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COMPARISON OF THE EXPRESSION LEVELS OF COX-2 IN COLORECTAL CANCER TISSUE AND ADJACENT NON-CANCEROUS TISSUES

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Abstract

Comparing the expression levels related to Cyclooxygenase (COX-2) in tissues of Colorectal Cancer (CRC) as well as (the adjacent non-cancerous tissues, and healthy control tissue from another individuals), also analyzing the relation of COX2 expression with the clinic pathological parameters which are related to CRC.

There are 80 specimens of tissues have been applied. COX2 expression in such clinical samples has been determined through immunohistochemical staining (IHC). The relation between COX2 expression as well as the clinic pathological parameters (age, gender, as well as intensity) has been evaluated through statistical analysis.

COX2 expression has been considerably increased in the tissues of CRC in comparison to the non-cancerous ones as well as to the healthy controls tissue from other individuals ($P < 0.001$). COX2 expression has been positively correlated with the tumor grade differentiation ($P < 0.001$), yet negative correlation has been identified between COX2 expression and age, gender in CRC ($P < 0.05$). Also, COX2 expression has been related with metastasis and invasions of CRC.

Conclusion: COX2 could be a marker of high importance to predict poor prognosis in the patients experiencing CRC and can be applied as possible therapeutic target in CRC.

Key words: Colorectal cancer, Cyclooxygenase, Immunohistochemistry, Invasion, metastases.

Introduction

CRC can be defined as extremely malignant tumor with regard to the digestive tract, approximately 1.2 million new conditions as well as 600000 mortality each year in the world (Raskov *et al.*, 2014). Recognized risk factors with regard to CRC are the familial history regarding colorectal as well as the other tumors, also the lifestyle factors, like smoking, obesity, high-fat diet, and inactivity (Becker, 2003). Identifying these risk factors resulted in importance on the primary prevention (Husain *et al.*, 2002), that involve dietary modifications as well as chemoprevention (Friedlich and Stern, 2000). Epidemiologic and experimental data suggesting the inhibitors regarding cyclooxygenase (COX, prostaglandin synthetase) system (Hershman, 1996), like NSAIDs, consisting of aspirin as well as the selective COX-2 inhibitors, suppressing the growth regarding intestinal tumors (Baron *et al.*, 2003; Chan *et al.*, 2004).

COX2, which is highly over-expressed in the inflammatory bowel disease likewise in the adenocarcinomas and the colorectal adenomas (Beissert *et al.*, 1989; Eberhart *et al.*, 1994; Wang and DuBois, 2010). Since the year of 1992 it was common that there are 2 iso-enzymes of that enzyme (COX), which are referred to as COX1 and COX2 (Vane *et al.*, 1998). It's now thought, in general, that the COX1 has been constitutively expressed in the majority of the tissues, and shows a "housekeeping-enzyme" features. On the other hand, COX2 results from an "immediate-early-gene" that is tightly regulated and quickly inducible. With regard to normal circumstances, COX2 expression has been extremely limited to particular organs, which include kidneys (Harris *et al.*, 1994), CNS (*i.e.* central nervous system) (Maihofner *et al.*, 2000), and eyes (Maihofner *et al.*, 2001), however, the expression of COX2 may

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increase dramatically in a variety of tissues, which follows initiating transcription via activating factors, which include various pro-inflammatory cytokines, tumor promoters, or growth factors (Vane *et al.*, 1998). What is interesting, is that COX2 has been discovered as highly expressed in specimens of tumor in patients that have Colorectal Cancer (Eberhart *et al.*, 1994; Kargman *et al.*, 1995; and Dimberg *et al.*, 1999). The activity of COX2 inhibition is mostly considered to be accounting for the activity of NSAIDs chemo prevention against FAP and Colorectal Cancer in humans (Sheng *et al.*, 1997; Steinbach *et al.*, 2000). There is an importance in noting that some cytokines which has a role in inducing COX2 were discovered to be expressed in Colorectal Cancer, in particular IL1-beta and IL6 (Kinoshita *et al.*, 1999; and Piancattelli *et al.*,1999).

