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METHYLATION OF P16 GENE DURING GASTRIC CARCINOGENESIS IN ASSOCIATION WITH *HELICOBACTER PYLORI* INFECTION

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ABSTRACT

Four Histo-examination patterns of gastric biopsies used to study methylation status of *p16* promoter upon *Helicobacter pylori* infections. The incidence of *H. pylori* in 120 samples was done by 23S ribosomal RNA gene. Statistical significant association (p= 0.03) have been showed between *H. pylori* and carcinogenesis progression, comprising normal gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer. Methyl-specific PCR showed that the methylation status of p16 promoter was increase during carcinogenesis progression (p=0.0015). Among 56 *H. pylori* infected cases, 34(60%) were p16 promoter methylated. Within each disease step all *H. pylori* infected cases showed higher p16 promoter methylation frequencies than *H. pylori* non-infected cases, with increase status of methylation during disease progression. It was 33.3% in normal gastritis cases and raised 82.3% in cancer cases. The highly association between *H. pylori* infection and hypermethylation of p16 promoter (p= 0.0009) confirms the role of *H. pylori* in silencing of p16 gene.

KEYWORDS: ***Helicobacter pylori,*** **P16, DNA methylation, tumor suppressor gene carcinogenesis.**

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INTRODUCTION

Helicobacter pylori is one of the most important human pathogen, infects the stomach of approximately 50% of the world's human population.[1,2] Abundant evidences were elucidated the strong association between gastric chronic infection of H. pylori and the progression of gastric cancer, then was accused as the causative agent of chronic atrophic gastritis, a precursor of gastric carcinomas via a series of steps comprising gastritis, atrophy, intestinal metaplasia, dysplasia and gastric cancer.[3] The cell progression to cancer requires stepwise up regulation or down regulation of critical genes. Which divided into genetic alteration and aberrant epigenetic modifications. Mutation, loss of heterozygosity, translocation, small and deletions have each been contributed toward these alterations in gene expression. Epigenetic abnormalities are DNA methylation, histone modification and chromatin remolding. Methylation of CpGs island of genes promoter is one of the most important genomic change which is associated with silencing of gene expression, usually tumor suppressor gene. Global hypomethylation of repetitive sequence (LINE and Alu) leads to genomic instability and activate oncogenes, consequently these events can alter cell phenotype.[4,5] Inactivation of many housekeeping genes especially tumor suppressor genes has been reported in gastric cancer such as cadherin 1 (CDH1), DAP-kinase, GSTP-1, MGMT, p14, RASSF1A, APC, COX-2, THBS1, TIMP2,RUNX3 and p16 cyclin-dependent kinase inhibitor 2A (CDKN2A) are more often inactivated due to methylation than from mutations or chromosomal deletion.[3,6,7,8,9] The aim of this study is to detect of aberrant DNA methylation of P16 promoter region in different outcomes of H. pylori infections progression. DNA methylation is molecular signatures including a panel of methylated CpGs may show the potential advantage in the screening and prognosis or early diagnosis or therapy response prediction.

