
Expression of Cyclooxygenase-2 (COX-2) in *Helicobacter Pylori* Premalignant and Malignant Gastric Lesion, the Association with Tumor Angiogenesis.

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Abstract

Background: Cyclooxygenase-2 (Cox-2) is a central mediator in inflammation and cancer. Expression of the Cox-2 gene is up-regulated in the gastric mucosa during *H. pylori* infection. Vascular endothelial growth factor (VEGF) has a potent angiogenic activity and cyclooxygenase-2 (COX-2) promotes angiogenesis by modulated production of angiogenic factors including VEGF.

Aim: investigate the distribution and intensity of COX-2 and VEGF expression in premalignant and malignant gastric lesions with *H. pylori*.

Material and Methods: Gastric biopsies from patients with chronic active gastritis and gastric adenocarcinoma, and control group were studied. Immunohistochemical analysis was used to examine the expression of COX-2 and VEGF in 81 cases of patients, including 30 cases with chronic gastritis infected with *H. pylori*, 51 cases of gastric cancer, 42 cases from GC have *H. pylori* and 30 cases with normal mucosa and none infected with *H. pylori* as control group.

Results: In *H. pylori*-infected patients, COX-2 expression was predominantly found in the epithelium and, to a lesser extent, in the lamina propria. In the non infected group, few cases demonstrated weak COX-2 expression. Intensity of COX-2 was significantly different between the chronic active gastritis, gastric adenocarcinoma groups and control group. The positive rate of COX-2 was increased from chronic gastritis (60%), to gastric cancer (88.09%) in patients with *H. pylori*, compared with negative ones (22.58%). COX-2 was expressed in 23 of 24 intestinal types and in 14 of 18 diffuse types' carcinomas. The negative VEGF carcinomas have turned positive for COX-2 only for 76.92% of the cases. Different from those, the positive VEGF carcinomas have associated COX-2 immunoreactivity in 93.10% of the cases.

Conclusions: These results suggest that COX-2 may play an important role in carcinogenesis by stimulating tumor angiogenesis. Also, show a relation between the expressions of COX-2 and VEGF in gastric carcinomas and the value significantly higher in the positive COX-2 carcinomas, suggesting an intense angiogenesis activity in that group of tumors.

Keywords: Gastric cancer, Cox-2, *H. pylori*, tumor angiogenesis, VEGF

Introduction

Gastric cancer is one of the most frequent and lethal malignancies in the world ⁽¹⁾. Early detection of stomach cancer is difficult, and in most western countries the 5-year survival rate is less than 20% ⁽²⁾. More than 90% of stomach cancers are adenocarcinoma, which are divided into intestinal and diffuse types by the Lauren classification ⁽¹⁾.

Helicobacter pylori are a spiral, microaerophilic, gram-negative bacterium that colonizes the gastric mucosa in 25-50% and 70-90% of the population in the developed and developing countries, respectively ⁽³⁾. *H. pylori* is believed to be the major contributing factor to the development of chronic gastritis and peptic ulcer diseases in human and strongly suggest that *H. pylori* infection increases the risk of adenocarcinoma in the distal stomach ^(4,5).

Helicobacter pylori induced COX-2 expression in human gastric mucosa ^(6,7). Cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase enzyme, which converts arachidonic acid to prostanooids, is strongly expressed in gastrointestinal tumors ⁽⁸⁾. Up regulation of this inducible COX isoform in malignancies has also been found to associate with increased invasiveness and metastatic potentials of the tumors ⁽⁹⁾. Cox-2 is considered "the immediate early gene", it is composed when the cell is stimulated and it takes part in many pathophysiologic processes, such as carcinogenesis and inflammation ⁽¹⁰⁾.

The COX-2 overexpression alters cell kinetics, suppresses programmed cell death, induces invasive phenotypes, supports tumor angiogenesis and influences cell adhesion to endothelial cells ⁽¹¹⁾. *H.*

pylori infection induces gastric COX-2 up regulation and cure of the infection reduces the COX-2 expression ⁽¹²⁾.

