Expression and prognostic significance of cyclin D1 and cyclooxygenase-2 in colorectal carcinoma: an immunohistochemical study

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Background
Colorectal cancer (CRC) is one of the most frequent cancers worldwide. Cyclin D1 (CNND1) and cyclooxygenase-2 (Cox-2) are expressed in a plethora of neoplastic tissues.
Aim
The present work was conducted to examine the immunohistochemical expression of CNND1 and Cox-2 in colorectal adenocarcinoma, compared with colonic adenoma to evaluate its association with various clinicopathological features.
Patients and methods
A total of 30 colorectal adenocarcinoma cases, 20 cases of colonic adenoma, and 10 normal colonic mucosal biopsies as controls were studied. Immunohistochemical technique was applied to detect CNND1 and Cox-2 expression and correlate them with clinicopathological findings.
Results
Both cytoplasmic high CNND1 and nuclear positive Cox-2 expression were significantly increased from normal colonic mucosa (0 and 10%, respectively) to CRC (80 and 83.3%, respectively) passing through colon adenoma (25 and 55%, respectively) ($P \leq 0.001$ for both). High CNND1 score was significantly related to lymph node spread and stage ($P \leq 0.001$ for both). A statistically significant difference was documented between Cox-2 and grade of differentiation ($P=0.017$), distant metastasis, and TNM stage ($P=0.033$, 0.003, respectively).
Conclusion
The present work suggests the oncogenic role of CNND1 and Cox-2 in CRC. Furthermore, overexpressions of CNND1 and Cox-2 are associated with poor prognostic factors, implicating their potentially prognostic role in CRC.

Keywords:
colorectal adenocarcinoma, cyclin D1, cyclooxygenase, immunohistochemistry

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Introduction

Colorectal cancer (CRC) is the third most common cancer, preceded by lung and breast. It is considered as the second leading cause of cancer-related death worldwide. Incidence of CRC is substantially higher in males, which is usually two-thirds of total incidence (Dekker *et al.*, 2019). After lung and prostate cancers, CRC is the third most common cancer in men. In female, CRC is the second most common cancer after breast cancer (Bray *et al.*, 2018). In Egypt, CRC is the seventh most commonly diagnosed cancer, accounting for 4.2%, and ranks fourth in female and seventh in males (Metwally *et al.*, 2018).

Its high mortality and occurrence incidence rates make CRC a global health challenge, besides being projected to substantially increase in developing countries. Therefore, continuous efforts are planned to reduce its incidence by studying its molecular features. Moreover, detection of more novel prognostic and therapeutic markers will be helpful to improve prognosis (Favoriti *et al.*, 2016).

The pathogenesis of CRC is characterized by a systemic fashion of progressive genetic abnormalities that disturb the cell cycle progress, and its assessment will lead to reduction in its incidence and improvement of clinical outcomes (Guren, 2019). The cell cycle controls the cell division and its checkpoints by an ordered series of events. These cascades of events are regulated by cyclins and cyclin-dependent kinases (Besson *et al.*, 2008). Cyclin D1 (CNND1), one of the members of this family, is an oncogene that controls G1-S phase progression by pRb phosphorylation, so it is supposed to participate in malignant progression

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and carcinogenesis. It is a 36-kDa protein encoded by CNND1 gene, mapped in chromosome 11q13. Alteration in CNND1 expression has been found in a plethora of malignancies, including CRC with unclear final established conclusions (Sheng *et al.*, 2020).

Cyclooxygenase (Cox) is an inducible enzyme, responsible for arachidonic acid metabolism, releasing prostaglandins. There are two isoforms (Cox-1 and Cox-2). Cox-2 is encoded by a gene that is located on chromosome 1 at q31.1. Various cytokines and cancer-promoting factors lead to increased Cox-2 level, which in turn has a role in many pathophysiological events, including apoptosis, cell proliferation, angiogenesis, and metastasis (Perisa *et al.*, 2017). Moreover, overexpression of Cox-2 was found in carcinomas of many organs, for example, cervix, gastric, ovary, and lung and was associated with dismal outcome (Jiang *et al.*, 2013). However, its role in CRC needs more clarification.

This study aimed to investigate the immunohistochemical expressions of both Cox-2 and CNND1 in CRC in comparison with colonic adenoma, highlighting their oncogenic role in CRC progression as well as their correlation to clinical parameters of CRC, revealing their prognostic values.