Materials and Methods

Specimens

Fresh specimens (80) of colorectal tissues were collected from patients with CRC (n = 42) beside healthy control individuals (n=10). Likewise; (n = 28) CRC samples from (n = 42) were matched with adjacent normal tissue (n = 28). All these specimens were obtained from patients underwent colonoscopy and segmental colonic resections, those that were diagnosed as having colorectal cancer with different grades or healthy tissues (proved by histopathological examination) were considered as a control group.

All these tissue samples were collected from patients; admitted maintained in different hospitals in Baghdad city during the period of March/2018 to April/2019.

All sought after datum on demographic and clinical histopathological parameters which was gained from the patient's medical records and designed information sheet. The exclusion criteria was on patients for CRC patients including chemotherapy or radiation treatments prior surgery, also excluded the patients who had another malignancies or polyps in another organs (Purcell *et al*., 2017). All participants received conventional bowel preparation without preoperative antibiotic administration.

Histopathological Examination

It has been performed on the basis of the approach regarding Galeazzi *et al.*, (1999). The histopathological examination of the colon tissues which were selected from the patients of CRC were determined after fixing, and sectioning the organs, then staining them as well as Immunohistostaining. All these were performed in the Educational Laboratories in the City of Medicine as follows:

Preparation of Histological Sections:

At the moment of eradication the part of colon tissue (ex: Ascending, Transverse, Descending, Sigmoid) or Rectum was removed and then washed with phosphate buffer saline (PBS), preserved and fixed in 10% formalin for (24- 48)hr,then the section was washed up with tap water and processed with a set of increasing ethanol concentrations (70%, 80%, 90% and absolute ethanol) for 2hr at each concentration. After that, the tissue was clarified in xylene for 2hr, then it was impregnated in melting paraffin at 60-70ºC for 1hr. The tissue was embedded in paraffin blocks and waited to be solidified, finally it was sectioned using a microtome at slices of (5-6μm) and they have been mounted on the slides. The sections of tissue have been stained with the eosin stains and hematoxylin, after that the cell morphology has been identified under light microscopy. The upcoming phase has been to prepare positive charge slides.

Immunohistochemically examination:

Specimen preparation for IHC staining:

Human cancer tissue sections were subjected to immunohistochemistry analysis with the use of Pathinsitu System (Panthinsitu / USA) and PolyExcel HRP/DAB Detection System (Pathinsitu / USA) in the basis of the instructions of the manufacturer.

Antibody dilutions:

COX-2, are concentrated antibodies, hence diluted them with antibodies diluent according to company (Pathinsitu) sheet enclosed with them:

COX-2 was diluted by the ratio of 1:50-1:100 (according to the sheet between 50 -100), by taking 1% bovine serum albumin (BSA) and 0.05% Sodium azide (NaN3). The antibody dilution as well as the protocol might be different on the basis of specimen preparation as well as specific application.

Positive Control markers:

Sample biopsies of Colon Ca which were known to be positive for the immune marker we intended to use, were prepared and used as positive control for COX-2 (Tissues recognized for expressing antigens of interest have been applied as positive controls).

Negative Control markers:

For the negative control, the primary antibody was replaced with PBS.

Immunohistostaining steps:

This was accomplished according to González-Quezada *et al.*, (2018).

Finally, slide's evaluation has been achieved through

pathologists blinded to the characteristics of patient. The slides have been scored in the following way on the basis of the approach suggested via Rasheed, (2012): the intensity regarding staining has been classified from 0 to 3, as 0(-) negative, 1(+) low or weak, 2(++) moderate, and 3(+++) high or strong. The extent regarding staining (score) indicated as the percentage related to positive cells in association to the whole tumor area, has been classified from 0 to 3, as 0(0%), 1(10-20%), 2(20-50%), 3(>50%).

Statistical analysis

Data analysis has been conducted with the use of available statistical package of SPSS-25. The data have been proved in simple measure of frequency, standard deviation, mean, as well as range (minimum-maximum values).

The significance of difference of different percentages (qualitative data) has been tested with the use of Pearson Chi-square test (c^2-test) with application regarding Yate's correction or Fisher Exact test when applicable. Statistical significance has been considered when P value has been equal or not more than 0.05.

Results

H&E stained

The results of intensity of the stains (grade of differentiation) was classified from 0 to 3, as 0(-) negative, 1(+) low ,weak, or poorly, 2(++) moderate, and 3(+++) high , strong, or well differentiated (Fig. 1).

