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RESEARCH ARTICLE

Differential expression of COX-2: A potential marker for clinical phenotypes of Gastric Cancer

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Abstract

COX-2 is a key enzyme in prostaglandin synthesis which can be induced by many growth factors and pro-inflammatory cytokines. COX-2 is related to inflammation and carcinogenesis. It's over expression leads to the accumulation of prostaglandins and thereby promoting carcinogenesis. By using the semi-quantitative reverse transcription PCR and immunohistochemistry, we show that COX-2 was frequently over expressed in gastric carcinoma. COX-2 was significantly differentially expressed within different clinical parameters such as tumor stages (I+II and III+IV), metastasis (lymph node and liver), tumor invasion types (serosal, and lymphatic), histological types (intestinal and diffuse), gender and age groups. We also demonstrate that *H. pylori* infected samples show significantly higher expression of COX-2 than non infected samples. We also demonstrate that *H. pylori* negative samples do not show differential expression of COX-2 in histology type, but *H. pylori* infected sample show significant differentially higher expression of COX-2 in intestinal histology type. Interestingly, we have also found that the level of COX-2 immunoreactivity gradually increases with tumor stages; I+II and III+IV similar to RT-PCR data. Thus, our data suggest that the COX-2 expression and its cross talk with *H. pylori* infection may be critical in the progression of gastric cancer.

Introduction

Gastric cancer (GC) is a multi factorial disease, characterized by highly malignant neoplasm in the gastric mucosa. Gastric cancer is the fifth most common cancer among males and seventh most common cancer among females in India (V Rao et al., 1998). The incidence of gastric cancer in Mizoram has been reported to be the highest in India. Globally high incidence of gastric cancer has been reported from Southeast Asia, most commonly from Japan, China, South Korea, Central and South America (Alberts et al., 2003; Ushijima et al., 2004). Cause of this malignancy has been attributed to the consumption of preserved food containing carcinogenic nitrates (Alberts et al., 2003). Environment associated risk factors include diet, snuff consumption, obesity and *Helicobacter pylori* (*H. pylori*) infection (Konturek et al., 2006). This malignancy is often diagnosed in advanced stages and is associated with poor prognosis. An in-depth understanding of the molecular mechanism of gastric cancer has lagged behind many other malignancies of similar incidence and morbidity. It has been well established that approximately 15-20% malignant gastric cancers are associated with chronic infection with *H. pylori* (Kuper et al., 2000) and Epstein-

Barr virus (EBV) (Parkin et al., 2006) infection. According to WHO, *H. pylori* is categorized as a class I carcinogen, infecting the stomach of about 50% of the world's population (Mantovani et al., 2008; Grivenikov et al., 2010). Recent genetic studies indicate that pro-inflammatory responses play an important role in cancer development regardless of whether they are caused by infectious or noninfectious stimuli (Mantovani et al., 2008; Grivenikov et al., 2010; Coussens et al., 2000). Among the various inflammatory networks involved in the tumor microenvironment, the cyclooxygenase-2 (COX-2) is increasingly being appreciated as a key player in tumorigenesis (Wang et al., 2010a; 2010b).

Of the two isoforms of COX, COX-1 is constitutively expressed in most tissues and appears to be responsible for the production of prostaglandin that mediates normal physiological functions, such as the maintenance of the integrity of gastric mucosa and regulation of renal blood flow while, COX-2 is undetectable in most normal tissues. COX-2 expression is induced by cytokines, growth factors, oncogenes and contributes to the synthesis of prostaglandins leading to malignant transformation (Fosslien et al., 2000). It also has been reported that activation of MMP-2 can be modulated by COX-2 and its constitutive expression can alter the metastatic potential of colorectal cancer cells (Tsujii et al., 1997).

COX-2 is an inducible rate limiting enzyme for prostaglandin biosynthesis and has been shown to play an essential role in inflammatory responses. COX-2 has been reported to be rapidly induced by various stimuli such as mitogens, lipopoly-saccharides and cytokines (Williams et al., 1996; Ristimaki et al., 1994). Chronic inflammation leads to increased expression of inflammatory cytokines that promotes COX-2 expression and the induction of epithelial mesenchymal transition through construction of niches for tumor initiating cells (Li et al., 2012). It has also been reported that COX-2-induced angiogenesis may be involved in the development of cancer (Uefuji et al., 2000; Uefuji et al., 2001; Rigas et al., 1993; Tsujii et al., 1998). Furthermore, CagA-positive *H. pylori* infection has also been associated with increased expression of COX-2 in human gastric cancer (Sawaoka et al., 1998).

The prognosis of gastric cancer in early stage and their related factors is still unknown. Only available standard treatment for the early stage disease is surgical resection. Therefore, identification of prognostic factors in early stage of gastric cancer may provide relatively better treatment and patient survival than available conventional therapy. Although the over expression of COX-2 has been documented in several studies (Sinicrope et al., 2004; Ranger et al., 2004; Wiliams et al., 1999; Hosomi et al., 2000) including gastric cancer (Murata et al., 1999), it remains unclear how COX-2 expression gradually promotes gastric cancer progression. In this article we have made an effort to explore clinical significance of COX-2 expression and *H. pylori* infection in gastric carcinogenesis

Materials and Methods

Samples collection

Primary tumor biopsy of 78 Gastric Carcinoma (GC) patients were collected from surgically removed tissue from patients after their informed consent, approved by the institutional ethical committee of Institute of Medical Sciences, Banaras Hindu University. Samples were stored at -80ºC and in formalin until further analysis. The clinical data of the patients were categorized according to Lauren classification as mentioned in Table1.

