0(0%)	00%)	10 (100%)
	Lymphocyte FAS	0(0%)	0(0%)	0(0%)	0(0%)	
Total no of GDU cases 21(100%)						

Table (4): Tissue versus lymphocytes insitu expression of FASL mRNA according to scoring system in Cag A^{positive}/Cag A ^{negative} - GDU Patients

CagA In situ	Marker CD95L (FASL)	Negative	Positive			Total No.
		Grade	Grade			
			Low	Intermediate	High	
		< 5%	5-25%	26-50%	>50%	
CagApositive	Tissue FASL	2(%)	7(%)	0(0%)	0(0%)	9(42.85%)
	Lymphocyte FASL	0(0%)	0(0%)	3(14.28%)	6(28.57%)	
CagAnegative	Tissue FASL	2(9.52%)	6(28.57%)	4(19.04%)	0(0%)	12(57.14%)
	Lymphocyte FASL	1(4.76%)	1(4.76%)	2(9.52%)	8(38.09%)	
Normal control	Tissue FASL	7(70%)	3(30%)	0(0%)	0(0%)	10 (100%)
	Lymphocyte FASL	0(0%)	0(0%)	0(0%)	0(0%)	
Total no of GDU cases		21(100%)				

Table (5): Tissue versus lymphocytes insitu expression of IL8 mRNA according to scoring system in Cag A positive/Cag Anegative GDU Patients.

CagA In situ	Marker IL8	Negative	Positive			Total No.
		Grade	Grade			
			Low	Intermediate	High	
		< 5%	5-25%	26-50%	>50%	
Cag A positive	Tissue IL8	0(0%)	3(14.28%)	4(19.04%)	2(9.52%)	9(42.85%)
	Lymphocyte IL8	0(0%)	0(0%)	2(9.52%)	7(33.33%)	
CagA negative	Tissue IL8	2(9.52%)	4 (19.04%)	4(19.04%)	2(9.52%)	12(57.14%)
	Lymphocyte IL8	1(4.76%)	0(0%)	5(23.80%)	6(28.57%)	
Normal control	Tissue IL8	7(70%)	3(30%)	0(0%)	0(0%)	10(100%)
	LymphocyteIL8	0(0%)	0(0%)	0(0%)	0(0%)	
Total no of GDU cases		21(100%)				

Table (6): Tissue versus lymphocytes insitu expression of FAS,FasL,IL8 mRNA in Cag Apositive/Cag Anegative GDU Patients

Marker mean ± SE*		Cag Apositive mean ± SE*	Cag Anegative mean ± SE*	T-Test p value	Normal Control H.pylori negative	T-Test p value
Fas mRNA	Tissue FAS	9(42.85%) 69 ±3.4238	12(57.14%) 54 ± 7.3978	(P<0.05)	3.6±0.45	(P<0.05)
	Lymphocyte FAS	9(42.85%) 35.6667 ± .2525	12(57.14% 28.2500 ± .4218	(p>0.05)	ND	ND
p- value		(P<0.05)	(P<0.05)		ND	ND
FasL mRNA	Tissue FASL	9(42.85%) 11.11±2.16	12(57.14%) 16.25±2.99	p>0.05	3.3±0.66	(P<0.05)
	Lymphocyte FASL	12(57.14%) 74.77±7.43	12(57.14%) 46.66±5.66	P<0.05	ND	ND
p- value		P<0.05	P<0.05		ND	ND
IL8 mRNA	Tissue IL8	9(42.85%) 51.44±9.59	12(57.14%) 25.50±5.17	p<0.05	3.2±0.61	(P<0.05)
	Lymphocyte IL8	9(42.85%) 80.33±6.48	12(57.14%) 46.75±5.94	p<0.05	ND	ND
p- value		p<0.05	p<0.05		ND	ND

** SE: standard error *N.D: not detected*

Table (7): Correlations among tissue and cellular markers of GDU cases

Parameters		Tissue FAS	Lymphocytes FASL	tissue FASL	ymphocytes IL8	tissue IL8	CagA
Lymphocytes FAS	Pearson Correlation	0.435*	0.301	0.052	0.213	0.101	0.083
	P value	0.049	0.185	0.824	0.355	0.664	0.719
Tissue FAS	Pearson Correlation		0.561**	0.282	0.529*	0.249	0.436*
	P value		0.008	0.215	0.014	0.277	0.048
Lymphocytes FASL	Pearson Correlation			0.272	0.650**	0.221	0.423
	P value			0.233	0.001	0.336	0.056
Tissue FASL	Pearson Correlation				0.035	0.207	0.281
	P value				0.879	0.369	0.218
Lymphocytes IL8	Pearson Correlation					0.160	0.383
	P value					0.488	0.087
Tissue IL8	Pearson Correlation						0.402
	P value						0.071