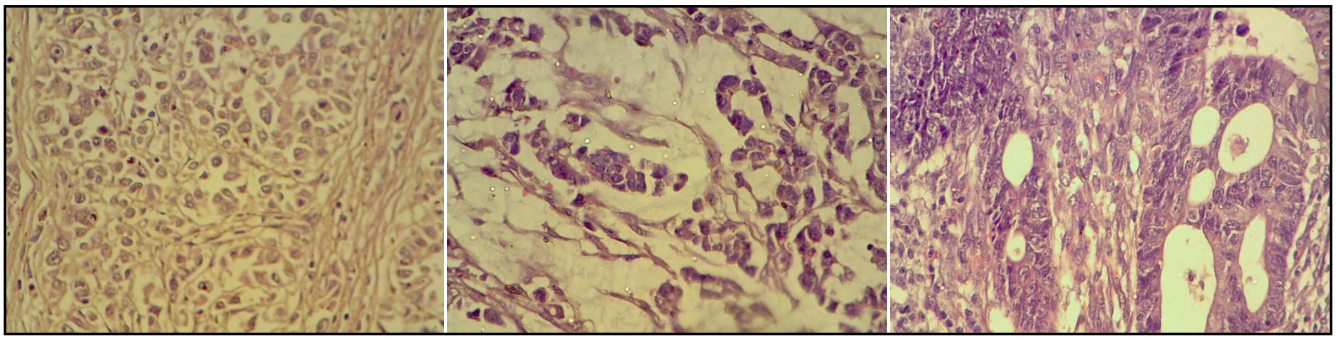
COX-2 expression in CRC

The results of cyclooxygenase COX2 illustrated an increased in COX2 expression [from score (+1, +2, +3)] Figures (2A), (2B), and (2C) respectively as well as positive stained cells have been seen in cancer cells of patient in comparison to healthy control, and adjacent normal tissues. Hence the results of statistical analysis indicated a significantly increased COX2 expression in CRC tissue compared with adjacent controls as well as healthy control individuals (Table 1), and (Table 2); while no significant association between adjacent normal tissue compared with healthy control tissue (Table 3). As well as no significant relation between COX-2 expression and other clinic pathological variables (age, gender) were present (Table 4).

Discussion

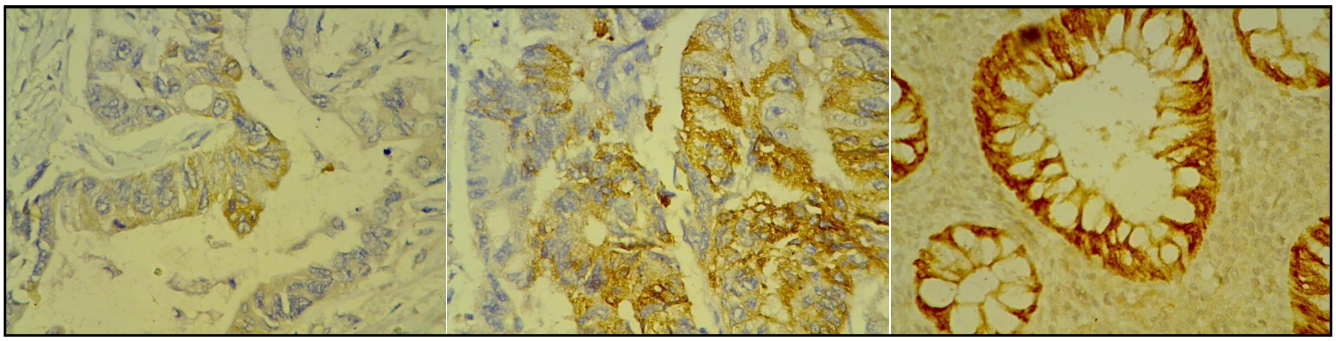
COX-2, that is considered to be highly over-expressed with regard to inflammatory bowel disease in addition to colorectal adenomas and adenocarcinomas (Eberhart *et al.*, 1994; Masuda *et al.*, 1995; Wang and Dubois, 2010). Other researches specified that elevated expression regarding COX-2 as well as elevated production with regard to the prostaglandins in the intestinal epithelial cells is protecting cells from apoptosis as well as stimulating creating angiogenic factors (Bruno *et al.*, 2010; Buchanan and DuBois, 2006).