Patients and methods

This retrospective study was carried out on 20 cases of colon adenoma and 30 cases of colorectal adenocarcinoma in the form of colectomy and endoscopic specimen with 10 cases of non-neoplastic colon tissue (taken at >5 cm away from tumor) taken as a control group. These cases were randomly collected from the archives of Pathology Department, Faculty of Medicine, Benha University, from 2016 to 2020. Demographic data of studied cases were obtained from archived files. Being a retrospective study, a written informed consent was not needed. The study was approved by the Research Ethics Committees of Faculty of Medicine, Benha University, Egypt. Hematoxylin and eosin sections were examined to confirm diagnosis and were reviewed for tumor type, differentiation, and nodal state. Cases were graded according to the WHO criteria (Rosty *et al.*, 2014).

Pathological staging of the studied CRC was determined according to the 8th edition of AJCC, Cancer Staging Manual, 2017 by using the TNM staging system (Tong *et al.*, 2018).

Cyclin D1 and cyclooxygenase-2 immunohistochemistry

For immunohistochemical staining of CNND1 and Cox-2, 4-mm sections were cut from paraffin blocks and placed on positive charged slides. The technique of avidin-biotin or peroxidase was used according to the manufacturer's instructions. The primary antibodies used were CNND1 (monoclonal rabbit anti-cyclin D1, Clone: SP4, Catalog Number: RM-9104-S1; Lab Vision, Los Angeles; California, USA) at a dilution of 1: 50 for 60 min/room temperature and Cox-2 (Monoclonal Mouse Anti-Human Cox-2 antibody, Clone CX-294; Dako, Glostrup, Denmark) at a dilution of 1/100 for 60 min. The detection kit was the Ultravision detection system (Cat #, TP-015-HD; Lab Vision). Antigen retrieval was done using 10 mmol/l citrate monohydrate buffer (pH 6.0) and heating for 15 min in a microwave. The color development was performed using 3′-diaminobenzidine tetrahydrochloride as a chromogen. Negative (cold phosphate-buffered saline) and positive controls (breast carcinoma for CNND1 and lung carcinoma for Cox-2) were enclosed in each run.

Interpretation

The percentage of nuclear staining of CNND1 was reported as follows: 0=less than 5%, 1=5–25%, 2=26–50%, 3=51–75%, and 4=more than 75%. The staining pattern was scaled from 0 to 3, where 0 was negative, 1 was weak, 2 was moderate, and 3 was strong. The final expression score was calculated as follows: – for score 0, + for scores 1–3, ++ for scores 4–6, and +++ for scores more than 6. For the purpose of statistical analysis, the cases that scored – and + as a low score were compared with the cases that scored ++ and +++ as a high score (Albasri *et al.*, 2019).

Cox-2 expression was mainly cytoplasmic in the tumor cells. Immunostaining evaluation was performed using a semi-quantitative scoring system by estimating the percentage of the tumor cells stained and staining intensity. The extent of staining was graded as follows: 0 – staining in less than 1% of tumor cells; 1 – staining in 1–20%; 2 – staining in 20–50%; and 3 – staining in more than 50%. Overall intensity of staining was also assessed as follows: 0 no staining; 1 – weak staining; 2 – moderate staining; and 3 – strong staining. Final scores (range, 0–9) were obtained by multiplying staining extents and intensities. Final scores were described as follows: 0=no expression, 1–3=weak expression, 4–6=moderate expression, and 7–9=strong expression. For statistical analysis, no expression and weak expression were combined and described as negative for expression, and moderate and strong expression were combined and described as positive for expression (Kazem *et al.*, 2014).

Statistical analysis

The program used was SPSS, version 26 (SPSS Inc., Chicago, Illinois, USA). Qualitative data were analyzed using frequency and percentage. χ^2 test and Fisher exact test were used to compare frequencies. *P* value was considered significant if it was less than or equal to 0.05.

Results

The age of examined cases ranged between 30 and 80 years, with a mean age of 55 years (mean±SD=55 ± 13.7). The clinicopathological data of patients with CRC are shown in Table 2.