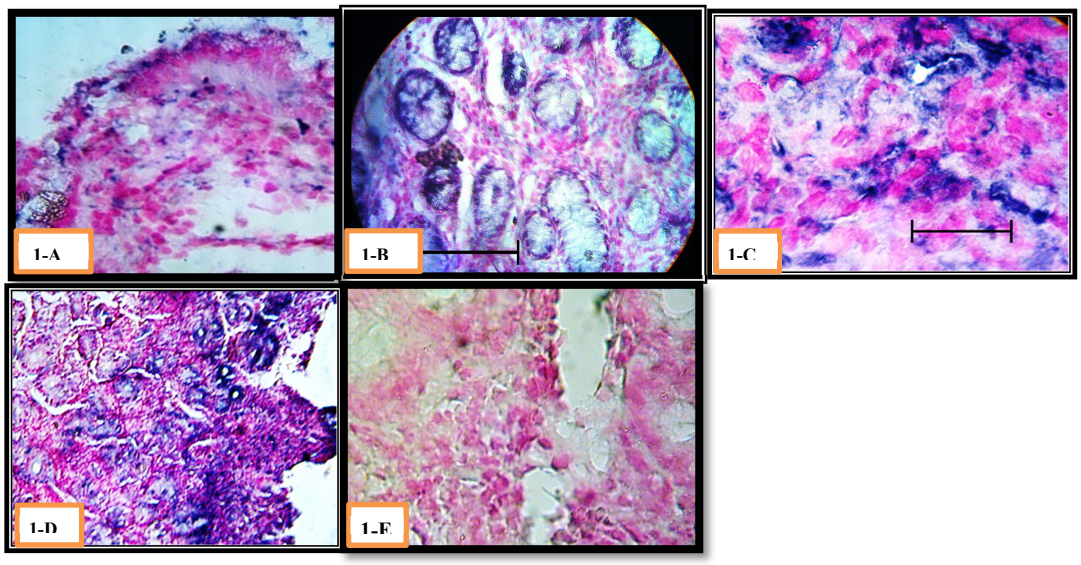

Figure (1): *In situ* hybridization for human gastric ulcer tissue section .staining by BCIP/NBT (bluish purple) counterstained with nuclear fast red. Bar size=50µm.
1-A: H.pylori Cag A mRNA expression in gastric ulcer tissue
1-B : Human Fas mRNA expression in gastric ulcer tissue
1-C: Human FasL mRNA expression in gastric ulcer tissue
1-D: Human IL8 mRNA expression in gastric ulcer tissue
1-E:Negative expressions of target genes (CagA,Fas,FasL,IL8) in normal gastric tissue section.

Discussion

In the present study, twenty-one patients were proved to have GDU by endoscopic examination. The prevalence of H.pylori associated GDU according to the age group were detected among (28-37) years age group , (28.57%) ;(48-57) and (68-77) years (23.8%). The result of the present study come in agreement with other studies [1, 13].These results suggest the distribution of H.pylori infection is pangastric in the younger patients.

In the present study (52.4%) of males under investigation had GDU and the rest (47.6%) were females. This result come in contrary with that reported by other studies in [14] found that the prevalence of GDU associated with H.pylori infection was detected more frequently among women (60.6%) compared with (42.9%) among male patients . The possible reason for such difference in results may be related to study design which was an epidemiological study and focus on the prevalence of H.pylori infection in relation with gender and the size of community under investigation that give a chance for difference in sample size of each gender type which is apparently larger in case of females in European communities .Emotional stress factor may have a role in susceptibility to H.pylori infection .Gender differences to H.pylori induced gastroduodenal diseases and in disease susceptibility may reflect differences in immune responses, hormonal effects and sex linked genetic

factors and differences in bacterial colonization [1]

