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Correlation Between Inducible Nitric Oxide Synthase and Cyclooxygenase-2 Expression in Human Colorectal Adenocarcinoma: A Cross-Sectional Study

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Abstract Cyclooxygenase-2 (COX-2) enzyme is believed to play a role in tumor angiogenesis, differentiation, and apoptosis. The inducible isoform of nitric oxide synthase (iNOS) also has the potential ability to damage DNA and conceivably contribute to tumor formation by a rise in nitric oxide production. Seventeen patients diagnosed with colorectal adenocarcinoma, who underwent surgical resection of the tumor, were enrolled in the study. Two macroscopic tissue samples, one from the tumor and the other from the tumor free surgical margin were collected from every patient as formalin fixed paraffin embedded blocks. Samples were analyzed for iNOS and COX-2 expression by immunohistochemistry and Western blotting. Results were digitized and semi-quantitatively analyzed. Immunohistochemistry revealed a similar pattern of expression for both iNOS and COX-2, as both were detected in tumor and epithelial cells. The mean iNOS and COX-2 levels determined by Western blotting method were significantly higher in tumor than in the tumor-free tissues (Wilcoxon signed-rank test, $p<0.001$ both for iNOS and COX-2). Patients with lymph node involvement had higher levels of both enzymes in tumors (Mann-Whitney U test, $p<0.05$). There was correlation between iNOS and COX-2 expression of tumor determined by immunohistochemistry and also by Western blotting (Spearman's rho test, $R=0.53$, $p=0.03$ and $R=0.57$, $p=0.02$, respectively). In conclusion, our results point out a relationship between iNOS and COX-2 expression in human colorectal adenocarcinomas and may also suggest a possible link between advanced stages of the disease and higher expression of iNOS and COX-2.

Keywords Colorectal cancer · Nitric oxide synthase · Cyclooxygenase-2 · Adenocarcinoma

Abbreviations

(COX-2)	Cyclooxygenase-2
(iNOS)	Inducible Nitric Oxide Synthase
(eNOS)	Endothelial Nitric Oxide Synthase
(nNOS)	Neuronal Nitric Oxide Synthase
(FFPE)	Formalin Fixed Paraffin Embedded
(NO)	Nitric Oxide

Background

Colorectal cancer, as one of the most prevalent malignancies in both male and female adults, is a leading cause of mortality in many countries [1, 2]. Several exogenous

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and/or endogenous factors are considered to predispose humans to large bowel cancer [3]. Recently, a great deal of attention has been given to endogenous factors as they appear to influence tumor growth, dissemination and invasion [4].

Preceding studies have suggested a role for NO, through the activation of COX-2 in motivating prostaglandin biosynthesis, especially in inflammatory models [5, 6]. Even though, expression of both iNOS and COX-2, and their correlation have been shown in malignancies such as hepatocellular carcinoma [7], non-small cell lung carcinoma [8], head and neck cancer [9], and breast cancer [10], the existence of a similar correlation in colorectal carcinoma remains controversial as only few studies have expressed such relationship. Furthermore, these reports contained divisive and conflicting information particularly on the basis of immunohistochemical expression [11, 12].

Nitric oxide production from L-arginine is mediated via three known isoforms of nitric oxide synthase (NOS). Endothelial (eNOS) and neuronal NOS enzymes (nNOS) are physiologically active and act in response to intracellular changes of calcium concentrations [13, 14], however, inducible isoform of NOS (iNOS) is produced in response to inflammatory cytokines and operates through a Ca^{2+} independent pathway. Other than causing DNA damage and inhibiting DNA repair [15], NO acts as an antiapoptotic agent through Caspase pathway embarrassment which is engaged in the process of apoptosis [16]. According to above, it may be supposed that NO, an endogenous factor, can facilitate the development and progression of malignancies especially as several studies have publicized increased level of iNOS in different malignancies such as ovarian, uterine, breast, brain [17–19] and colorectal cancer [20, 21].