RNA extraction and Reverse Transcriptase-PCR (RT-PCR)

RNA was isolated from tissue samples using TRI reagent (Sigma, USA) according to the manufacturer's protocol. The first strand cDNA were synthesized by reverse transcriptase using High capacity cDNA kit (ABI, USA). The expression of COX-2 were checked using gene specific primer Forward: 5'-GTTCCCACCCATGTCAAAAC-3', (Reverse: 5'-CAACGTTCCAAAATCCCTTG-3' and normalized with expression levels of β-actin (internal control) primer Forward: 5'-AAATCTGGCACCACACCTTC-3', Reverse: 5'-AGCACAGCCTGGATAGCAAC-3'. The PCR product was electrophorized in 2% agarose gel and stained with ethidium bromide. Differential expression was calculated as Mean of Normal ±2xSD.

Immunohistochemical staining

Tissue sections (4µm thickness) of formalin-fixed paraffin embedded (FFPE) primary tumor biopsy were mounted onto slides and dried 12-24 hours at 37ºc. Tissue sections were then deparaffinized in xylene and rehydrated. For the antigen retrieval, the tissue sections were heated for 20 min in 10mM of sodium citrate buffer pH 6.0 at 95-97ºC. Endogenous peroxidase activity was blocked with 3% H_2O_2 in Tris-buffered Saline (TBS) followed by incubation

with anti-human rabbit monoclonal anti-COX-2 antibody diluted in PBS (pH 7.6) containing 1% BSA and 0.09% sodium azide as per recommended dilution by manufacture's protocol (Biogenex Laboratories, USA). After the incubation, immunodetection was done with super sensitive biotinylated anti-rabbit immunoglobulins and streptavidin peroxidase complex. The brown color developed by DAB Chromagen solution followed by counterstaining with Mayer's hematoxylin was considered as positive staining.

Evaluation of immunoreactivity

COX-2 expression was scored semi-quantitatively according to the percentage of positively stained tumor cells. The evaluation of COX-2 expression was quantified and scored by assessing a percentage of brown positive stained tumor cells and assigned intensity score according to Allred scoring protocol (Kulkarni et al., 2001). The intensity score assigned represented the average intensity as follows: none (0), weak (+1), moderate (+2), strong (+3) and grade of positive cells 0 = no expression, 1 = expression in < 10% cells, 2 = expression in 10–50% cells and 3 = expression in >50% cells. The proportion of stained positive cells and intensity score was combined to obtain total score for each sample.

Detection of *H. pylori*

H. pylori detection was done by rapid urease test (RUT) in the biopsy samples/haematoxylin-eosin staining in FFPE tissue sections and/or by nested PCR. Briefly, for RUT, biopsy samples were inoculated immediately after collection in 2ml of urea broth at 37ºC in an incubator for 90 minutes. The change in color of the broth from pale yellow to deep pink was taken as a positive test. FFPE tissue sections (4-µm thick) were stained with haematoxylin and eosin to observe the presence of curved rod shaped bacteria on the mucosal surface. Confirmation of H. pylori was done at a molecular level by nested PCR targeting the conserved HSP60 and its restriction fragment length polymorphism. The reaction was performed in 25µl final volume containing 50 ng of DNA, 1U of Taq polymerase (Bangalore Genie, India), 200 mmol/L (each) deoxynucleotide triphosphate (MBI, Fermentas, USA) and 1.5 mmol/L $MgCl_2$ in standard PCR buffer and 10 pmol of each primer as described by Singh et al (Singh et al., 2005).

Statistical analysis

All statistical analysis was performed by GraphPad Prism 5. Student t test was used for the compression of COX-2 expression between the groups as compare to control. The $p \leq 0.05$ were considered as statistical significant.

Results

Expression of COX-2 in primary tumor and normal mucosal biopsies of gastric cancer

A total of 78 patient biopsies and 25 normal mucosal samples were included in our study of age ranges 20-80 years, among which 40-60 year age group (47.4%) patients with gastric cancer was more frequent.