MATERIALS AND METHODS

The sources of samples were gastric biopsies (n=120). Samples were collected during the period 10/2/2015 to 20/3/2016. Upper gastrointestinal tract disease patients were diagnosed clinically and the disease was evaluated by specialist physicians, presented with dyspepsia referred to the Esophagus Gastro-duodenum Scope Unit for upper endoscopy at many Iraqi hospitals. Three tissue biopsies were obtained from antrum. Rapid urease test was performed for the first antral biopsies at the time of endoscopy. The other biopsy specimens placed in 1 ml of formalin was examined under microscope after formalin embedding. Another biopsy specimen was preserved immediately at −60°C for molecular analysis. The gene of 23S rRNA was used to detection of H. pylori, amplification and melting conditions were optimized for the PCR using specific primer set forward GGTCTCAGCAAAGAGTCCCT, and reverse CCCACCAAGCATTGTCCT for 41 cycles at 75°C annealing temperature for 35 sec, the product was 493 bp.[10] Bisulfite conversion of genomic DNA was conducted according Herman at al.,[11] one microgram of genomic DNA was denatured in 0.2 M NaOH for 10 minutes at 37°C. The denatured DNA was diluted in 500 µl of freshly prepared solution of 10 mM hydroquinone and 3 M sodium bisulfite and incubated for 16 h at 50°C. After incubation, the DNA sample was desalinized through a column (Wizard DNA Clean-Up System; Promega, Madison, WI, USA), treated with 0.3 M NaOH for 10 minutes at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was suspended in 40 µL of H_2O. Two sets of primers described by Herman at al.,[11] were used for methyl-specific PCR (MSP): for methylated cases (TTATTAGAGGGTG-GGGCGGATCGC) forward and (GACCCCGAACCGCGACCGTAA) reverse (150bp product) treated by 40 reaction cycle at 65C° annealing temperatures. The unmethylated cases detected by using primers (TTATTAGAGGGTGGGGTGGATTGT) forward and (CAACCCCAA-ACCACAACCATAA) reverse (151bp) treated by 41 reaction cycle at 60C° annealing temperatures.

STATISTICAL ANALYSIS

The association relationships were carried out by Fisher's exact test according to ©2016 GraphPad Software (http://graphpad.com /quickcalcs/contingency1.cfm). P-value was calculated with Two-tailed as recommended in this software.

RESULTS AND DISCUSSION

One hundred twenty patients were enrolled in this study. Four groups were identified according to tissue differentiation during disease development: normal, atrophic gastritis, intestinal metaplasia and gastric, respectively Table (1). During development of intestinal-type adenocarcinoma, series of well-defined histological progression steps have been identified in several examinations,[12] initiated by the transition from normal mucosa to chronic superficial gastritis, which followed by multifocal atrophic gastritis then intestinal metaplasia, finally the tissue could be transformed into adenocarcinoma.[13]

Table 1

Studied cases

Cases groups of disease development	No. of cases	Percentage	Gender		Mean of age (years)	Range of age (years)
			Male	Female		
Normal	31	25.8 %	11	20	41.2 ± 1.1	13 to 81
Atrophic Gastritis	30	25 %	12	18		
Intestinal Metaplasia	32	26.6 %	16	16		
Gastric Cancer	27	22.5 %	14	13		
Total	120	100 %	53	67		

Specific primer sequences were designed for 23S ribosomal RNA gene (493bp) for the purpose of PCR assay, Figure (1). Accordingly, 56 (46.6%) of patients were consider *H. pylori* infected in this study, while 64 (53.3%) patients were PCR negative.

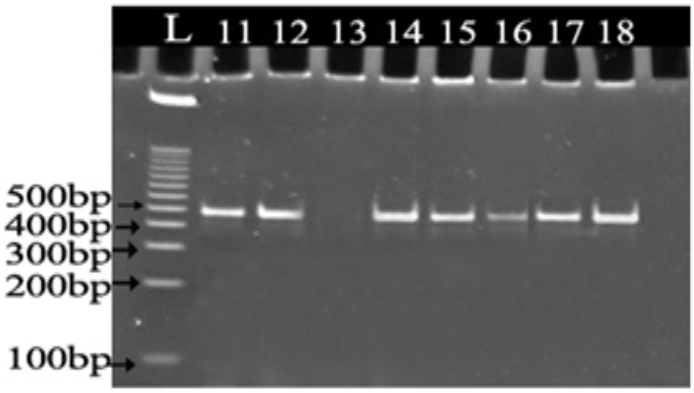

Figure 1
Gel electrophoresis analysis of PCR product of H. pylori 23S ribosomal RNA gene (493bp), lane1:size marker, lane 4: negative control, whereas other lanes are positive result, using 5% polyacryl amide gel at 6 volt /cm for 1.5 hour