The contribution of COX-2 at the tumor angiogenesis includes the growth of the vascular endothelial growth factor (VEGF: a key factor for induction of tumor angiogenesis) expression, the production of E prostaglandin (PGE) 2 and I prostaglandin (PGI) 2, which may stimulate directly the migration of the endothelial cells and the angiogenesis induced by the growth factors, as well as the endothelial cells' inhibition by Bcl-2 stimulation ^(13,14).

Vascular endothelial growth factor, the well-characterized angiogenic factor, is known to play a major role in the multistep process leading to the reconstruction of normal mucosa architecture. This process is believed to be mediated through angiogenesis, ensuring an adequate supply of nutrients to the healing tissue ⁽¹⁵⁾. Moreover, VEGF also plays a vital role in tumor-associated microvascular invasion ⁽¹⁶⁾. In human gastric cancers, VEGF has been found to be over-expressed, and, VEGF expression has been reported to be upregulated by *H. pylori* through a COX-2 dependent mechanism ⁽¹⁷⁾.

The aim of this study was to evaluate the expression of Cox-2 in human chronic gastritis and gastric carcinoma in the presence of *H. pylori* infection and examine the relationship between their expression and angiogenesis in gastric carcinogenesis.

Materials and Methods:

Patients and Specimens:

The studied cases included 81 cases of patients, (45 men and 38 women) ranged between 47 and 82 years (mean 67.8 years). Who attended to Baghdad Teaching Hospital, AL-Yarmouk Teaching Hospital and Gastroenterology and Hepatology Teaching Hospital. The patients including 30 cases of chronic gastritis (CG) infected with *H. pylori*, 51 cases of gastric cancer (GC), 42 cases from GC have *H. pylori*. The control group included 30 cases with normal mucosa (N) and none infected with *H. pylori*. All cases of CG were endoscopically resected biopsies while all the studied GC cases were surgically resected gastrectomy specimens or endoscopically resected biopsy.

Classification, grading and both pathological staging and stage grouping of GC cases were performed according to WHO. Patients were divided into two groups according to the age, those ≥60 years and others <60. *H. pylori* were confirmed by the rapid urease test and histology. None of these patients was taking aspirin or NSAID. Scoring was done by two independent investigators and the mean score was taken in each case. Antral biopsies were taken from the greater and lesser curvature within 2 to 3cm from the pylorus for assessment of COX-2 staining and VEGF.

Each specimen was fixed in 10% phosphate-buffered formalin immediately after resection, embedded in paraffin and cut into 4µm-thick sections for immunohistochemical study and routine histological examination.

Immunohistochemistry:

Mucosal biopsies were immunostaining with polyclonal antibodies to COX-2 (polyclonal, J1602, 1:50dilution; Santa Cruz Biotechnology, Inc.), VEGF (polyclonal, L2702, 1:50 dilution; Santa Cruz Biotechnology, Inc.). The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary antibody will form a complex

with the peroxidase-conjugated streptavidin and by adding the substrate, which contains 3,3-diaminobenzidine (DAB) in a chromogen solution, counterstained with Mayer hematoxylin, and mounted.

Evaluation of immunostaining:

Counting the number of positive cells which gave brown cytoplasmic staining system under light microscope. The extent of the IHC signal was determined in 10 fields (X100 magnification).

In each field the total number of cells was counted and the extent of cytoplasmic staining cells was determined as a percent. The total staining score was divided by the number of whole cells per field in 10 fields, so the percentage of positively stained cells in the 10 fields was calculated for each case by taking the mean of the percentage of the positively stained cell in the 10 fields.

To evaluate COX-2 and VEGF expression, a score was established. Percentage of positive cells (0 = 0% immunopositive cells, 1 = < 25% positive cells, 2 = 26%-50% positive cells, and 3 = > 50% positive cells (2, 7).