Semi-quantitative RT-PCR: Our semi-quantitative RT-PCR data show significant over expression of COX-2 in 60.25% (p=0.0004, n=78) tumors as compared to normal mucosa **(Fig. 1a, 1b; Table1**). Differential expression of COX-2 was significant in tumors with different clinical parameters. Among the tumor having the over expression of COX-2, III+IV tumor stage show significantly higher expression than I+II tumor stage (p=<0.0001, **Fig.1b**). Patients with lymph node metastasis shows significantly higher expression of COX-2 than patients with no metastasis (p=0.006, **Fig. 1c**), Among the patients with *H. pylori* infection COX-2 expression was significantly high in sample having both lymph node and liver metastasis than lymph node only (p=0.024, **Fig. 1c**), but in sample with no *H. pylori* infection there was no significant difference. Samples with both serosal and lymphatic invasion has significantly higher expression of COX-2 than non invasive samples (p=0.038, **Fig. 2b**) although there was no significant change with serosal invasion (p= 0.769, **Fig 2b**). However, there was no significant difference with the site of growth of tumor (distal and proximal) (p=0.226, **Fig. 3b**) and histological types (intestinal and diffuse) of tumor (p=0.263, **Fig. 3a**), if not considering the *H. pylori* infection. Samples having the infection of *H. pylori* show significantly high expression of COX-2 than *H. pylori* negative gastric carcinoma samples (p=0.004, **Fig. 2a**). In addition, COX-2 expression in *H. pylori* infected samples was significantly higher in intestinal than diffuse type of tumor histology (p=0.021, **Fig. 2a**), But there was no significant change in expression of COX-2 in case of *H. pylori* negative samples (p=0.480, **Fig. 2a)**. Male shows significantly higher expression of COX-2 than female (p=0.013, **Fig. 2c**). Although middle age patient had increased expression of COX-2 but it was not significant at mRNA level.

Immunoreactivity of COX-2: Similar to RT-PCR results, immunoreactivity of COX-2 was positive in 65% of the tumors (p=<0.0001, n=78) (**Fig. 4a, 4b, 5a** and Table1) compare to normal mucosal samples. Significant differential

immunoreactivity of COX-2 was also observed between tumor stages; I+II (98.57±26.78; n=18) and III+IV (179.3±14.98; n=60) (p=0.013, **Fig. 5a**). Immunopositivity of COX-2 increases significantly with advancing grades of the tumor. It gradually increases from weak (+1) in 21% (n=51) tumors of stage I+II to moderate (+2) in 34% (n=51) tumors of stage III and strong (+3) positivity in 45% (n=51) tumors of stage IV (**Fig. 6e**). Patients with lymph node metastasis shows significantly higher expression of COX-2 (125±15.13; n=63) than patients with no metastasis, (61.43±18.31; n=10) (p=0.035, **Fig. 4b**), Among the patients with *H. pylori* infection expression of COX-2, similar to RT PCR result were increased in samples having both lymph node and liver metastasis than lymph node only but it was not significant (P=0.273, **Fig. 5b**). Similar to RT-PCR result sample with no *H. pylori* infection also show no significant difference in expression of COX-2 (p=0.261, **Fig. 5b**). Unlike RT-PCR result at protein level there was no significant change in either of combination of invasion type (no invasion: serosal, p=0.384, serosal: serosal +lymphatic, p=0.100, no invasion: serosal + lymphatic, p=0.451, **Fig. 5d**). Similar to RT-PCR result there was no significant difference with the site of growth of tumor (distal and proximal) (p=0.249, **Fig. 6d**) and histological types (intestinal and diffuse) of tumor (p=0.285, **Fig. 6c**), if not considering the *H. pylori* infection. Samples having the infection of *H. pylori* show high expression of COX-2 (145±20; n=16) than *H. pylori* negative (137.4±15; n= 62) gastric carcinoma samples but unlike RT-PCR result it was not significant (p=0.412, **Fig. 5c**). In addition, similar to RT-PCR result COX-2 expression in *H. pylori* infected samples was significantly higher in intestinal (120.0±30; n=10) than diffuse type (48.0±21; n=6) tumor histology (p=0.025, **Fig 5c**), But there was no significant change in expression of COX-2 in case of *H. pylori* negative samples (P=0.450, **Fig 5c**). Male shows significantly higher expression of COX-2 than female, male (138±16.29; n=30) and female (87.39±17.62; n=21) (p=0.019, **Fig 6a**). Middle age (40-60 yrs) patient has significantly higher expression of COX-2 than older age (> 60 yrs) (age group; 41-60 years (148.5±21.42; n=26) and >60 years (103±19.96; n=17) p=0.012, **Fig. 6b**).

Table 1: Different clinicopathological parameters showing correlation with COX-2 expression in gastric cancer

Parameter	**Clinicopathological variables (n)**	**Up regulation of COX-2 n (%)**		**Immunoreactivity of COX-2 n (%)**	
		n (%)	**p-value**	**n (%)**	**p-value**
Tumor/Normal	Tumor (78)	47 (60.25)	0.0004*	51 (65)	≤0.0001*
	Normal (25)				
Sex	Male (47)	29(61.7)	0.013*	31 (65.9)	0.019*
	Female (31)	18 (58)		20 (64.5)	
Age group (Years)	20-40 (19)	12 (63.1)	0.179	08 (42)	0.012*
	41-60 (37)	22 (59.4)		26 (70.2)	
	>60 (22)	13 (59)		17 (77.2)	
Histological Type	Intestinal (44)	27 (61.3)	0.263	32 (72.2)	0.285
	Diffuse (34)	20 (58.8)		19 (55.8)	
Sites of growth	Distal (44)	28 (63.6)	0.483	33 (75)	0.198
	Proximal (15)	07 (46.6)		11 (73.3)	
	Body (19)	11 (57.8)		07 (36.8)	
Tumor Invasion	No Invasion (15)	09 (60)	0.038*	08 (53.3)	0.451
	Serosal (49)	34 (69.3)		35(71.4)	
	Serosal + Lymphatic (14)	10 (71.4)		08 (57.1)	
Tumor Stage	I+II (18)	10 (55.5)	<0.0001*	11 (61.1)	0.013*
	III+IV (60)	37 (61.6)		40 (66.6)	
Metastasis	No Metastasis (10)	05 (50)	0.006*	05 (50)	
	Lymph node (63)	42 (66.6)		46 (73)	0.035*