Patients with gastric cancer pattern showed the highest frequency of *H. pylori* infected (63%), while lower rate was observed in normal patients (29%) Figure (2). Previous studies revealed that *H. pylori* prevalence increased with development of carcinogenesis, severe atrophic gastritis, corpus-predominant gastritis, or both, along with intestinal metaplasia are at high risk for intestinal-type gastric cancer with incidence of *H. pylori* infections.[14]It has been shown that intestinal-type gastric cancer developed in *H. pylori*-positive patients who have severe multifocal atrophic gastritis in association with intestinal metaplasia.[15] So, atrophic gastritis can be causes by *H. pylori* infection.

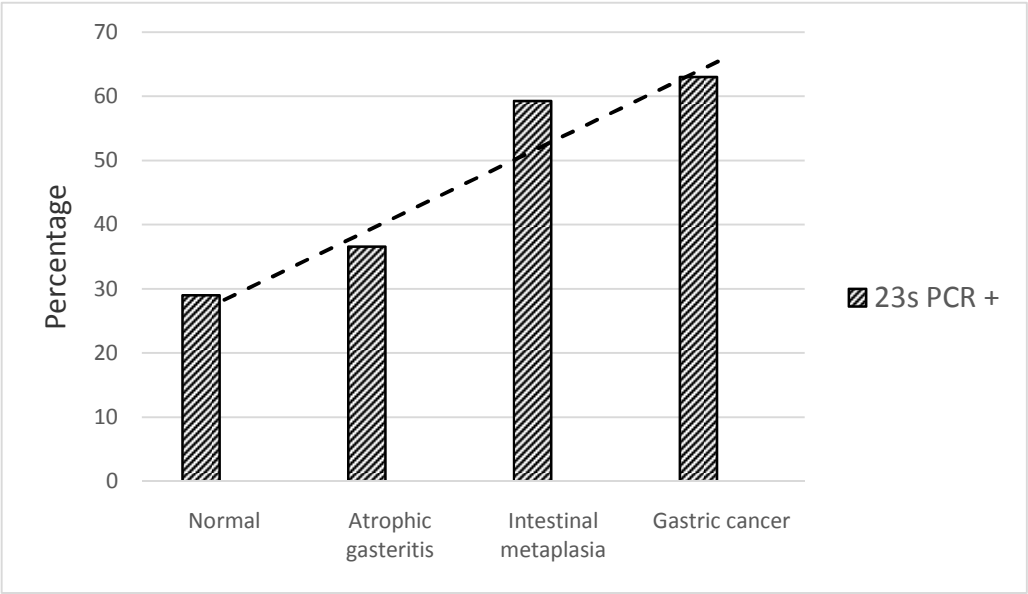

Figure 2
Distribution of patients according PCR assay upon disease progression(p=0.03).

The relationship between *H. pylori* and total patterns of patients considered statically significant (p= 0.03). This results confirm the previous studies which proved that *H. pylori* is the causative agent of gastric cancer progression.[3] For several years, the relationship between *H. pylori* and intestinal type gastric carcinoma was a matter of debate among scientists. However, abundant studies in last two decades provided clear evidences that this bacterium was significantly increases gastric cancer risk, one of such result revealed in a study of *H. pylori* infection in 1,526 Japanese patients,[16]by comparing to uninfected patients. Another study showed that about 3% of *H. pylori* infected patients developed intestinal type gastric cancer.[16] Corresponding studies demonstrated that eradication of *H. pylori* was decreases the risk of intestinal type gastric carcinoma in patients without premalignant lesions. That provided a clear evidence that *H. pylori* have an effect on early stages of gastric carcinogenesis.[17,18] Other studies revealed that the eradication of *H. pylori* by antibiotics attenuated the progression of gastric cancer.[19,20] Taken together the current study with others they support an unequivocal role for *H. pylori* in the development of gastric cancer.