The ability of H.pylori to induce GDU in thirty-six selected patients in the present study was related to several factors mainly H.pylori related virulence factors that contribute in dissimilar ways to gastric mucosal damage. Among these factors are those that known to be required for the colonization and survival of H. pylori in the human stomach. These factors are urease and flagella that are expressed by all Helicobacter species. H. pylori manufacture large amounts of urease that helps to break down urea into carbon dioxide and ammonium, which neutralizes the acid during colonization of the stomach. The protection of H.pylori by this enzyme also induces apoptosis of gastric cells in vitro and inhibits gastric somatostatin release in animals, which could have consequences for the physiology of digestion in general([15, 16]. On the other hand, the chronic nature of GDU may reflect the vital role of bacterial flagellar motility of H.pylori which has essential role for successful colonization of gastric mucosa. Flagella allow the bacterium to swim across the viscous gastric mucus and reach the more neutral pH underneath the mucus. This property also enables the bacterium to resist the muscular contractions that regularly empty the stomach. Adhesins utilized by H.pylori for adhesion with gastroduodenal epithelial cells and stimulate pedestal formation and develop attaching-effacing lesions [16].

Our results come in agreement with earlier studies that focused on the possible role of H.pylori in the development of GDU . High detection level of H.pylori infection in present study come in concordance with that of Nirag et al.,(2003), they found that (68.65%) of gastric biopsy specimens obtained from DU cases were h.pylori positive in Haematoxylin and Eosin stain as well as immunohistochemistry . Cutler et al, (1995) reported recorded that immunoreactivity for H.pylori in the lamina propria was detected in 25% of their gastric biopsy specimens.

Our result come in agreement with [13],they detect H.pylori in (78%) of GU cases using Haematoxylin and Eosin(H&E) stain and H.pylori –specific antibody immune staining. The use of H&E stain which was considered as a common histopathologically utilized stain when used for detection of H.pylori infection in tissue section it was less sensitive than our ISH molecular technique beside the effect of visualization experience of pathologist which play a great role in missing of large number of positive cases.

We agree with opinion which was delivered after a series of clinical and molecular analysis by [17]they stated that H.pylori and CagA antibodies were strongly associated with atrophic antral gastritis and Cag ApositiveH.pylori strains cause more sever gastritis than CagA negative strains, in addition patients infected with Cag Apositive strain may also be more susceptible to peptic ulcer[18]. Our results come in concordance with [19], they found that (88.2%) of patients with GDU have Cag A positive infective strain in their tissue biopsy using PCR technique. The differences most likely related to the sensitivity of PCR technique that used for detection of the infective strain even it was present in a very limited numbers inside the infected tissue and the ability of amplification of target CagA gene for hundred times which we lack in our ISH technique beside other possible factors such as the sample size of population under investigation as well as genetic environmental factors, economic and social habits which may have a role in high percentage of infection .

The result of the present study agree with that of [20]they conclude that there was a strong correlation between infection with type I strains(CagApositive ,VacA positive) and occurrence of severe gastroduodenal diseases, whereas type II strains (CagA negative, VacA negative) with attenuated virulence do not induce dramatic change in the gastric mucosa. Thus, H. pylori strains that express CagA cause more extensive inflammation of the gastric mucosa. Infections with these strains have been reported to be more likely to result in peptic ulceration, atrophic gastritis and gastric adenocarcinoma [21]

Fas Ag has been shown to be constitutively expressed at low levels in normal gastrointestinal tract tissue while in areas of inflammation, peripherally activated lymphocyte s express both the Fas Ag and the Fas ligand (Fas L) on their cell surface, and secrete a soluble form of FasL .In the gastrointestinal tract, Fas receptor normally is constitutively expressed in epithelial cells. By contrast, there is little or no detectable Fas ligand expression, except on the surface of Paneth cells .Interaction between Fas Ag and Fas L is one of the early steps in the classical Fas-mediated apoptotic program. Constitutive expression of basal levels of Fas Ag in gastric and duodenal tissues suggests the inherent sensitivity of this tissue to Fas-mediated apoptosis ([22, 23]