Cyclooxygenase with two known isoforms (COX-1 and COX-2) acts in the prostanoids biosynthesis pathway as a rate limiting enzyme [22]. It is assumed that COX-2 enzyme plays a role in tumor angiogenesis, differentiation, and apoptosis [23–25], and similar to iNOS, its expression is easily induced by different cytokines and is up regulated in various malignancies [26]. Different groups have studied and compared the expression of COX-2 in colorectal cancer and normal tissue. High level of COX-2 expression is correlated with advanced stages of colorectal cancer [27–31].

The aim of the following study was to evaluate the expression of iNOS and COX-2 determined by immunohistochemistry and Western blotting in human colorectal adenocarcinomas of Iranian patients, to seek for an association in the microscopic site of expression and also to look for a correlation between the tissue content of these enzymes.

Materials and Methods

Patients

Male and female patients who attended the cancer clinic located in Tehran Cancer Institute between April 2006 and June 2008 and were suspected to have colorectal cancer were chosen for this study. Patients who underwent surgical tumor resection due to colorectal adenocarcinoma were enrolled in the study. Samples of the tumor and from the tumor-free surgical margin as controls were taken by surgeons and immediately transferred to the department of pathology to be fixed for further studies. The samples consisted of formalin fixed paraffin embedded (FFPE) tissue blocks. All block sections were reviewed by a well trained pathologist and were confirmed to be involved with or free from tumor.

A total number of 17 patients with pathologic diagnosis of adenocarcinoma within the colorectal area in the resected tissue were selected. Patients were carefully interviewed and a detailed medical history was taken by trained physicians focusing on any condition known to be associated with alterations of COX-2 or iNOS expression. Patients with inflammatory bowel disease, lichen planus, leukokoria, human oral carcinoma or history of chemotherapy and radiotherapy were excluded from the study.Patients who had consumed selective or non-selective COX inhibitors during the past three months were also excluded. Antiviral drugs, antibiotic and antiparasite medication usage during the two weeks prior to the surgery were also an exclusion criterion. In addition, patients whom surgical margin was not free from tumor were excluded from the study.

Data on demographic issues, lymph node involvement, staging, tumor type and location, differentiation and grading were collected and all selected blocks were transferred to the cellular and molecular laboratory for further analysis. Staging was performed based on available clinical and paraclinical data. Dukes' and TNM system were applied for staging the tumor [32]. According to the level of tumor cell differentiation, tumoral samples were graded by a pathologist and confirmed by a second pathologist.

Immunohistochemistry

Formalin fixed paraffin embedded blocks were cut into 5 μm slices. The sections were deparaffinized with xylene and then dehydrated in a three graded ethanol baths ranging from 97 to 70%. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in absolute methanol for 30 minutes at room temperature. The iNOS and COX-2 expression was detected using iNOS polyclonal antibody

(Invitrogen, USA) and COX-2 monoclonal antibody (Dako, Denmark) at 1:50 dilutions and EnVision™G|2 System/HRP, Rabbit/Mouse kit (Dako, Denmark). The sections were lightly counterstained with Mayer's hematoxylin (Sigma, USA). After drying and attaching lamellas using enthallen, all slides were studied by a pathologist for pattern of expression and these results were confirmed by another pathologist.

For semi-quantitative analysis of expression in tumoral cells, the samples were observed using microscope (Nikon Corp, Japan) and color density of DAB chromogen was scored using Scion image (Scion Corp, USA) in at least three different fields. The mean values for three measurements, after deduction of the background, were reported.