Association between methylation status of p16 promoter and progression of disease.

The results of methyl-specific PCR for p16 promoter region showed that there are 53 (44.2%) methylated of 120 cases Figure (3). The methylation frequency of p16 promoter increased while the disease progressing, starting with normal, atrophic gastritis, intestinal metaplasia to cancer as 19.3%,33.3%,53.1% and 74% respectively Figure (4), which means that the degree of *p16* promoter hypermethylation increased with the increasing severity of the disease. The relationship between increasing of p16 promoter hypermethylation and progression of disease was considered statistically significant (p= 0.0015) Table (2).

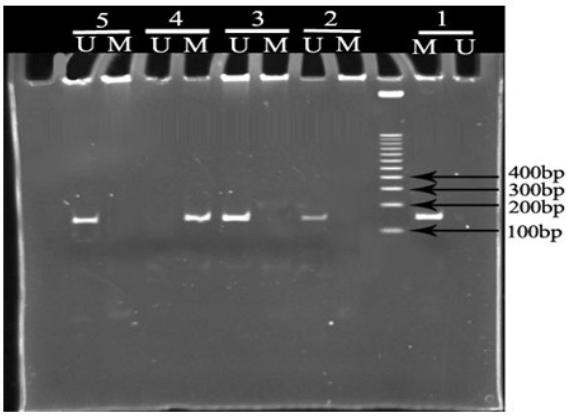

Figure 3
Gel electrophoresis of MSP product of p16 promoter using 5% polyacryl amide gel at 6volt/cm for 1 hour
M: Product amplified with methylated-specific primer (151bp, U: product amplified with unmethylated-specific primer (150bp) Lane1 : positive methylated control, Lane5: positive unmethylated control, Lane 2,3 and 4: samples

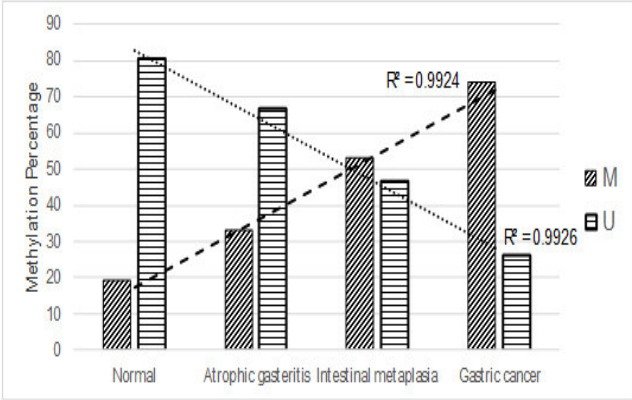

Figure 4
Distribution of patients according p16 promoter Methylation upon disease progression,
were M= methylated frequency, U= unmethylated frequency

Table 2
Distribution with statistical analysis of patients between patterns and *p16* promoter methylation

Cases	P16 M (%)	P16 U (%)	p-value (normal vs each pattern)	p-value (normal vs total patterns)
Normal 31	6 (19.3%)	25 (80.7%)		0.0015
Atrophic gastritis 30	10 (33.3%)	20 (66.6%)	0.25	
Intestinal metaplasia 32	17 (53.1%)	15 (46.9%)	0.0085	
Gastric cancer 27	20 (74%)	7 (26%)	0.0001	
Total 120	53 (44.2%)	67 (55.8%)		

M corresponding to methylated cases, U corresponding to unmethylated cases.