Statistical analysis:

Data were analyzed by SPSS. The associations between expressions of COX-2, VEGF and clinic pathological parameters were assessed by the Chi-square test. The COX proportional hazard model was used for multivariate analysis of prognostic factors. *P*<0.05 was considered statistically significant.

Results:

Clinical and pathological data for the studied CG cases are represented in (Table-1). Our results do not show a significant difference between COX-2 expression and the sex and age of the patients with gastric cancer. The expression of COX-2 with the carcinoma group with *H. pylori* had significantly higher than carcinoma group without *H. pylori* (*p*<0.001).

Table -1: Relation between Cox-2 expressions in selected characteristics and risk factors in subjects with gastric cancer.

Variables	Cox-2 expression		Cox-2 Positive rate (%)	P-value
	-(n=9)	+(n=42)		
<u>Age</u> ≥60	3	23	54.76	>0.05
<60	6	19	45.23	Ns
<u>Sex</u> Male	2	22	52.38	>0.05
Female	7	20	47.61	Ns
<u>H. pylori</u> infection Yes	5	37	88.09	<0.001**
No	4	5	11.90	

** Highly significant difference (*P*<0.01), Ns: No significant difference (*P*>0.05)

Cox-2 expression was found in 42 from 81 gastric biopsies of *H. pylori*- infected patients displayed positive epithelial Cox-2 expression, including 88.09% of GC cases were positive , 60%

of chronic gastritis positive only 7 from 31 of non-infected control the difference was statistically significant. (*P*<0.001) (Table -2).

Table -2: Expression of Cox-2 in normal mucosa without *H pylori* and chronic gastritis and gastric cancer with *H. pylori* infection

variable	Groups	No	Cox-2 Expression		Cox-2 Positive rate (%)	P-value
			-	+		
Cox-2	N	31	24	7	22.58	0.001**
	CG	30	12	18	60	
	GC	42	5	37	88.09	

**=significant difference ($P < 0.001$)

Cox-2 immunostaining:

Cox-2 positivity showed diffuse brown cytoplasmic staining in epithelial cells of both GC and CG cases. The intensity scoring ranged from 0-3 *H. pylori* associated gastritis exhibited strong expression of COX-2 in foveolar and glandular epithelium (Figure-1).

In (Table -3) Chi-square test of significant was conducted to examine the association between COX-2 in normal mucosa, chronic gastritis and gastric cancer protein expression in gastric tissue. It was found that highly significant association ($P < 0.001$) between them in the three scoring levels, the results showed that percentage of COX-2 were elevated in the gastric cancer group and chronic gastritis respectively than in normal control.



Figure - 1: Gastric adenocarcinoma with strongly positive COX-2 immunoreactions (X 400). Staining by DAB chromagen (dark brown) counterstained with H & E.

Table-3: Scoring of COX-2 expression in non infected patients (control) and *H. pylori*-infected patients with chronic gastritis (CG), and gastric cancer (GC).

Variable	Score ^a	Groups			Total N=98	P value
		N.(n=31) No. (%)	CG(n=30) No (%)	GC(n=37) No. (%)		
Cox-2	0	24(77.41)	6(20)	0(0)	30	0.01
	1	5(16.12)	7(23.33)	9(24.32)	21	
	2	2(6.45)	13(43.33)	11(29.72)	26	
	3	0(0)	4(13.33)	17(45.94)	21	

Score^a: 0 = 0% immunopositive cells, 1 = < 25% positive cells, 2 = 26%-50% positive cells, and 3 = > 50% positive cells.

The COX-2 immunoreactions have become significantly positive more frequently in gastric carcinomas of intestinal type (95.83%), in

comparison to the carcinomas of diffuse type (77.77%) (Table-4).

Table-4: Expression of Cox-2 in gastric carcinoma with *H. pylori* patients according to Lauren classification.