Western Blotting

Three 20 μm slices from each block were cut and transferred into micro tubes (1.5 ml). According to the method developed by Shi et al. [33] n-octane and methanol (Merck, Germany) were used for effective deparaffinization of the FFPE slices then 150 μl of the extraction buffer (20 mM Tris-HCl buffer containing 2% SDS, at pH=9.0) was added to the tubes followed by 10 s vortexing. Afterwards, the samples were heated at 100°C in water bath for 30 minutes and incubated at 80°C with mild agitation for 3 h. After centrifugation, the supernatant was removed, mixed with loading dye and heated at 95°C for 10 minutes. The samples were loaded (30 μg protein) onto SDS-polyacrylamide gel and run at 120 V and 14 mA for 1.5 h. The gels were blotted on PVDF membrane and the blots were incubated overnight at 4°C with COX-2 monoclonal antibody (Dako, Denmark) at 1/500 dilution or iNOS polyclonal antibody (Invitrogen, USA) at 1/250 dilution. Beta-actin monoclonal antibody (Santa Cruz, USA) at 1/1000 dilution was used as internal control. Detection was performed by BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche, Germany) and secondary anti-gout and anti-rabbit antibody with the dilution of 1/10,000 from the same kit was applied for visualization. The bands were digitized using Scion image (Scion corp, USA) and the data obtained for iNOS and COX-2 was normalized to beta-actin bands.

Statistical Analysis

Data were analyzed using SPSS software (version 11.5). All data are expressed as mean ± SD. Spearman's rho test was used for correlational analysis and Wilcoxon signed-rank test for paired group analysis. Independent group statistics were analyzed by Mann-Whitney *U* test. Multiple comparisons were performed using Kruskal-Wallis H test. *P* value less than 0.05 was considered as statistically significant. Binary logistic regression analysis was performed for evaluating the independency of iNOS and COX-2 in predicting the normal versus tumoral status considering the level of expression for iNOS and COX-2 as covariates.

Results

General Characteristics

Thirty four samples were obtained from seventeen patients (two samples from each). The study population consisted of 8 males (47.1%) and 9 females (52.9%) with the mean age of 52.53±17.92. According to the Dukes' staging system, 9 patients were in B1 and B2 stages while the other 8 patients had higher Dukes' stages (C and D).

Five lesions were located in the cecum and right hemi-colon, one in the left hemi-colon, three in the sigmoid area and eight in the rectum. According to the tumor type, tumors were divided into 2 groups, 8 were ulcerative and the rest were non-ulcerative. As can be inferred from Dukes' staging in 8 patients, the resected lymph nodes showed tumoral invasion; however, in the other 9 cases, the nodes were intact or only had reactive changes. Table 1 demonstrates the clinical and pathologic information of the study samples.

Immunohistochemistry

Qualitative Analysis

Amongst normal samples, basal areas of the epithelial cells were the most positive areas for COX-2 enzyme while inflammatory cells were weakly positive. In the slides obtained from tumor involved tissue, epithelial tumor cells were strongly positive while tumor-free tissue and inflammatory cells were positive for COX-2 (Fig. 1).

Studying iNOS expression in the normal tissue the slides showed epithelial cells positively stained with iNOS antibody, however, weak expression of iNOS in inflammatory cells, fibroblasts and extracellular matrix was evident. Likewise, the strongest expression in the tumor tissues was seen in the epithelial tumor cells while weaker expression was seen in the inflammatory cells, fibroblasts and extracellular matrix. Comparing the obtained results it was revealed the same pattern of expression for both enzymes in epithelial cells (Fig. 1).

Semi-Quantitative Analysis

Independent analysis of tumor samples according to the tumor type (ulcerative or non-ulcerative), gender and tumor location could not demonstrate any noticeable differences.

Table 1 Clinical and pathologic characteristics of patients

Characteristics	N
Gender	
Male	8
Female	9
Age	
Median (range)	49 (21–89)
Differentiation	
Well	8
Moderate	6
Poor	3
TNM staging	
Stage 1	3
Stage 2	6
Stage 3	4
Stage 4	4
Dukes' stage	
B1	3
B2	6
C	4
D	4
Tumor type	
Ulcerative	8
Non-ulcerative	9
Tumor location	
Cecum	5
Descending colon	1
Sigmoid	3
Rectum	8

Both studied enzymes (COX-2 and iNOS) were expressed in higher proportions in patients with lymph node involvement and higher Dukes' stages (C and D) (Table 2).