The results of the present study give a primary indication about the vital role of H.pylori as we notice that there was obvious effect on the grade of expression of FAS& FASL in tissue and infiltrating lymphocytes among cases that harbored this pathogen in gastroduodenal biopsies compared with normal apparently healthy control. This could be related to several factors belongs to molecular interaction between pathogen and mucosal surface of its host. Our results come in concordance with [16, 24]who explain that functions of adherent factors which are utilized by H.pylori for its adhesion to the gastric epithelial cells possibly inhibited or enhanced by other bacterial virulence factors during the processes of adhesin synthesis, transportation, secretion and activation mainly via Cag PAI encoding genes, this collectively play a central role in the final scenario of interaction between host and pathogen. Whenever pathogen have potent Cag PAI, bacterial adhesion, and good signals will be received by the gastric epithelial cells which reflect its response by increasing of expression of MHCII to play as antigen presenting cell (APC) and strong Th1 response will be occur with obvious IFNγ

secretion which act as good stimulator for up-regulation of FAS and even FASL in gastroduodenal tissue (GDT) and tissue infiltrating lymphocytes (TILs)[16].

In the present study, the mean of tissue FAS m RNA expression greater than the mean of tissue FASL m RNA (69±3.4 V 11.11± 2.16) among Cag A positive GDU cases and (54±7.3V 16.25± 2.99) among Cag A negative GDU. On the other hand the mean of lymphocyte FAS m RNA expression lesser than lymphocyte FASL m RNA among Cag A positive GDU cases (35.66±4.25 V 74.77±7.43) and (28.25±.42 V 46.66±5.66) among Cag A negative GDU cases.The results of the above comparison indicate that fratricide happened in lesser extent than suicide in cases of tissue and infiltrating lymphocytes in our GDU cases under investigation. These results come in agreement with [23, 25] who mentioned that gastric epithelial cell apoptosis may be induced not only by CD95L-expressing lymphocytes but also by CD95L-expressing epithelial cells and CD95L might be expressed as a membrane-bound form on epithelial cells and mediate apoptosis by "fratricide" interacting with CD95 on neighboring epithelial cells or through suicide of the CD95L-CD95–expressing cell itself.

The result of our work come in concordance with others stated that upregulation of CD95 receptor and FasL expression and induction of apoptosis in gastric epithelial cell line was achieved with the cytotoxic (Cag A positive & VacA positive) H. pylori strain [22, 23, 26]. Cytotoxic H. pylori strains produce high concentrations of VacA and induce vacuolation in gastric epithelial cells in vitro. These strains are more virulent and are associated with more sever disease such as peptic ulceration [23, 27, 28]. Upregulation of Fas-FasL in gastric tissue may be related to the cytokine microenvironment or H.pylori factors other than Cag, Vac .this opinion was come in agreement with [23, 29] , they stated that CagA producing H. pylori strains are associated with increased cytokine production On the other hand lipopolysaccharide (LPS) isolated from a cytotoxic H. pylori strain was found to be responsible for induction of apoptosis in rat gastric epithelial cells for this we can consider H.pylori LPS as one of apoptogenic factors but not the universal one[30]. Mucosal colonization by the bacterium ignites a cascade of events that would be expected to result in large increases in inflammatory cytokines in the infected tissue which were originated from the gastric mucosa as well as from infiltrating inflammatory cells. Among these inflammatory cytokines, IL-1, IL-2, TNF-α, and IFNγ that have been shown to up-regulate the expression of Fas Ag in cell lines and in gastroduodenal biopsies that were studied using ISH detection technique .They elucidate that the organism per se does not directly up-regulate Fas Ag in gastroduodenal tissue culture system, and they suggest that it may act in vivo through its effect on the immune system ([23, 31] . The possibility of cytokine up-regulation of Fas Ag in this tissue would make it an attractive target for FasL expressed by invading inflammatory cells [32].These data strongly support the important pathogenic and functional role of the CD95 receptor and ligand system in H. pylori–induced apoptosis.

Several independent approaches have suggested that T-helper type 1 (Th1) cells are selectively increased during H.pylori infection [33] .Th1 cytokines, such as IFNγ and TNF-α, can increase the release of proinflammatory cytokines, such as IL-8 from the epithelium as well as Fas and Fas ligand (FasL) [34] .Furthermore, these cytokines can also increase the expression of MHC class II molecules by gastric epithelial cells, thereby increasing the binding of H. pylori to the gastric epithelium [35].