Histological type	No.	Cox-2 expression		Cox-2 positive Rate (%)	P-valve
		-	+		
Intestinal type	24	1	23	95.83	0.01
Diffuse type	18	4	14	77.77	

In order to evaluate a relation between the tumor angiogenesis and the immunohistochemical expression of COX-2 we have evaluated the VEGF expression in the two gastric carcinomas groups: negative COX-2 and positive COX-2, (Table- 5). Strong expression was also found on intestinal epithelium of gastric cancer, (Figure -2). The VEGF value was significantly higher in COX-2 positive carcinomas, suggesting an invasive angiogenesis activity within the group of tumors.

The VEGF negative carcinomas were positive for COX-2 only in 76.92% of the cases. Unlike these, the VEGF positive carcinomas have associated immunoreactivity for COX-2 in 93.10% of the cases (Table-5).

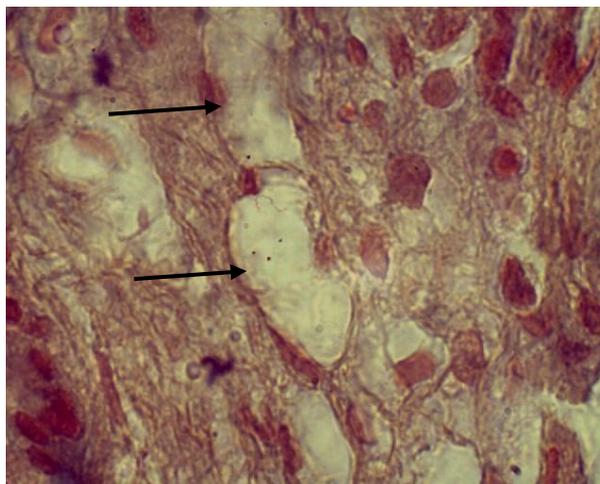


Figure (2): Immunohistochemical staining (IHC) of VEGF protein in tissue of gastric carcinoma. Staining by DAB chromogen (dark brown) counterstained with H& E (X400).

Table-5: Relation between Cox-2 and VEGF in gastric carcinoma with *H. pylori* patients.

Parameters	Cox-2 expression		Positive rate (%)	P valve
	- n=5	+ n=37		
VEGF negative n=13	3	10	76.92	0.01
VEGF positive n=29	2	27	93.10	

Discussion:

Tumor-induced activity of Cox-2 and related downstream enzymes has been implicated to play a crucial role in enhanced tumor invasion and metastasis ⁽¹⁾, angiogenesis, decreased host immunity, and apoptosis resistance. Although both Cox-1 and Cox-2 have been shown to be constitutively expressed in many cell types in several organ systems, Cox-2 is known to be the mitogen-inducible isoform ⁽¹⁸⁾.

In this study, we have shown that COX-2 is expressed in the epithelial lining of the stomach in *H. pylori*-associated gastric carcinogenesis pathway in chronic gastritis, and gastric cancer. This finding implies that COX-2 might be involved in the early stages of gastric cancer development i.e., gastric atrophy and intestinal metaplasia ⁽⁸⁾, which was in keeping with that reported by Sawaoka *et al* ⁽¹⁹⁾. On the contrary, Fu *et al* ⁽²⁰⁾ reported that in *H. pylori*-positive gastritis, COX-2 staining is primarily localized in the mononuclear cells in the lamina propria with no detectable staining in the epithelium. The difference in localization of COX-2 staining could be the result of variations in antibodies used in different studies and patient heterogeneity.

In the current study, COX-2 protein overexpression by immunostaining was found increased in cancer tissue which is identical to the data published by ^(10, 21, 22). One possibly for this result is high incidence of *H. pylori* infection. *H. pylori* have been to contribute to initiating mucosal injury in the stomach and subsequent development