Besides increased expression of the COX-2 and iNOS in patients with lymph node involvement, Kruskal-Wallis H test revealed that COX-2 expression in TNM stage 1 significantly differs from that of other stages (p=0.01). Similar differences of iNOS expression was found in different stages (p=0.03) (Data not shown). In spite of relatively higher expressions toward well differentiated tumors, no significant differences were detected in iNOS and COX-2 immunohistochemichal expressions analized according to the differentiation (Table 2).

Spearman's rho test showed correlation between iNOS and COX-2 expression in tumoral tissues (R=0.53, p=0.03) while no significant correlation was found in normal samples (R=-0.21, p=0.42) (Fig. 3). By reanalyzing all samples (tumors and normals) a stronger relationship was revealed (R=0.72, p<0.001). The results of regression analysis for immunohistochemistry are shown in Fig. 3. According to the results of binary logistic regression elevated expression of both iNOS (R-square=0.896, p=0.031, Exp(B)=1.353) and COX-2 (R-square=0.725, p=0.005, Exp(B)=1.175) could independently and significantly differentiate between tumor and normal samples.

Western Blotting

The mean COX-2 expression ratio to beta-actin, in the normal and tumor samples was 0.04±0.03 and 1.06±0.68, respectively. The analysis revealed that the difference is statistically significant (Wilcoxon signed-rank test, p< 0.001). Likewise, iNOS expression showed the same pattern as its expression was significantly increased in tumor samples compared to that of normal ones (Wilcoxon signed-rank test, p<0.001). The mean value for iNOS in normal tissue and tumor samples was 0.09±0.09 and 0.98± 0.51, respectively.

According to an independent analysis, higher production of both enzymes in patients with lymph node involvement and high Dukes' stages (C, D) was confirmed by Western blotting method (Mann-Whitney U test, P=0.04 and P=0.01 for iNOS and COX-2, respectively). In contrast, tumor type (ulcerative or non-ulcerative), gender and tumor location had no effect on the expression of this enzymes in tumor tissues (Table 2).

Multiple comparisons of Western blotting results showed significant difference in COX-2 expression between stage 1, 2 and higher TNM stages (Kruskal-Wallis test, p=0.02) (Data not shown).

The correlations between iNOS and COX-2 expressions determined by immunohistochemistry were also confirmed by Western blotting (Figs. 2 and 3). The Spearman's rho test evidenced an association between iNOS and COX-2 expression in tumor tissues (R=0.569, p=0.02) and in normal samples (R=0.620, p<0.01). Analyzing all samples (normals and tumors) a similar correlation (correlation coefficient=0.72, p<0.001) was found. Regression analysis of Western blotting results is shown in Fig. 3. According to the result of binary logistic regression (R-square=0.921, p=0.047, Exp(B)=2.659) elevated expression of COX-2, but not iNOS (R-square=0.925, p=0.066) could independently and significantly differentiate between tumors from normal tissue specimens.

Disscussion

The interconnection between expression and activity of iNOS and COX-2 and their contribution in oncogenesis has been studied before. It has been shown that increase in the activity of iNOS and production of prostanoids is paralleled with increased expression of COX-2 [10] and presence of