	HP +ve Lymph node (07)	05 (71.4)	0.024*	04 (57.1)	0.273
	HP +ve Lymph node+ Liver (09)	08 (88.8)		08 (88.8)	
	HP –ve Lymph node (56)	35 (62.5)	0.169	37 (66)	0.261
	HP –ve Lymph node + Liver (07)	06 (85.7)		05 (71.4)	
H. pylori	HP -ve (62)	33 (53.2)	0.004*	39 (62.9)	0.412
	HP +ve (16)	14 (87.5)		12 (75)	
	HP +ve Intestinal Type (10)	08 (80)	0.021*	0 7(70)	0.025*
	HP +ve Diffuse Type (06)	04 (66.6)		04 (66.6)	
	HP –ve Intestinal Type (35)	20 (57.1)	0.480	22 (62.8)	0.450
	HP –ve Diffuse Type (25)	13 (52)		13 (52)	

***significant p- value; n = number of samples, HP –ve= non infected *H. pylori*, HP +ve= *H. pylori* infected sample**

Fig. 1a Representative RT-PCR analyses COX-2 mRNA expression in the normal gastric mucosa and tumor tissue using biopsy specimens. The expression of beta-actin is shown as an internal control. b. Normalized expression level of COX-2 in tumor biopsies is compared to normal; And relative expression of COX-2 between stage I+II and III+IV. c. Relative expression of COX-2 between tumor having no lymp node metastasis (LN -Ve) and lymp node metastasis, *H. pylori* positive with LN and *H. pylori* positive with (LN+Liver) metastasis, *H. pylori* negative with LN and *H. pylori* negative with (LN+Liver) metastasis

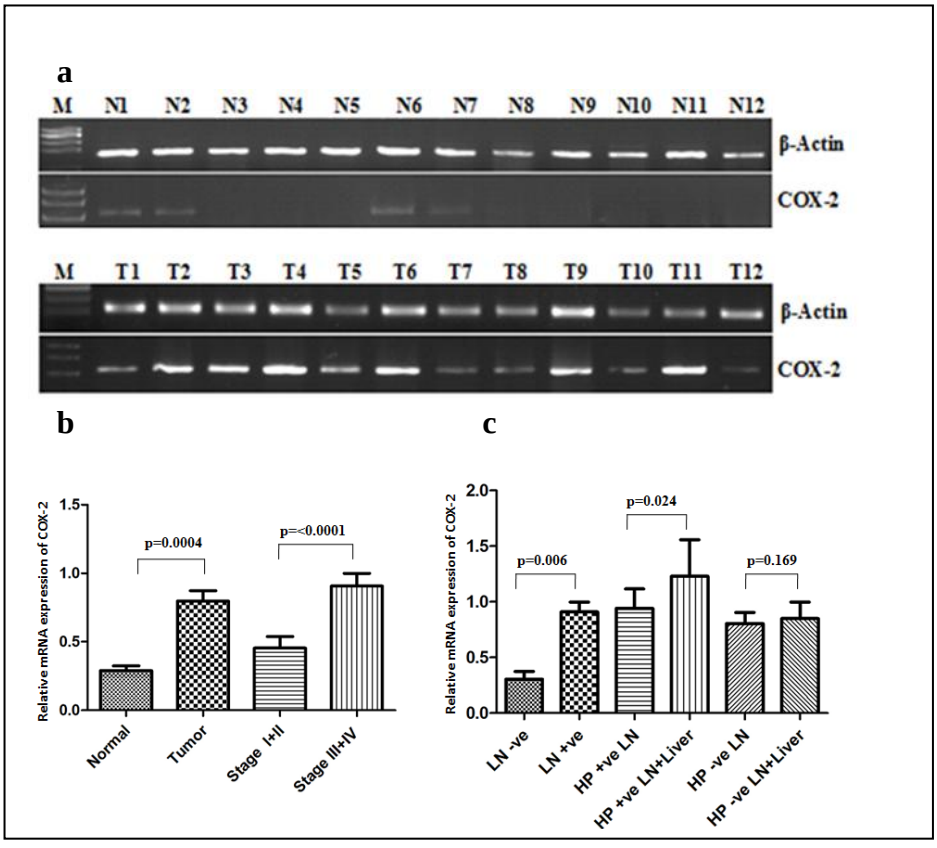

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Fig. 2a Relative expression of COX-2 between tumor having *H. pylori* negative and *H. pylori* positive, *H. pylori* positive with Intestinal type and Diffuse type, *H. pylori* negative with Intestinal and diffuse type. b. Relative expression of COX-2 between tumor with no invasion and serosal invasion, tumor with no invasion and (serosal +lymphatic) invasion. C. Relative expression of COX-2 between Male and Female. d. Relative expression of COX-2 between age group; 20-40 Yrs and 41-60 Yrs, 41-60 Yrs and >60 Yrs.