Cyclin D1 and cyclooxygenase-2 immunoexpressions in studied groups

High CNND1 score was observed in 24 (80%) cases and five (25%) cases in adenocarcinoma and adenoma, respectively, compared with low expression in six (20%) cases of adenocarcinoma, 15 (75%) cases of adenoma, and all non-neoplastic colonic mucosa (100%) (P≤0.001) (Fig. 1a–c). Cox-2 expression appeared as yellow–brown cytoplasmic staining. Its expression showed a highly significant difference among the studied three groups, as 25 (83.3%) cases of CRC were Cox-2 positive, compared with nine (45%) cases of adenoma and only one (10%) case of control group (P≤0.001) (Fig. 2a–c, Table 1).

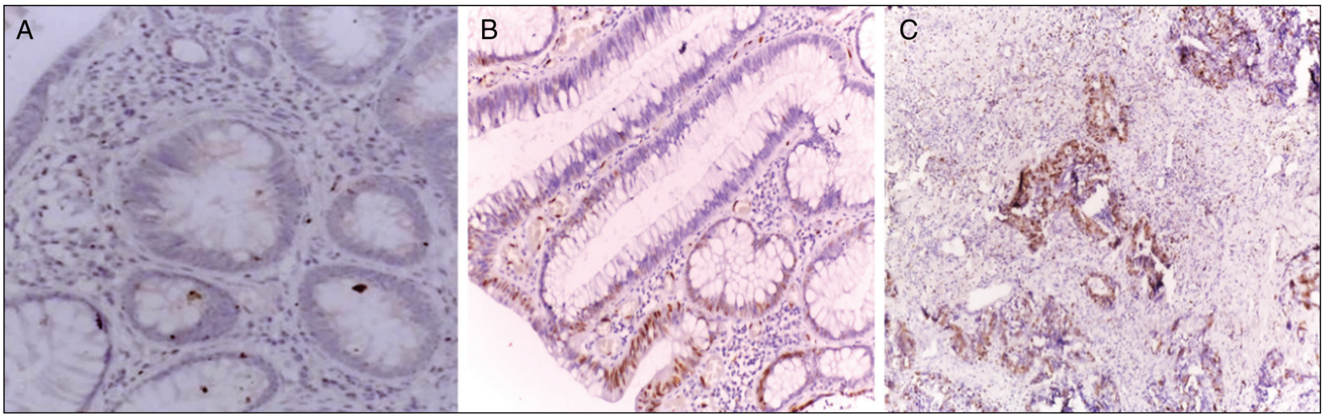
Relation of immunoexpression of cyclin D1 to clinicopathological features

CNND1 staining was significantly correlated with TNM stage (P=0.000) and lymph node metastasis (P≤0.001). No statistically significant correlation was observed between CNND1 immunoexpression and other clinicopathological characteristics, including age of the patient, sex, size of the tumor, and tumor grade (P>0.05) (Table 2, Fig. 3a,b).

Relation of immunoexpression of cyclooxygenase-2 to clinicopathological features

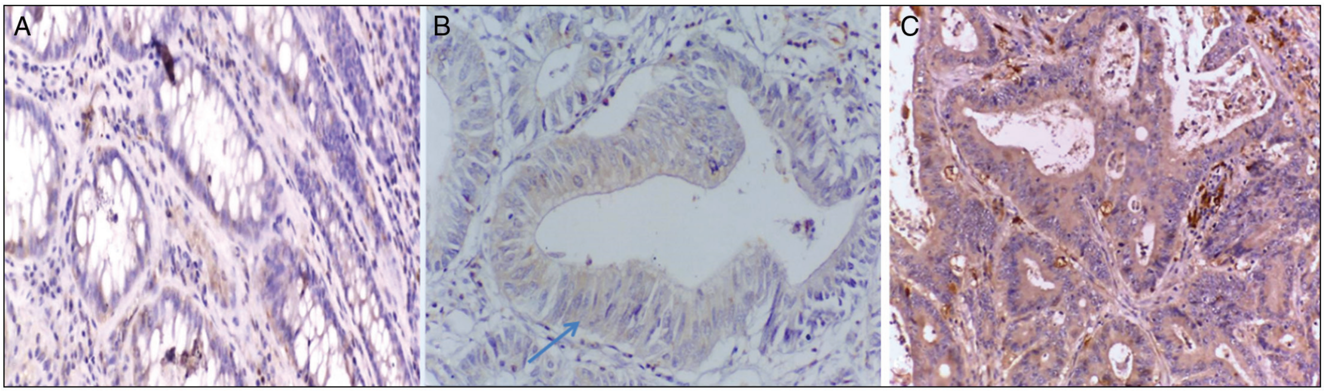
The associations between Cox-2 expression and the tumor grade, TNM stage, and distant metastasis showed a statistically significant difference (P=0.017, 0.003, and 0.033, respectively), with higher expression being more common in advanced tumors (P=0.003). However, sex, age, lymph node, depth of invasion, or tumor location had no significant relationship with