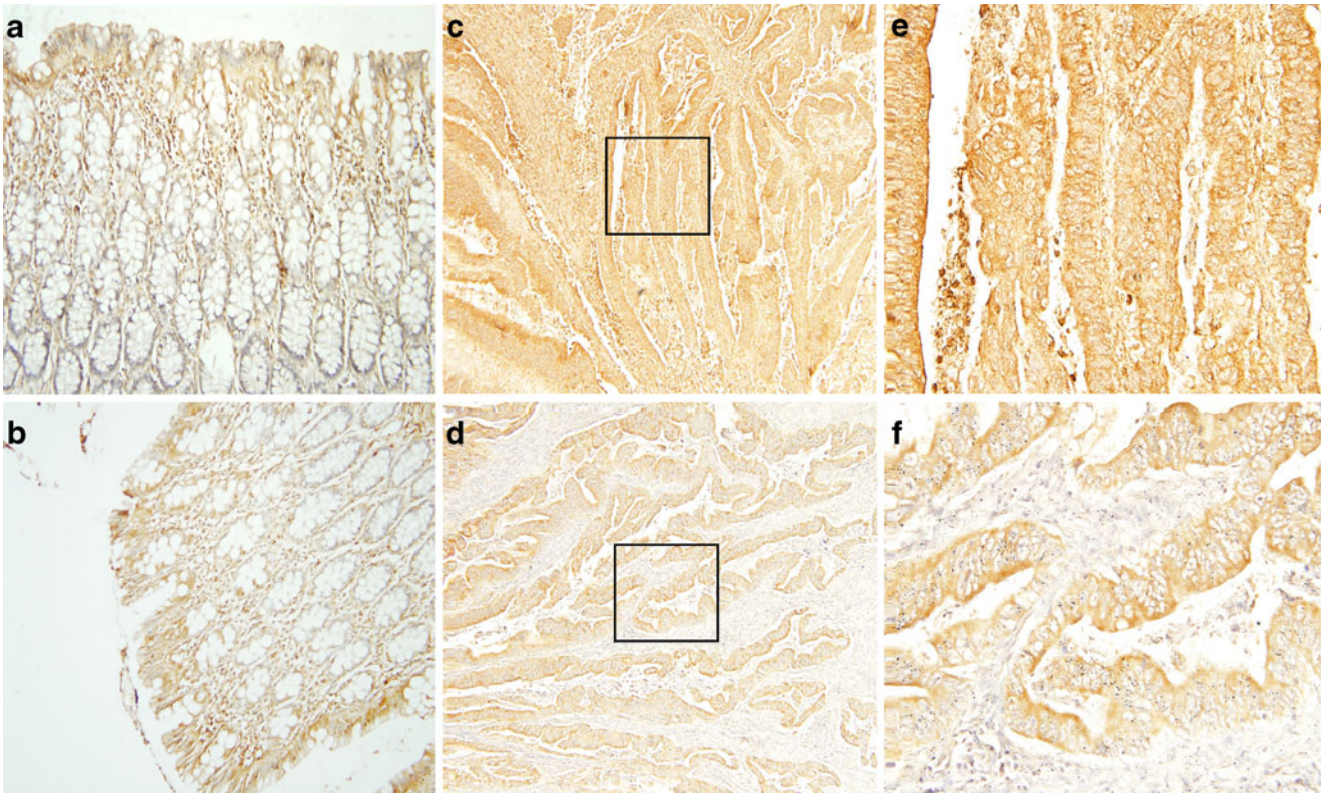

Fig. 1 Inducible nitric oxide synthase (*iNOS*), and cyclooxygenase (*COX*)-2 immunostaining. Consecutive sections of a normal specimen with a low density of iNOS **a** and COX-2 **b** positive epithelial cells. iNOS **c**, and COX-2 **d** immunostaining for tumor samples shows strongly positive differentiated epithelial cells. iNOS **e** and COX-2 **f** expression within same samples with greater magnification (area shown in the square). Both iNOS and COX-2 were detected in tumor epithelal cells. Hematoxylin counterstain; original magnification in **a–d** ×100, **e** and **d**×400

Table 2 Immunohistochemistry and Western blotting results for iNOS and COX-2 expression in tumor samples according to the tumor location, gender, type, differentiation and stage. Data are presented as mean ± SD