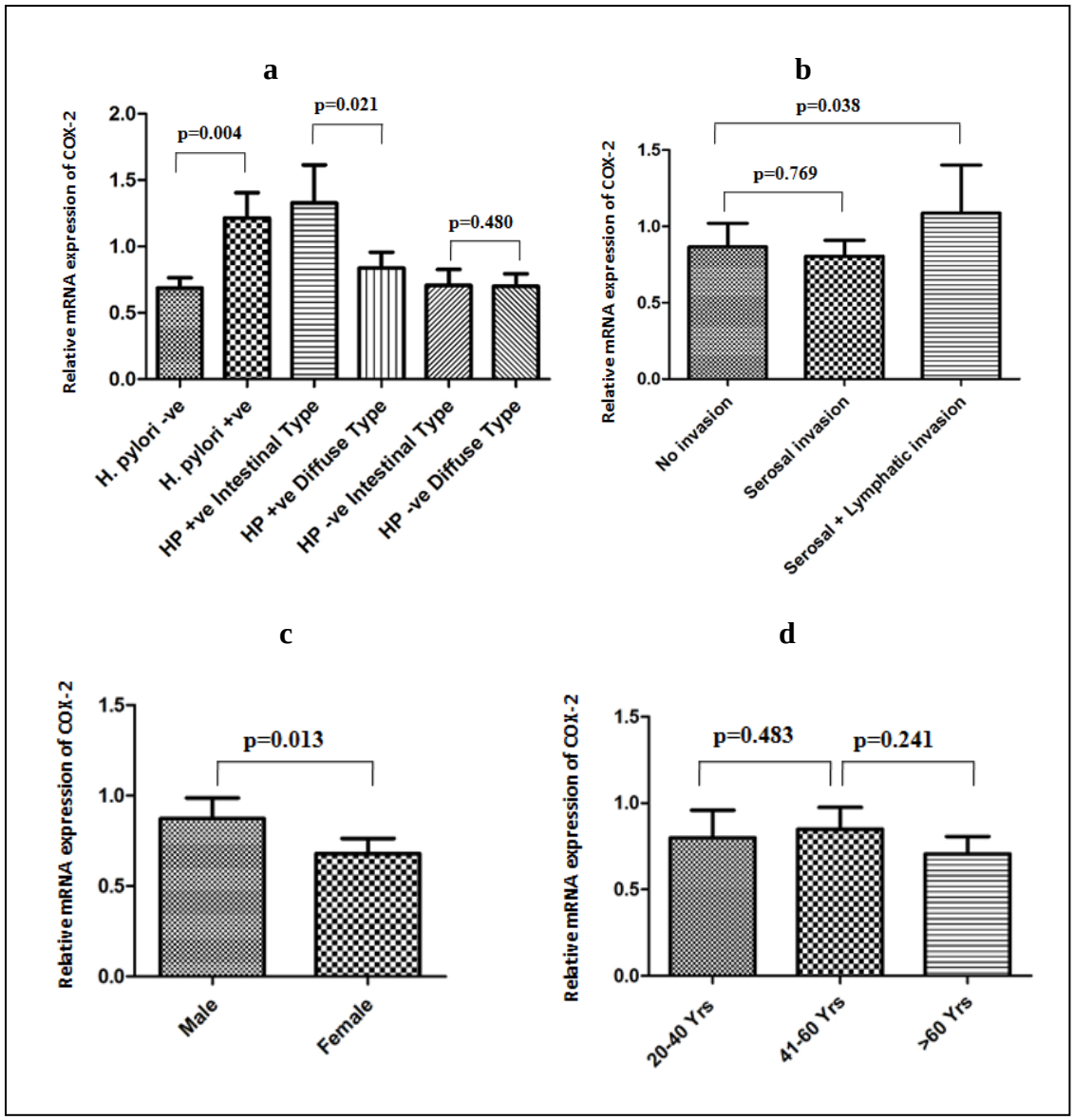


Fig. 3a. Relative expression of COX-2 between Intestinal and diffuse histology type. b. Relative expression of COX-2 between site of growth Distal and proximal type.

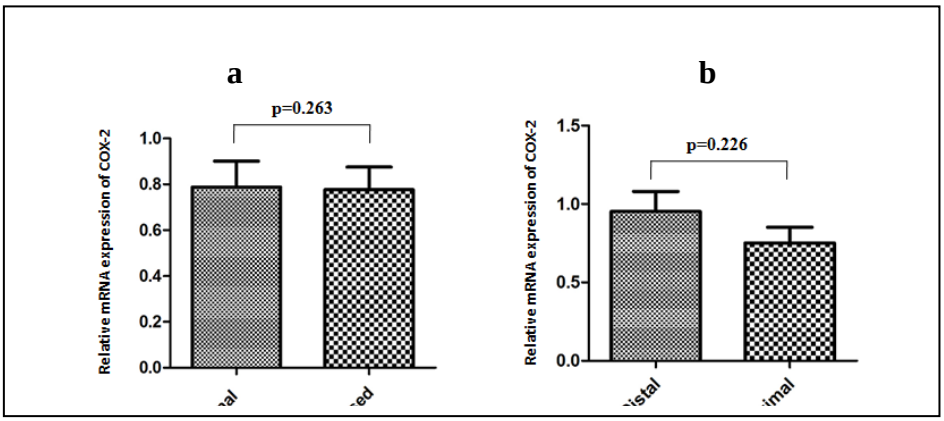


Fig. 4a. Immunoreactivity of COX-2 in normal gastric mucosa. b. Relative Immunoreactivity of COX-2 between stage I, II, III and IV. Gradually increases with tumor stages as compared to normal gastric mucosa.