As Th1 cells are associated with cell-mediated immune responses, they may also play a role in damaging gastric tissues directly by triggering apoptosis in gastric epithelial cells. One mechanism by which T cells kill their targets is expression of FasL and its binding to the Fas receptor. This mechanism of killing can be achieved by both CD8+ cytotoxic T lymphocytes, as well as CD4+ Th1 cells ([36]. FasL expression was increased in gastric tissue and on mononuclear cells adjacent to the epithelium and in lamina propria [23, 37]. Th1 cells can also express higher levels of FasL than Th2 cells ([38].In addition, some cytokines, such as TNF-α or IFNγ, can induce apoptosis directly, as well as augment the effects of the bacteria alone [23, 39].

Furthermore multiple immunohistochemical evidence suggests that cytokine-producing Th1 cells predominate in the gastric antral mucosa during infection [35].In addition, Th1 cells also dominate in the healthy uninfected gastric mucosa ([23]. or in a state of gastritis for other reasons ([38]. Th1 cytokines including IFNγ and TNF- α, and H. pylori infection activate transcription factors in gastric epithelial cells that bind to the NF-kB and AP-1 regulatory sites[40] . Since these regulatory sites are located in the 59 region of the Fas gene, it is possible that both can contribute to the induction of Fas expression. However, our results showed that H. pylori strain, which lacks the cag PAI, induced Fas expression, although to a lesser degree [16, 23].

H.pylori associated inflammatory reaction is characterized by a massive mucosal infiltration of polymorphonuclear leukocytes (PMN), T cells, macrophages, and plasma cells. These cellular changes are associated with enhanced production of cytokines, which are believed to contribute to maintaining the gastric inflammation and causing epithelial cell damage[23] Considerable evidence has been accumulated to indicate that IL-8, the major human PMN chemoatractant, plays a major role in the H. pylori associated acute inflammatory response [23]. Both IL-8 mRNA and protein levels strictly correlate with H. pylori density[41].IL-8 expression has been associated with significantly more sever infiltration of PMN, and down-regulation of mucosal IL-8 synthesis, induced by H. pylori eradication, is paralleled by a resolution of the PMN infiltration[42] .During H. pylori infection, epithelial cells are the major producers of IL-8. Although several studies have documented the

ability of H. pylori to directly stimulate IL-8 synthesis, there is evidence that T cell-derived cytokines may modulate epithelial cell IL-8 gene expression[23] . The results of the present study come in concordance with[43] who found that antral mucosal biopsies from DU cases in Japanese patients infected with Cag positive H.pylori strain have high level of insitu IL8 expression compared with patient have DU and infected with CagA negative H.pylori strain with obvious significant difference between two groups. The result of the present work agree with that recoded by [44, 45] who found that the level of insitu IL8 expression was higher among DU. They found that positive rate of antral IL8 mRNA expression in gastric epithelial cells was significantly higher in CagApositive GDU H.pylori compared with CagAnegative H.pylori with concurrent increase in the inflammatory cell infiltrates of mononuclear cell and polymorphonuclear cell types . They proved that the level of IL8 m RNA expression was reduced after successful eradication of CagA positive H.pylori. Several studies indicated that the infection with H.pylori induced the expression and production of various cytokines in gastric mucosa, and the cytokines contribute to the pathogenesis of H.pylori associated gastro- duodenal disease but the contribution of human gastric epithelial cells to the cytokine induction was put under focus very rarely. It was proposed that the ability to stimulate cytokine induction is associated with several gene products of H.pylori but the mechanism of cytokine induction via H.pylori still remains unclear, though the Cag PAI was found to be closely related to IL8 induction. Since H.pylori exists in gastric mucosal layer overlying the epithelium and does not invade the epithelial tissue, the production of urease from noninvasive bacteria stimulate the induction of proinflammatory cytokines and subsequently apoptotic process in gastric mucosa [23, 46]. The Cag independent pathway of cytokine induction also may involve inducing mucosal inflammation and this fact come in agreement with the result of the present study in which IL8 mRNA was detected insitu among CagA negative GDU cases although the level of expression was significantly lower than that detected among CagApositive GDU/CAG cases but still the level of IL8 mRNA expression was higher among H.pylori positive cases compared with control group uninfected with H.pylori [23].