N		Immunohistochemistry				Western blotting			
		iNOS	p	COX-2	p	iNOS	p	COX-2	p
Gender									
Male	8	70.7±9.8	0.20	81.1±21.8	0.81	1.1±0.5	0.24	0.9±0.6	0.37
Female	9	81.5±18.3		84.2±28.7		0.9±0.6		1.2±0.7	
Tumor location									
Right-sided	5	71.6±10.2	0.57	80.7±35.1	0.54	0.9±0.5	0.88	1.2±0.4	0.28
Left-sided	12	78.4±17.3		83.6±21.3		1.0±0.5		1.0±0.8	
Tumor type									
Ulcerative	8	76.2±16.9	0.96	87.8±24.1	0.37	1.0±0.5	0.96	1.0±0.7	0.88
Non-ulcerative	9	76.7±15.3		78.3±26.2		1.0±0.5		1.1±0.7	
Differentiation									
Well	8	81.9±18.0	0.43	80.10±23.8	0.71	1.14±0.5	0.12	1.31±0.8	0.36
Moderate	6	74.2±13.5		91.2±31.3		1.0±0.5		0.9±0.4	
Poor	3	66.3±7.5		73.0±13.0		0.5±0.2		0.7±0.8	
Dukes' stage									
B1, B2	9	67.4±12.1	0.02	66.9±10.8	0.01	0.7±0.4	0.02	0.7±0.6	0.01
C, D	8	86.6±12.9		100.6±24.5		1.3±0.5		1.5±0.5	

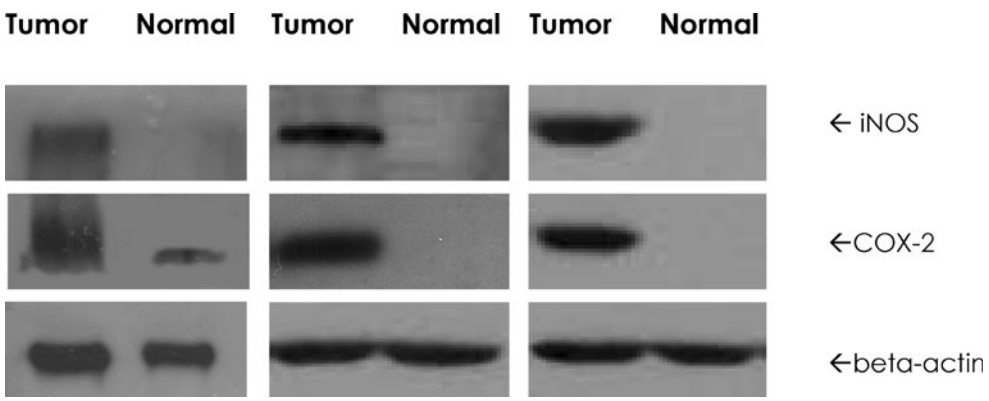

Fig. 2 Western blot analysis of tumor samples and normal tissue. Expression of inducible nitric oxide synthase (*iNOS*), cyclooxygenase (*COX*)-2 and beta-actin in 3 representative paired adenocarcinoma and adjacent normal mucosa samples. The protein extracts were prepared from tumor and tumor-free parts and resolved on SDS-PAGE followed by blotting on PVDF membrane. The bands were detected by specific antibodies and detected by chemoluminescence

TP53 mutants in tumors is associated with higher levels of both enzymes compared to the wild type TP53 [34]. Moreover, induction of COX-2 has been reported in the presence of nitric oxide [35] and it has been suggested that COX-2 expression can result in iNOS up-regulation through cAMP-dependant pathway [36]. Increased levels of both enzymes were detected in our samples, though, assuming an interconnection in expression can explain the high COX-2 levels in the presence of elevated iNOS and subsequently nitric oxide levels.

A retrospective study in Malaysia revealed high expression of iNOS and COX-2 in a significant number of tumor samples, but they could not show a significant difference between different stages of the disease [37]. Cianchi et al. reported significant correlation in immunohistochemical expression of iNOS and COX-2. Their results showed that both enzymes are mostly expressed in epithelial tumor cells [11]. In contrast, Ohta et al. in their study reported the same pattern for iNOS, but COX-2 was described to be expressed in stromal cells of the tumor [12]. In the current report; we demonstrated that the immunohistochemical expression of iNOS is interconnected considerably with that of COX-2 and the co-expression of these enzymes is seen within the tumor epithelial cells.