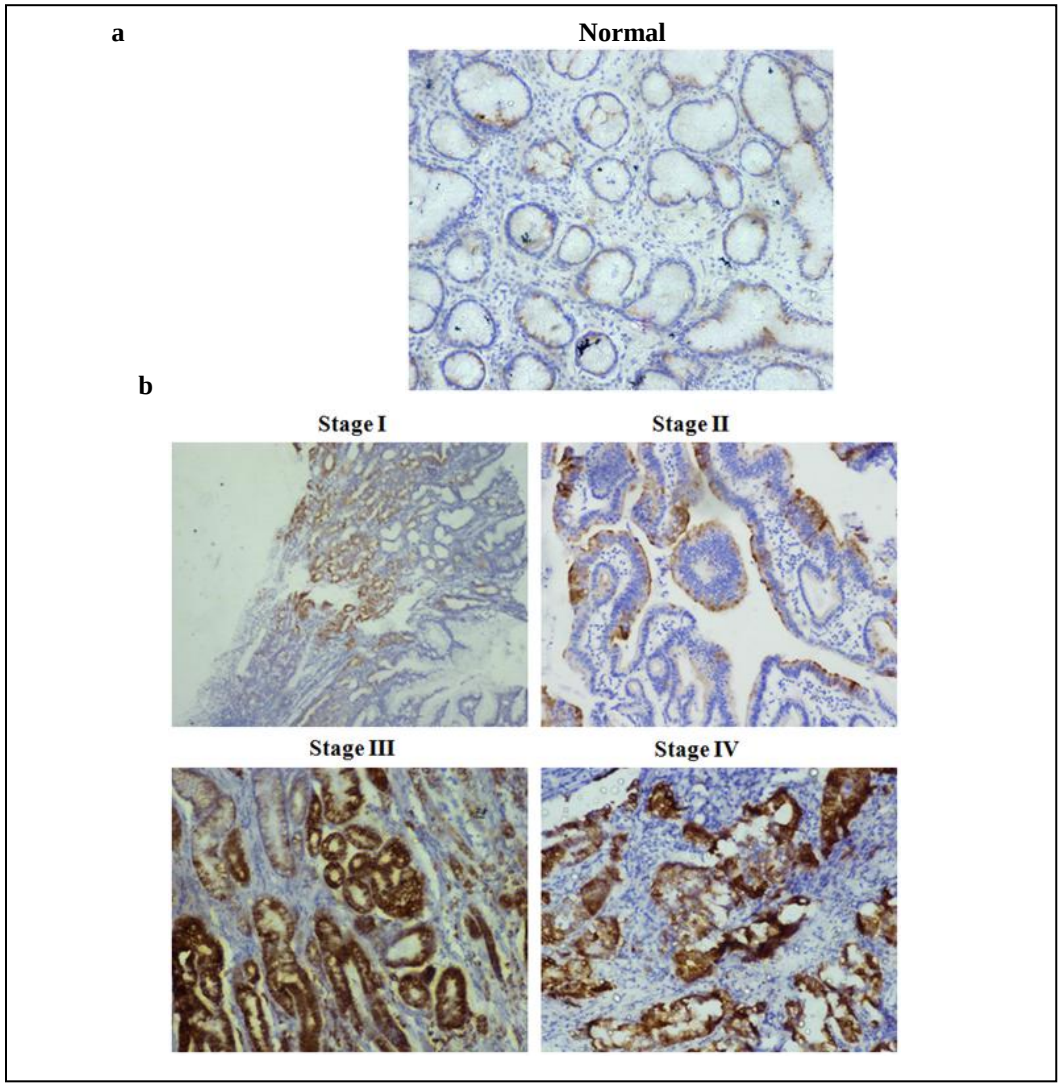


Fig. 5a. Normalized Immunoreactivity of COX-2 in tumor biopsies is compared to normal; And relative Immunoreactivity of COX-2 between stage I+II and III+IV. b. Relative Immunoreactivity of COX-2 between tumor having no lymp node metastasis (LN -Ve) and lymph node metastasis, *H. pylori* positive with LN and *H. pylori* positive with (LN+Liver) metastasis, *H. pylori* negative with LN and *H. pylori* negative with (LN+Liver) metastasis. c. Relative Immunoreactivity of COX-2 between tumor having *H. pylori* negative and *H. pylori* positive, *H. pylori* positive with Intestinal type and Diffuse type, *H. pylori* negative with Intestinal and diffuse type. d. Relative expression of COX-2 between tumor with no invasion and serosal invasion, tumor with no invasion and (serosal +lymphatic) invasion.