A study implemented via Bertagnolli *et al.*, (2006) indicated considerable elevation in the COX-2 protein expression regarding tumor tissue in comparison to paired normal mucosa that has been accompanied with elevated levels of mRNA specified via semiquantitative reverse transcriptase PCR. Furthermore, a study conducted via Cianchi *et al.*, (2001) examined up-regulation related to the COX-2 protein expression (over-expression) with regard to the tumor specimens in comparison to the adjacent normal mucosa. Furthermore, COX-2 has been over-expressed in tumor tissue in comparison to normal colonic mucosa (Dimberg *et al.*, 1999). Results obtained from a study by Kazem *et al.*, (2014) indicated that in the normal adjacent colonic mucosa, COX2 has been totally absent in addition to Kazem and coworkers (2014) provided statistically considerable relation COX2 positivity scores and pathological grade, being at high end for badly differentiated tumors; that is in accordance with the results of the presented study. A study conducted via Wu and



A **B** **C**

Fig. 1: Colonic cancer tissue biopsy shows: **A-** Poorly differentiation, H&E stain (400x), **B-** Moderately differentiated stained in H&E (400x). **C-** Well differentiated stained in H&E (400 x).



A **B** **C**

Fig. 2: COX-2 IHC Scores expression: **A-** COX-2 IHC Score +1 expression (400x)., **B-** COX-2 IHC Score +2 expression (400x)., **C-** COX-2 IHC Score +3 expression (400x).

Table 1: The data regarding the significant expression of COX2scoring and intensity of stain in patient's cancer tissue compared to healthy control individual's tissue.

		Patient "Cancer tissue"(n=42)		Healthy control(n=10)	
	No	**%**	**No**	**%**	
COX2 Score	[0]	-	-	7	70.0
	[+1]	6	14.3	3	30.0
	[+2]	13	31.0	-	-
	[+3]	23	54.8	-	-
P value compared to healthy control tissue		0.0001*		-	
COX2 Intensity	[0]	-	-	7	70.0
	[+]	11	26.2	3	30.0
	[++]	13	31.0	-	-
	[+++]	18	42.9	-	-
P value compared to patient normal tissue		0.0001*		-	
*Significant difference between proportions using Pearson Chi-square test at 0.05 level.					

Table 2: The data regarding the significant expression of COX2 scoring and intensity in patient's cancer tissue compare to patient's adjacent tissue.

		Patient "Cancer tissue"(n=28)		Patient "Adjacent normal tissue" (n=10)	
	No	**%**	**No**	**%**	
COX2 Score	[0]	-	-	16	57.1
	[+1]	5	17.9	9	32.1
	[+2]	8	28.6	3	10.7
	[+3]	15	53.6	-	-
P value compared to patient adjacent tissue		0.0001*			
COX2 Intensity	[0]	-	-	16	57.1
	[+]	6	21.4	9	32.1
	[++]	9	32.1	2	7.1
	[+++]	13	46.4	1	3.6
P value compared to patient adjacent tissue		0.0001*			
*Significant difference between proportions using Pearson Chi-square test at 0.05 level.					

Sun, (2015) assumed that high COX2 protein expression has been considerably related with the tumor differentiation. COX-2 has been upregulated in approximately 85-90% regarding primary human CRCs (Hull *et al.*, 2000). Method's statistical analysis (malignant vs normal tissue from same patient) samples indicated no considerable difference in respective intensity scores which are related to COX-2 in malignant and normal. COX-2 expression has been considerably high in malignant epithelial cells in the case of comparison with the adjacent normal epithelium (P = 0.003) (Charalambous *et al.*, 2009). Nuclear factor-*k*B can be considered as inducible

Table 3: The data regarding of no significant association of expression COX2 scoring and intensity between the adjacent normal tissue of the patients and healthy control individuals.

		Patient "Adjacent normal tissue"(n=28)		Healthy control (n=10)	
	No	**%**	**No**	**%**	
COX2 Score	[0]	16	57.1	7	70.0
	[+1]	9	32.1	3	30.0
	[+2]	3	10.7	-	-
	[+3]	-	-	-	-
P value compared to healthy control tissue		0.526		-	
COX2 Intensity	[0]	16	57.1	7	70.0
	[+]	9	32.1	3	30.0
	[++]	2	7.1	-	-
	[+++]	1	3.6	-	-
P value compared to healthy control tissue		0.733		-	
*Significant difference between proportions using Pearson Chi-square test at 0.05 level.					