More recently, Cianchi et al. proved that there is a statistical significant difference between tumor center and invasive front in protein expression of COX-2 and iNOS and showed that the invasive front is most active site in relation to angiogenesis [38]. These new findings can be considered consistent with our findings which show higher level of expression in samples with lymph node and distant metastasis and again, highlights the role for COX-2 and iNOS in angiogenesis and tumor spread.

Regarding iNOS, controversial findings have been reported. It had been suggested that activity and expression of iNOS is diminished significantly in human colorectal malignant cells [20, 21], however other studies reported amplified expression of iNOS in colorectal tumor [11, 12, 39, 40]. Previous reports focused on the relation between differentiation grade and iNOS levels reported higher levels in well differentiated adenocarcinoma tumors compared to moderate and poor-differentiated tumor cells [41]. However, our data could not demonstrate such a relationship. As reported before, iNOS is mostly localized in epithelial tumor cells which are regularly seen in well differentiated adenocarcinomas [11, 12]; likewise, our findings were in agreement with these studies. Furthermore, in our study only weak expression of iNOS was observed in inflammatory cells. These results perhaps suggest a stronger role of iNOS in epithelial cells compared to inflammatory cells.

In the previous studies, it has been suggested that tumor angiogenesis might be induced by NOS [42]. This could raise the question that whether iNOS correlates with lymph node involvement and tumor metastasis. Lower survival, lymph node involvement and metastasis in association with iNOS expression have been reported previously [43, 44]. In our study a higher expression of iNOS was observed in patients of whom lymph node involvement was proven. Based on these results, one can consider iNOS expression as a possible marker for advanced stages of colon cancer.

Up regulation of COX-2 from normal cells to primary tumors and to metastases has been suggested in previous studies, and relates to proliferative activity and Dukes' stage. Additionally, longer survival has been documented for patients with lower COX-2 expression [30]. Another study performed by Otah et al. also revealed that higher stages of colorectal cancers are accompanied by higher levels of COX-2. Our findings are concordant with their results as higher level of COX-2 in advanced stages of colorectal adenocarcinoma was confirmed.

Due to growing evidences, correlation between iNOS and COX-2 expression is very important for their role in