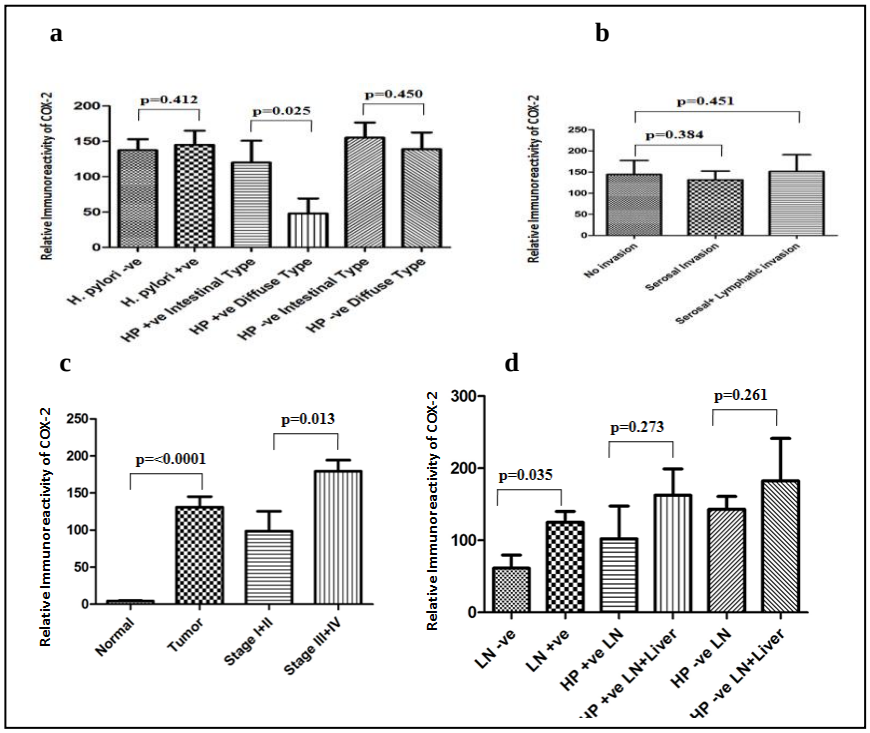
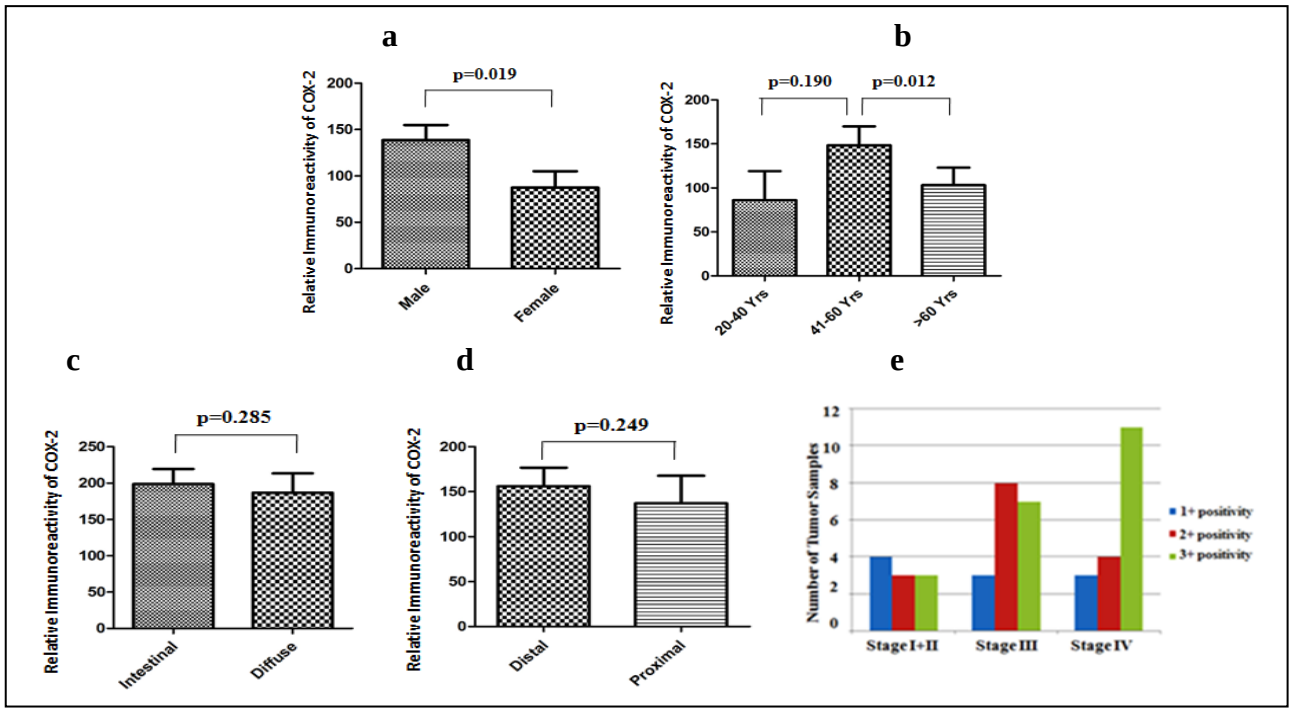


Fig. 6a. Relative Immunoreactivity of COX-2 between Male and Female. b. Relative Immunoreactivity of COX-2 between age group; 20-40 Yrs and 41-60 Yrs, 41-60 Yrs and >60 Yrs. c. Relative Immunoreactivity of COX-2 between Intestinal and diffuse histology type. d. Relative Immunoreactivity of COX-2 between site of growth Distal and proximal. e. Relative Immunopositivity (+1, +2, +3) between stages; I+II, III and IV.