Table 4: The data regarding no significant association between the expressions of COX2 scoring compared with age and gender

		COX2 for Patient "Cancer tissue"(n=42)						COX2 for Healthy controls(n=10)					
		[0&+1]		[+2]		[+3]		[0&+1]		[+2]		[+3]	
		No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Age	<50y	2	33.3	4	30.8	6	26.1	5	50.0	-	-	-	-
(years)	50—59	4	66.7	5	38.5	11	47.8	2	20.0	-	-	-	-
	60—69	-	-	4	30.8	3	13.0	2	20.0	-	-	-	-
	=>70y	-	-	-	-	3	13.0	1	10.0	-	-	-	-
	P value	0.066											
Gender	Male	4	66.7	8	61.5	15	65.2	4	40.0	-	-	-	-
	Female	2	33.3	5	38.5	8	34.8	6	60.0	-	-	-	-
	P value	0.967											
*Significant difference between proportions using Pearson Chi-square test at 0.05 level.													

eukaryotic transcription factor that is of high importance in regulating the expression of many genes included in the inflammatory and immune responses (Bowie and O'Neil, 2000; Sha, 1998). Actually, NF-κB isn't single protein, yet small family regarding closely associated protein dimers, that are binding to common sequence motif referred to as *k*B site (Karin and Lin, 2002). There are 2 regulatory pathways which were specified to control protein's activity: canonical NF-κB pathway, that is typically triggered in response to the viral as well as microbial infections and being exposed to certain pro-inflammatory cytokines; and alternative pathway, that is triggered through specific members regarding TNF cytokine family (Karin and Lin, 2002). As soon as being in nucleus, NF-*k*B will have the ability of regulating many genes, such as COX2. A study conducted via Wu and Sun, (2015) indicated no considerable relation with COX2 expression and age or sex, yet the results indicated there have been considerable significant increase in the positive expression regarding COX2 in CRC in comparison to the normal colorectal tissues. COX2 expression has been considerably related with the lymph node metastasis (Brown and DuBois, 2005). This might be due the fact that COX2 have the ability of: (1) increasing the production regarding prostaglandins as well as inhibiting the immune response of the body; (2) inhibiting the tumor cell apoptosis as well as promoting cell proliferation; (3) regulating the progression of cell cycle; (4) promoting the tumor angiogenesis; (5) increasing the expression related to matrix metalloproteinases in tumor cells; and (6) inducing activation regarding precursors that are related to the carcinogenic substances. Due to the fact that high expression of COX2 present in precancerous lesions as well as carcinoma *in situ* also being considerably high in comparison to normal tissue, it has been typically suggested that higher expression of COX2 is early event regarding the tumorigenesis. A study by Zhang *et al*., (2002) specified the COX2 expression in 64 normal mucosal specimens, 116 primary CRC specimens, as well as 16 colon cancer metastases, and they indicated that

positive rate related to the COX2 expression has been about 12% in normal mucosal tissues, 72% in the primary tumors, as well as about 100% in the colon cancer metastases. This work indicates that 2- and 4-year survival rates have been considerably low in COX2-positive group in comparison to COX2-negative group, which will indicate that COX2 over-expression has been positively related with metastasis and recurrence regarding CRC, and negatively with CRC's prognosis. Statistical analysis showed considerably high rating related to respective intensity scores for epithelium in CRC in comparison to controls (Mailhöfner *et al*., 2003). In agreement with others (Joo *et al*., 2002; Kazem *et al*., 2014; Wu and Sun, 2015), no considerably relation has been indicated in this study, between COX2 expression scores and other clinic pathological parameters, such as age and gender.

Conclusion

Detecting the diffuse COX-2 cytoplasmic expression in the colorectal carcinoma as well as the increased expression scores in the high tumor grades implicating that COX-2 could be early marker regarding the neoplastic transformation involved in progression or initiation of colorectal carcinoma. COX-2 could be marker of high importance to predict poor prognosis in patients with the CRC and could be utilized as possible therapeutic target in the CRC.

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