Figure 1



(a) Negative CNND1 expression in colonic mucosa (IHC, ×400) compared with (b) weak nuclear CNNDI expression in colonic adenoma and (c) moderate nuclear CNND1 expression in colorectal carcinoma (IHC, ×200). CNND1, cyclin D1; IHC, immunohistochemistry.

Figure 2



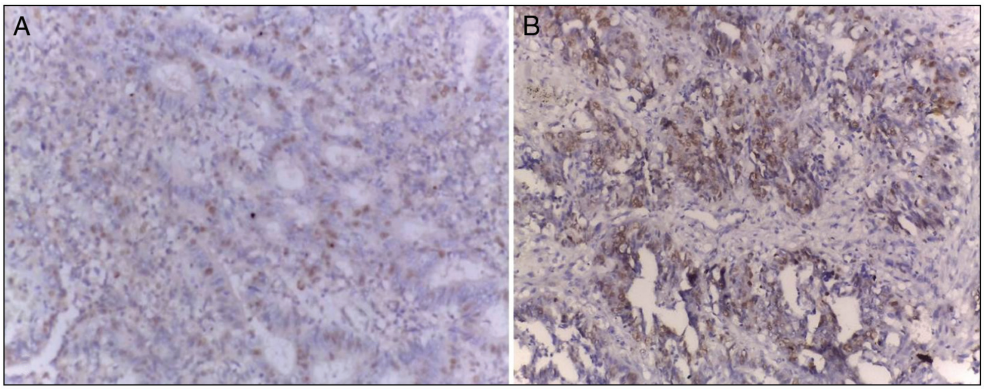
(a) Negative Cox-2 expression in colonic mucosa (IHC, ×400). (b) Low score of cytoplasmic Cox-2 expression in colonic adenoma (blue arrow) (IHC, ×400). (c) High score of cytoplasmic Cox-2 expression in colorectal carcinoma (IHC, ×200). CNND1, cyclin D1; Cox-2, cyclooxygenase-2; IHC, immunohistochemistry.

Table 1 Comparison between the studied groups according to cyclin D1 and cyclooxygenase-2

	Control group (*N*=10) [*n* (%)]	Adenoma (*N*=20) [*n* (%)]	Adenocarcinoma (*N*=30) [*n* (%)]	χ^2	*P* value
CNND1					
Low score	10 (100)	15 (75.0)	6 (20.0)	25.76	<0.001
High score	0	5 (25.0)	24 (80.0)		
Cox-2					
Positive	1 (10.0)	9 (45.0)	25 (83.3)	18.78	<0.001
Negative	9 (90.0)	11 (55.0)	5 (16.7)		

Cox-2, cyclooxygenase-2; CNND1, cyclin D1, χ^2, χ^2 test.

Figure 3



(a) Weak nuclear CNND1 expression in well-differentiated CRC. (b) Moderate nuclear CNND1 expression in moderately differentiated CRC (IHC, ×200). CNND1, cyclin D1; CRC, colorectal cancer; IHC, immunohistochemistry.

the expression of Cox-2 (*P*>0.05 for all) (Table 2, Fig. 4a,b).

Discussion

Multiple genetic alterations are responsible for the pathogenesis and progression of CRC in a systemic fashion (Chen *et al.*, 2007). Advances in studying molecular carcinogenesis have demonstrated important signaling pathways of the cell cycle in CRC pathogenesis (Yasui *et al.*, 2006). Thus, CNND1, one of these cell cycle markers, was involved in the study.