As a consequence of infection with H.pylori mainly Cag positive strains that has consistently been associated with enhanced neutrophil infiltration, more than CagAnegative , the activated neutrophils may be act as an important contributors of gastroduodenal mucosal damage via the generation of reactive oxygen metabolites and the release of proteolytic enzymes ([47]. The possible explanation for the gastroduodenal damage may be related to the fact that many components of H.pylori have been shown to induce neutrophil chemotaxis or activation. Stimulation of neutrophil myeloperoxidase, reactive oxygen metabolites, and changes in adhesion molecule expression result following exposure to components of H. pylori[23].

A neutrophil-activating protein of 150kDa (HP-NAP) that has been purified from water extracts of H. pylori and the napA gene, which encodes for this protein, is present in all strains. HP-NAP may have multiple functions, being induced in the bacterium by acid stress [23]. Evidence shows that the ability of H. pylori to directly activate neutrophils is independent of both toxin production and the Cag PAI[9] . Neutrophil infiltration will also be induced by host inflammatory mediators. Particular interest has focused on the C-X-C chemokine family of peptides, which have neutrophil-activating and chemotactic properties. Infection with Cag positive strains is associated with increased gastric IL-8 mRNA and IL-8 protein and increased IL-1α/IL-1β .Transcription of other C-X-C chemokines, such asENA-78 and GRO-α in the antral mucosa, is also increased in Cag positive infection[45].

The gastric epithelium is a major source of chemokines such as IL-8 in H. pylori infection. In vitro studies have shown that strains with the Cag PAI directly stimulateIL-8 secretion in gastric epithelial cells and multiple genes in the Cag PAI are essential for induction of this response[23] . The chemokine response is associated with NF-kB activation and tyrosine phosphorylation of host proteins. Although most in vivo studies assessing chemokine responses and bacterial genotype have been undertaken on gastric biopsies, the response of infected duodenal mucosa is likely to be similar. In duodenitis, enhanced IL-8 immunoreactivity in the epithelium of the duodenal bulb mucosa is evident. The induction of chemokines in the epithelium of the gastro duodenal mucosa by Cag positive strains of H. pylori is likely to be an important factor contributing to enhance polymorphonuclear cell infiltration[9].

In the present study we demonstrated that among CagApositive cases of GDU ,the level of IL8 produce via tissue infiltrating lymphocytes in a level higher than that detected in tissue which reflect the fact that not only the infected tissue play a role in the inflammatory process . The degree of inflammatory infiltrate appears to be affected by the strain of the infective pathogen whether it is CagA positive or CagAnegative. Generally, GDU cases infected with CagApositive strain characterized by a higher degree of inflammatory infiltrate when compared with CagAnegative cases[23].

Neutrophils themselves localized within gastric epithelium, foveolar lumen and mucosal erosion produced Groα and IL-8 during H. pylori infection, which may enhance their own recruitment. By this autocrine mechanism gastritis activity and mucosal damage may be amplified[23]. we propose that both chemokines IL-8 and Groα modulate gastric activity through stimulation of neutrophil recruitment.

Based on the concept of multistep navigation and combinatorial control of leukocyte chemotaxis by[48] ,

it may be speculated that a spatial and temporal change in Groα /IL-8 expression builds up a step by step gradient, which navigates neutrophil migration from the mucosal vessel into the gastric epithelium. According to our IL-8 expression pattern, the following scenario for neutrophil diapedesis and migration in H. pylori gastritis is proposed. Initially, neutrophil diapedesis is forced by Groα from endothelial cells of small gastric mucosal vessels[23]. In the next step, perivascular lamina propria macrophages express Groα and IL-8, directing neutrophils into the gastric mucosa. Then, Groα and IL-8 are up-regulated in the gastric epithelium, whereas lamina propria macrophages down-regulate these chemokines. By this mechanism, a chemokine gradient is built up, leading to migration of neutrophils from the vessels across the lamina propria into the gastric epithelium. Finally, neutrophils themselves localized within gastric epithelium, foveolar lumen and mucosal erosion produce Groα and IL-8 and may support their own recruitment .This speculation give a theoretical support to the facts find out in the present study that histological sections prepared from GDU cases infected with CagApositive H.pylori strain characterized by high grade of inflammatory cell infiltration accompanied by high grade of IL8 m RNA expression from gastric epithelia and in a higher degree from inflammatory cells mainly lymphocytes and neutrophils and this was positively correlated with density of H.pylori that infect the tissue , the virulence of the infective strain as well as the possibility of infection with toxigenic and non-toxigenic strain at the same time [9, 23].

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