Hypermethylation of CpG islands of gene promoter is an important epigenetic molecular mechanism for gene silencing, which confers tumor cell growth advantage. Hypermethylation of CpG islands of promoter genes has been revealed for many genes that associated with loss of expression of many potential key genes in cancer cells.[21] Methylation of cytosine residues at CpG sites in *p16* gene promotor, resulting in silencing of *p16* expression, which has been reported in many cell lines, including AGS, and some primary carcinomas in different organs, such as colon, brain, breast, bladder, ovary, lung, and myeloma and others.[22,23] Abbaszadegan *et al.,*[24] demonstrated that the methylation of *p16* is one of the most frequently methylated genes in gastric cancer. Their finding in cancer tissues and the corresponding serum obtained from gastric cancer patients. Aberrant methylation of the *p16* promoter was detected in 44.2% less than current finding 74%, whereas all normal gastric mucosa samples (*n* = 50) were unmethylated. The results of the present study are in corresponded with the previously examined silencing of *p16* gene by promoter hypermethylation in colorectal cancer. Thirty-two cases were diagnosed in Stage I and Stage II of the disease. Among these cases, 18 were hypermethylated and this supports the result of methylation frequencies of intestinal metaplasia pattern of this study. When the frequency of *p16* promoter hypermethylation was compared with the clinical staging of the disease, *p16* promoter hypermethylation was found to be higher in Stage III/IV (83.33%) compared to Stage I/ II (56.25%) and that closely related with methylation of *p16* in pattern of cancer in this study (74%).[25] Gastric tumor tissue in another study, revealed that the aberrant methylation status of *p16* was 30% (6/20). The difference of promoter methylation of these genes between tumor tissues and control group was significantly observed (p=0.02). So, the present result conformed this finding with more association ratio (p=0.0015).[26] The sensitive restriction endonuclease combined with PCR technique, was used to detect the aberrant methylation at CpGs of *p16* promoter in gastric carcinoma tissue. This technique revealed that abnormal methylation was present in 12 of 20 cancer cases, and confirmed the current results which recorded 20 methylated cases of 27 cancer cases. This suggests that there are associations between methylation of *p16* gene and gastric carcinogenesis.[27] As shown in the colon carcinoma cells, without homozygous deletion, methylation was occurred at both alleles of *p16* gene, and was related to the entire deactivation of this gene.[28] Based on the fact that the alteration of methylation status of *p16* gene and other genes occur in many kinds of carcinomas lacking of mutation and deletion, methylation is the key molecular mechanism of deactivation of tumor suppressor genes in primary carcinoma.

Association between disease progression and methylation status of p16 promoter upon Helicobacter pylori infection

The current study revealed that 34(60%) cases were p16 promoter methylated among 56 suspected as *H. pylori* infected, Figure (5).

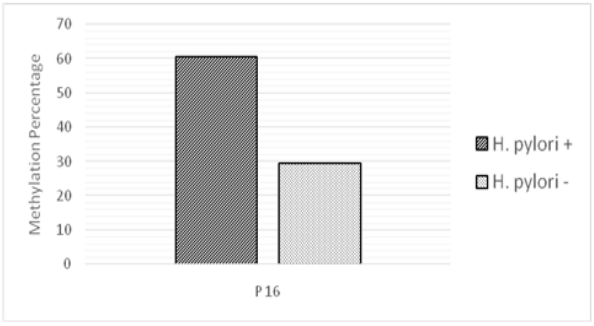

Figure 5
Distribution of patients between H. pylori infection and p16 promoter methylation status

Figure (6) shows the correlation between methylation status of *p16* and *H. pylori* infection within each pattern, starting from normal, atrophy, metaplasia to cancer. All patient suspected as *H. pylori* infected were showed higher *p16* methylation frequencies, than non-infected patients. *p16* methylation of the infected patients were 33.3% in normal patients and became 82.3% in cancer patients, while non-infected patients were 13.6% and 60% in normal and cancer respectively