In our study, all normal colonic mucosa (100%) and 75% of colon adenoma showed low CNND1 score, whereas high nuclear CNND1 expression was observed in 80% of CRC with significant difference (*P*≤0.001). These findings support the oncogenic role of CCDN1 in inducing pathological events, which precede malignant changes during CRC tumorigenesis, which was in accordance with published research studies (Jiang *et al.*, 2006; Li *et al.*, 2014).

Regarding the pattern of CNND1 expression in the current study, it was exclusively nuclear as previous studies (Al-Maghrabi *et al.*, 2015).

However, in the study of Holland *et al.* (2001), nuclear expression was in 17% of CRC compared with 40% with cytoplasmic expression and remaining 43% with mixed patterns. Interestingly, the higher nuclear CNND1 staining was found with poorly differentiated CRC, as our results. This variability could be owing to different anti-CNND1 antibody clones and using different immunostaining scoring, besides variability in studied cases and used techniques.

Low expression of CCDN1 was shown in 20% of studied CRC and a higher score in 80% of CRC cases. There was somewhat wide range of variable expression in previous studies recording as low as 23% to as high as 100%, which may be owing to different tumor types included, variable techniques, and scoring system (Myklebust *et al.*, 2012).

The present study reported that the maximum cases (62.5%) with CNND1 high score belonged to moderate differentiated CRC, followed by well differentiated (20.8%). This was parallel to the studies done by Al-Maghrabi *et al.* (2015) and Sharma *et al.* (2021), which reported no a significant difference in CNND1 expression between grades of CRC differentiation.

However, previous published studies documented almost twice the expression of CNND1 in poorly differentiated CRC (Albasri *et al.*, 2019), as well as in other tumors like breast carcinoma (Lundberg *et al.*, 2019) and lung cancer (Sterlacci *et al.*, 2010). This is in contrast to the present study, which may be due to

Table 2 Comparison between cyclin D1 and cyclooxygenase-2 and clinicopathological parameters in studied cases

Parameters	*n* (%)	CNND1 [*n* (%)]				Cox-2			
		Low score (*N*=6)	High score (*N*=24)	FET	*P* value	Positive (*N*=25)	Negative (*N*=5)	FET	*P* value
Age (years)									
<60	16 (53.3)	5 (83.3)	11 (45.8)	FET=1.42	0.18	13 (52)	3 (60)	FET=0.0	1.0
≥60	14 (46.7)	1 (16.7)	13 (54.2)			12 (48)	2 (40)		
Sex									
Female	14 (46.7)	4 (66.7)	10 (41.7)	FET=0.41	0.38	13 (52)	1 (20)	FET=0.67	0.34
Male	16 (53.3)	2 (33.3)	14 (58.3)			12 (48)	4 (80)		
LN									
No	9 (30)	6 (100)	3 (12.5)	FET=13.58	<0.001	7 (28)	2 (40)	FET=0.0	1.0
Yes	21 (70)	0	21 (87.5)			18 (72)	3 (60)		
Distant metastasis									
No	21 (70)	6 (100)	15 (60)	FET=2.11	0.15	20 (80)	1 (20)	FET=4.57	0.033
Yes	9 (30)	0	9 (40)			5 (20)	4 (80)		
TNM stage									
Stages I and II	10 (33.3)	6 (100)	4 (16.7)	FET=11.48	<0.001	5 (20)	5 (100)	FET=8.67	0.003
Stages III and IV	20 (66.7)	0	20 (83.3)			20 (80)	0		
Tumor grade									
Well	7 (23.3)	2 (33.3)	5 (20.8)	FET=1.09	0.82	7 (28)	0	FET=8.08	0.017
Moderate	19 (63.3)	4 (66.7)	15 (62.5)			17 (68)	2 (40)		
Poor	4 (13.3)	0	4 (16.7)			1 (4.0)	3 (60)		
Tumor location									
Right	12 (40)	2 (33.3)	10 (41.7)	FET=0.0	1.0	11 (44)	1 (20)	FET=0.25	0.62
Left	18 (60)	4 (66.7)	14 (58.3)			14 (56)	4 (80)		
Depth of invasion									
T1	1 (3.3)	1 (16.7)	0	FET=3.71	0.42	1 (4)	0	FET=2.3	0.69
T2	6 (20)	1 (16.7)	5 (20.8)			6 (24)	0		
T3	22 (73.3)	4 (66.7)	18 (75)			17 (68)	5 (100)		
T4	1 (3.3)	0	1 (4.2)			1 (4)	0		
Recurrence									
No	13 (43.3)	5 (83.3)	8 (33.3)	FET=5.78	0.041	11 (44)	2 (40)	FET=2.46	0.35
Yes	12 (40)	0	12 (50)			11 (44)	1 (20)		
Unknown	5 (16.7)	1 (16.7)	4 (16.7)			3 (12)	2 (40)		