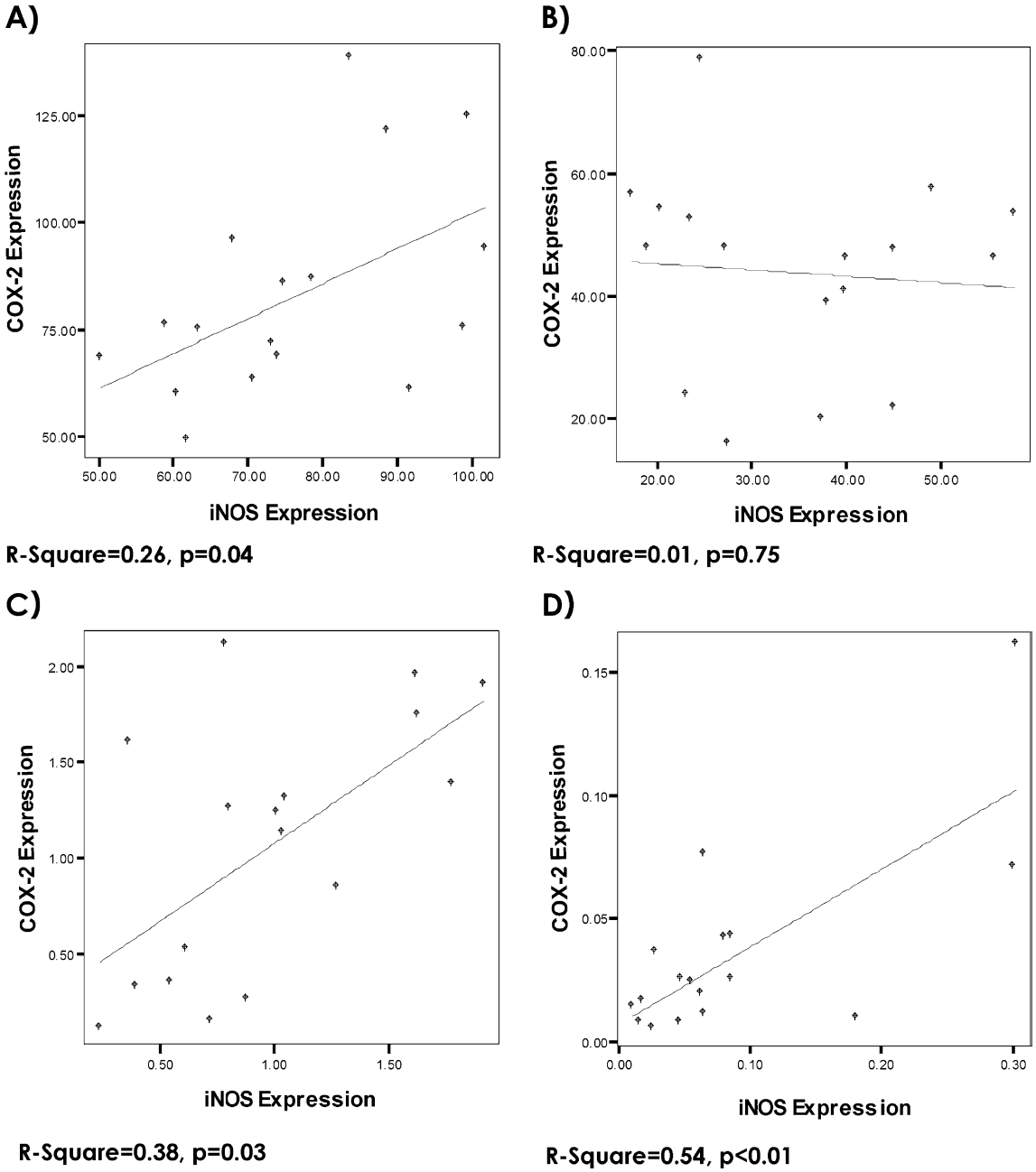

Fig. 3 Regression analysis for different groups according to iNOS and COX-2 expression determined by immunohistochemistry **a**, **b** and Western blotting **c**, **d**

chemoprevention. As mentioned above, literature supports the role of iNOS in colorectal cancer development, but is not clear whether chemoprophylaxis against iNOS expression and activity should be considered to the "at risk population". In this research work, we have demonstrated a correlation between iNOS and COX-2 expression. Our results indicated that increased expression of iNOS correlates well with enhanced COX-2 expression in tumor cells. This correlation is also clearly seen in higher stages. This finding has greater importance if one considers augmentation and metastasis in the tumor cells. Little evidence is available on the correlation between iNOS and COX-2. Previous findings showed that proangiogenic effect of iNOS is enhanced by COX-2 [11]. However, it is not clear whether COX-2 expression can induce iNOS expression or vice versa. Taken to account the use of COX or iNOS inhibitors, the correlation between iNOS and COX-2 could open a therapeutic or chemopreventive approach in colorectal tumors. Further research must be performed to find out if such relation exists

and to clarify whether chemoprophylaxis using COX inhibitors is sufficient or NOS inhibition should be added to the current strategies.

In conclusion, our results point towards a relationship between iNOS and COX-2 expression in human colorectal adenocarcinomas and may propose a relation of high expressions for both with advanced disease.

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Competing Interest The authors declare that they do not have any competing interests.

Conflict of interest None

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