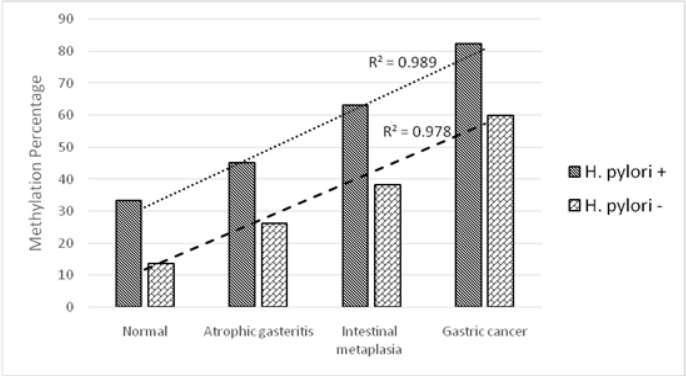

Figure 6
Distribution of patients between H. pylori infection and methylation status of p16 promoter during disease progression.

The results revealed a strong association between *H. pylori* infection and hypermethylation of *p16* promoter during disease progressing, statistically, its considered extreme significant (p-value 0.0009) (Table 3).A similar corresponding study revealed that the association between *H. pylori* infection and aberrant methylation status was exanimated in CpGs islands obtained from gastric mucosa, including normal control gastric biopsies from 45 outpatients, gastric dysplasia, and 80 paired sporadic gastric carcinomas (SGC) as well as the adjacent non-neoplastic gastric tissues. Methyl-specific PCR analysis showed that the methylation level in these islands were 61.3% and their corresponding normal tissues as a control 41.3% methylation in both normal gastric mucosa.[29] This confirmed the association of methylation frequencies with *H. pylori* infection in this study.

Table 3

Distribution with statistical analysis of cases between patterns and p16 promoter methylation upon H. pylori infection patients.M corresponding to methylated cases, U corresponding to unmethylated cases.

Cases	23S *H. pylori*	P16 M	P16 U	p-value
Normal	+9	3 (33.3%)	6 (66.6%)	0.32
31	-22	3 (13.6%)	19 (86.3%)	
Atrophic gastritis	+11	5 (45.1%)	6 (54.5%)	0.42
30	-19	5 (26.3%)	14 (73.6%)	
Intestinal metaplasia	+19	12 (63.1%)	7 (36.8%)	0.28
32	-13	5 (38.4%)	8 (61.5%)	
Gastric cancer	+17	14 (82.3%)	3 (17.6%)	0.36
27	-10	6 (60%)	4 (40%)	
Total	56 +	34 (60.7%)	22 (39.2%)	0.0009
120	64 -	19 (29.6%)	45 (70.3%)	

H. pylori infection is thought to contribute to its induction in gastric mucosa. However, the underling mechanisms are not completely clear, despite the belief that increased activity or expression of DNA methyltransferases (DNMTs), or decreased demethylation activity may contribute to the excessive methylation status. Promoter methylation have been revealed in p16 and other tumor suppresser genes in gastric cancer, among which Epithelial-cadherin (Ecadherin), Mut L homologue 1 (MLH1), Runt-related transcription factor 3 (RUNX3), adenomatous polyposis coli (APC), O(6)-methylguanine-DNA methyltransferase (MGMT), Ras association domain family 1A (RASSF1A) and death associated protein kinase (DAPK) have been extensively studied.[30,31] In conclusion advances in the molecular medicine have not only clarified the carcinogenesis progression of gastric cancer, but also offered new novel approaches regarding prevention, prognosis, diagnosis and therapeutic intervention, taking advantages from the gradient alteration of DNA methylation status upon disease progression. Most of epigenetic biomarkers of cancer DNA methylation are well-known tumor suppressor genes silenced in primary tumor tissues.[23]

CONCLUSION

Status of p16 promoter methylation increased with carcinogenesis progression starting from atrophy, intestinal metaplasia to gastric cancer. There is a strong association between *H. pylori* infection and increase of p16 promoter methylation status during carcinogenesis of gastric cancer (p=0.0009) revealed that *H. pylori* infection is responsible for inactivating of p16 gene.

CONFLICT OF INTEREST

Conflict of interest declared none.

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