Cox-2, cyclooxygenase-2; FET, Fisher exact test; LN, lymph node.
T1: tumor involves submucosa.
T2: tumor involves muscularis propria.
T3: tumor crosses through the muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissues.
T4: tumor directly involves other organs/structures, and/or perforates visceral peritoneum.

different number of studied cases, especially of poor differentiated tumors, which affects statistical outcome.

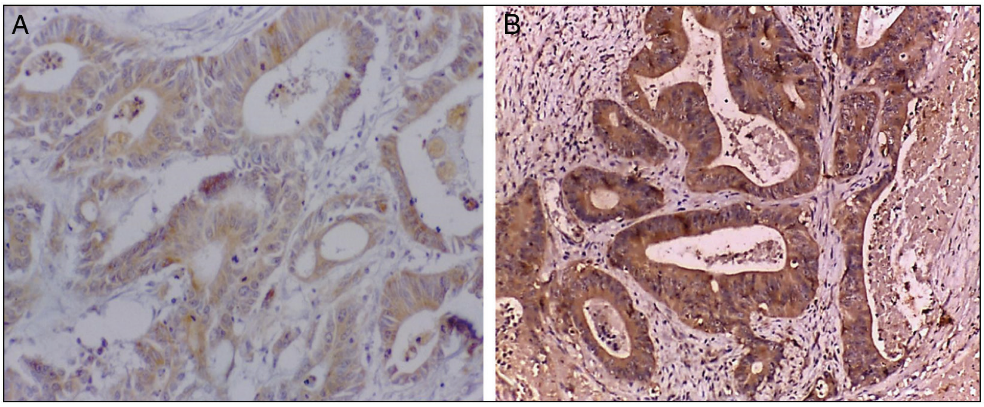
Results of Bahnassy *et al.* (2004) matched our results concerning a significant correlation with LN metastasis status (*P*=0.001).In the same study, CNND1 showed no significant correlation with stage (*P*=0.175), which is contradictory to the present work.

CNND1 overexpression was significantly correlated with advanced stage of CRC. Similarly, previous studies found a significant correlation between pathological stage and CCDN1 overexpression (Asaad *et al.*, 2010; Balcerczak *et al.*, 2005). Wangefjord *et al.* (2011) agreed with our findings as their study noted a significant correlation between high CNND1 and advanced CRC.

This study found a significant correlation between lymphatic spread in CRC and high CNND1 expression (*P*≤0.001) but did not find the same correlation concerning distant metastasis (*P*=0.15) and tumor grade (*P*=0.82). This was in parallel with the study done by Al-Maghrabi *et al.* (2015). In contrast, the same study found no significant correlation with pathological stage.

Holland *et al.* (2001) and Ogino *et al.* (2009) have found that CRC tumors with high CNND1 score are less aggressive than tumors with decreased CNND1 expression. Moreover, Lehn *et al.* (2010) concluded the same findings in breast cancer. This favorable prognostic role of CNND1 in their studies may be explained by the better response of tumors with higher CNND1 score to adjuvant chemotherapy and differences in procedures of staining and methods of evaluation.

Figure 4



(a) Weak Cox-2 expression in well-differentiated CRC (IHC, ×400). (b) Strong cytoplasmic Cox-2 expression in moderately differentiated CRC (IHC, ×400). CRC, colorectal cancer; Cox-2, cyclooxygenase-2; IHC, immunohistochemistry.

The current study found no significant correlation with age, tumor size, and location. This was supported by the studies of Jiang *et al.* (2006) and Li *et al.* (2014), where the same clinicopathological parameters were not correlated with CNND1.

Cox-2 is an inducible enzyme that catalyzes AA to PGs, which are involved in inflammatory reaction and carcinogenesis (Wang and Dubois, 2010). The carcinogenic role of Cox-2 has been elucidated in various organs, such as breast (Denkert *et al.*, 2004), liver (Bayomi *et al.*, 2015), and urinary bladder (Hammam *et al.*, 2008). As its role in CRC is still controversial, its expression was evaluated in the current study.