of chronic atrophic gastritis ⁽⁵⁾. *H. pylori* up-regulate COX-2 expression and stimulate the release of prostaglandin E in gastric cancer cells. Prostaglandin E shows a potent immunosuppression effect by inhibiting the T-cell or natural killer cell activity ⁽²⁾. PGs thus provide a selective advantage for cancer cell survival ^(19,20). The COX-2 expression was increased from chronic gastritis; to gastric cancer this finding shows higher expression of COX-2 protein, supporting that overexpression of COX-2 may play an important role in gastric cancer development, because the COX-2 expression may occur earlier than that of histologic changes in the gastric mucosa, suggesting that COX-2 expression can be used as a surrogate end point in an intervention trial to inhibit the progression of gastric lesions ⁽²³⁾. The COX-2 immunoreactions have been observed no significant association between the expression of the COX-2 and the age and sex of patients group, Similarly with Sun WH *et al*. did not emphasize a significant between the COX-2 expression and the age, sex and tumor localization, ⁽²⁴⁾. The expression of COX-2 in patients infected by *H. pylori* has become positive in our study much more frequently in the gastric carcinomas of intestinal type in comparison with the carcinomas of diffuse type. The obtained data are suggestive for the predominant expression of COX-2 in the carcinomas of intestinal type and in their forerunning lesions (the epithelial dysplasia and in smaller amount, the intestinal metaplasia).

The COX-2 immunopositivation appears in our study as a precocious event in the sequence involved in the gastric carcinoma of intestinal types development^(25,26). The overexpression of COX-2 may induce an aggressive biological behavior of the neoplasm, involved in the invasion and metastasis process^(27, 28).

It was recently reported that a Cox-2 promoter construct, which is silent in non-malignant cells without an exogenous stimulation,⁽²⁹⁾. This may suggest that transcription of the Cox-2 gene is activated in some malignant cells, which may be due to activation of oncogenes or inactivation of antioncogenes⁽²⁴⁾. Because Cox-2 is very unstable and its stability can be regulated^(19,27), it is possible that dysregulations of post-transcriptional processing of Cox-2 transcripts is also associated with carcinogenesis. Prostanoids produced by Cox-2 may facilitate tumor progression by several mechanisms: they may act as growth and differentiation factors, as immunosuppressors, and as angiogenic agents^(30, 31).

The tumor growth and metastasis is dependent upon the new blood vessel formation. Tumor angiogenesis is associated with the production of highly permeable and poorly formed vasculature thereby facilitates the metastasis of tumor cells via the bloodstream⁽³²⁾. VEGF in the COX-2- positive tissues was markedly higher than that in the COX-2-negative tissues, thereby suggesting an association between COX-2 expression and VEGF in gastric carcinomas⁽²⁾. Tumor angiogenesis is controlled by a balance between angiogenic and angiostatic regulators involved in multiple pathways that result in endothelial proliferation, differentiation and organization into a functional network of vascular channels⁽¹⁵⁾.

In our study, VEGF was over-expressed in gastric cancer tissues. The results suggested that VEGF might be mainly involved in the progression of gastric carcinoma. The mean value of VEGF positive tumors was significantly higher than that of VEGF negative tumors, suggesting that VEGF may facilitate tumor progression by promoting tumor angiogenesis⁽³³⁾. These results were confirmed by other study, we have reported that COX-2 expression levels, correlated well with VEGF levels. It has also been shown that host COX-2 expression in cells plays an important role in gastric cancer progression^(34,35).

The application of the COX-2 and VEGF immunostainings on the endobiotic fragments before the surgical treatment could be used in the prediction of the clinical evolution and the pre-surgical selection of the adjuvant therapy in gastric cancer patients. Regarding this, the COX-2 activity inhibition could have an important therapeutic effect in the control of the gastric neoplasm.

Conclusions:

The COX-2 immunoreactions have been significantly more frequent noticed in the gastric carcinomas with *H. pylori*, than in the normal peritumoral mucosa non-infected with *H. pylori*, an association between the immunohistochemical expressions of COX-2 and VEGF in gastric carcinomas, leads to increased angiogenesis, which may be the mechanisms underlying the development of gastric cancer. VEGF might play a main role in the COX-2 angiogenic pathway. Inhibition of angiogenesis or COX-2, VEGF activity may have an important therapeutic benefit in the control of gastric cancer.

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