Discussion

COX-2 plays an important role in the development of human tumors (Eibl et al. 2005; Yazawa et al. 2005). The higher expression of COX-2 is associated with the early process of carcinogenesis (Soslow et al. 2000). It also has been shown in different malignancy that expression of COX-2 is differential in different clinical parameters such as gross type, pathologic type, TNM stage and lymph-node metastasis (Marshall et al. 2005; Khunamornpong et al. 2009) including Gastric Cancer (Mrena et al. 2005). Moreover, the association of COX-2 expression with different clinical parameter and with manifestation of *H. pylori* infection is still remains poorly defined in Gastric Cancer progression. In the present study, we have demonstrated the over expression of COX-2 in tumors compared to normal mucosa at both transcription and protein level. Our result is concordant with previous literature demonstrating the over-expression of COX-2 (Murata H et al. 1999) (**Fig. 1b**, **5a**). Our results demonstrate that the expression of COX-2 increase with advancement of tumor stage which is also concordant with previous report (Khunamornpong et al. 2009; Laga et al. 2005) **(Fig. 1b, 5a**). *H. pylori* infection is one of the important factors for the Gastric Carcinoma. The expression of COX-2 gene is the result of direct response to *H. pylori* infection (Sun et al. 2005; Fu S et al. 1999). Concordant with previous report (McCarthy et al. 1999) COX-2 expression was significantly higher in *H. pylori* infected patient (**Fig. 2a, 5c**). Ahead of this, our results demonstrate that the patient without *H. pylori* infection, do not shows differential expression of COX-2 (p=0.480, p=0.450, **Fig. 2a, 5c**) in histology type (intestinal and diffuse type). Patient having *H. pylori* infection show significantly higher expression of COX-2 in intestinal type Gastric Carcinoma than diffuse type, (p= 0.021, p= 0.025, **Fig 2a, 5c**) both at mRNA and protein level. Intestinal type Gastric Carcinoma is less advanced and more differentiated (Ushiku et al. 2013), so we can speculate that this result indicate towards negative role of COX-2 in *H. pylori* associated initial stage of Gastric Carcinoma, although it will need further validation and study in more number of sample. Another report supporting our speculation stating that overall survival of patient with high copy number of *H. pylori* was higher than the patient having low copy number, although it was not statistically significant (Hai-Bo et al. 2010). It is possible that the induced COX-2 in positive *H. pylori* gastric mucosal lesions plays a significant role in deregulation of cell differentiation process during gastric carcinogenesis. Positive *H. pylori* group was significantly higher than that in the negative *H. pylori* group. It indicate that there are more cellular-biological behaviour of malignant tumor in gastric mucosal lesions with *H. pylori* infection.

Concordant with previous literature we also found differentially and significantly higher expression of COX-2 in sample having lymph node metastasis irrespective of *H. pylori* infection (P=0.006, p=0.035, **Fig. 1c, 5b**) (Murata H et al. 1999). Ahead of this we also found that samples without *H. pylori* infection there is no significant difference in expression of COX-2 in sample having both lymph node and liver metastasis (more advanced) than the sample having only lymph node metastasis (less advance) (**Fig. 1c, 5b**). But sample that have *H. pylori* infection shows significantly higher expression of COX-2 in sample with both lymph node and liver metastasis at transcription level but it was not significant at protein level (p=0.024, p=0.273, **Fig. 1c, 5b**). So these results do not have clear indication that in advanced stage of tumor (having both lymph node and liver metastasis) COX-2 promotes the tumor advancement with *H. pylori* infection, although to be confirm it will need experiments with more number of samples. Similar to metastases another tumor promoting event, also the expression of COX-2 increases with advancement of invasion type although it was not significant (serosal<serosal + lymphatic) (p=0.027, p= 0.451, **Fig. 2b, 5d**). Age of patient also have effect on expression of COX-2. Patient with middle age have higher expression of COX-2, this may be due to change in expression profile of chemokines with age, difference in the genetic constitution and/or difference of environmental factors among two populations. It has been previously reported that chemokines network greatly influenced the expression of COX-2 (Du Bois et al. 1998). Concordant with previous report (Kato et al. 2004) our results also demonstrate that COX-2 mediate Gastric Carcinoma predominate in male patient (p=0.013, 0.019, **Fig. 2c, 6a**). Although frequency of *H. pylori* infection were found to be more in male (75%, n=16) but there was no significant difference of COX-2 expression in *H. pylori* infected patient.

Thus we conclude that the COX-2 is a potential mediator in progression of Gastric Carcinoma. Although it will need further study in more number of samples, but our study indicate that cross talk between COX-2 mediated mechanism and *H. pylori* infection it may be the milestone for gastric carcinogenesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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