In the present study, positive Cox-2 expression was found in 83.3% of CRC, whereas it significantly deceased in the control group (10%) and 45% of adenoma (χ^2=18.78; P<0.001). Its expression was statistically upregulated from normal to carcinoma passing through adenomatous changes, supporting its pathological role in colorectal carcinogenesis, which was in keeping with previous studies (Brown and and DuBois, 2005; Arber *et al.*, 2006). There was also a significant difference in Cox-2 expression at the molecular level between CRC and normal colonic epithelium (Wu *et al.*, 2004).

In the current work, Cox-2 showed a potential prognostic value. Its expression was significantly correlated with grade (P=0.017), stage (P=0.003), and distant metastasis (P=0.003).

Similarly, Cox-2 expressions was expressed in 12% of normal mucosa, compared with 72% of CRC in a study done by Smakman *et al.* (2005).

These findings were confirmed by previous reports in which the normal colonic mucosa had Cox-2 negative expression (Singer *et al.*, 1998), and 29% of adenoma (Chapple *et al.*, 2000) and 77.9% of CRC (Roelofs *et al.*, 2014) were Cox-2 positive. At the same time, CRC with higher stage and distant metastasis showed significantly increased Cox-2 expression (Elzagheid *et al.*, 2013).

Elzagheid *et al.* (2013) demonstrated a significant correlation between Cox-2 overexpression and CRC stage (P<0.05). Increased Cox-2 activity promotes CRC progression, elucidating its prognostic value, which matched our results. Stage and pathological grade of CRC were significant correlation between Cox-2 overexpression in previous studies (Dimberg *et al.*, 1999), which supported the current results, suggesting a role of Cox-2 in tumor invasiveness.

In the same line, published research studies proved that at follow-up, colorectal adenoma with elevated Cox-2 before Cox-2 inhibitors were likely to have fewer colon adenoma among patients having history of CRC and/or adenoma. Moreover, Cox-2 inhibitors reduce the mortality and morbidity of CRC (Ezenkwa *et al.*, 2021). Therefore, CRC with Cox-2 overexpression has more favorable response to anti-Cox-2 therapies (Negi *et al.*, 2019). This was in concordance with our study, proving the precancerous significance of Cox-2 overexpression in colonic adenoma-carcinoma sequence.

In contrast, Lin *et al.* (2013) observed lower Cox 2 expression in CRC than adjacent normal mucosa. This discrepancy is different used a scoring scale as Lin *et al.* (2013) considered that Cox-2 was positive if more than 10% of the tumor cells showed membranous expression. In addition, defining a cutoff point for the nearby normal mucosa from the tumor may shed more light in this concern. Concerning this study, the nearby mucosa was taken at more than 5 cm away from tumors.

However, the normal mucosa, adjacent to CRC, showed negative Cox-2 staining in previous studies, which is parallel to the present work (Elzagheid *et al.*, 2013).

In the present study, there was a significant correlation between Cox-2 expression and distant metastasis status (*P*=0.003), which was in parallel with other studies (Xiong *et al.*, 2005; Shin *et al.*, 2014; Wan *et al.*, 2009); however, there was no correlation with lymphatic spread, as previously published (Okudur *et al.*, 2008).

Lim *et al.* (2008) and Mahmoud *et al.* (2014) disagreed with us, as they detected no significant association between tumor stage and Cox-2 expression.

No correlation was found between other parameters (age, sex, and size of tumors) and Cox-2 expression, which was in agreement with other results (Wu and Sun, 2015). Based on that, Cox-2 expression in CRC may be considered a poor prognostic marker in CRC as it was associated with poor prognosis, which was approved by other studies (Al-Maghrabi *et al.*, 2012). In contrast, Fux *et al.* (2005) and Lim *et al.* (2008) demonstrated little prognostic effect of Cox-2 in CRC.

Conclusion

The present work suggested the oncogenic role of CNND1 and Cox-2 in CRC. Furthermore, overexpressions of CNND1 and Cox-2 are associated with poor prognostic factors, implicating their potentially prognostic role in CRC.

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Nil.

Conflicts of interest

No conflict of interest.

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