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ORIGINAL ARTICLE

Immunohistochemical Expression of SOX2 and EpCAM in Colorectal Cancer and Its Precancerous lesions

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ABSTRACT

Background: There is an actual need for novel prognostic biomarkers to improve colorectal cancer (CRC) patient's outcome. We aim to evaluate SOX2 and EpCAM immunohistochemical expression in 40 cases of CRC and premalignant lesions. **Methods:** The immunohistochemical expression was done according to Envision polymer technique on 40 cases of CRC besides twenty specimens of premalignant lesions. Furthermore, their clinicopathological significance was statistically investigated. **Results:** High SOX2 immunoexpression was detected in 57.5 % of CRC cases and was significantly associated with tumor size, high tumor grade, LVI, LN involvement, advanced tumor stage, and tumor budding (P=0.04, P<0.001, p=0.02, p=0.001, p=0.001, p=0.001, respectively). Negative SOX2 immunoexpression was observed in 70 % of premalignant lesions with no statistically significant difference. High EpCAM immunoexpression was noted in 21 52.5% of the malignant lesions, and was significantly associated with high tumor grade, LVI, LN involvement, advanced tumor stage, and tumor budding (P=0.005, p =0.002, p<0.001, p<0.001, p=0.005, respectively). A statistically highly significant association between low and moderate EpCAM expression and stromal lymphocytic infiltration (P<0.001). Low EpCAM expression was noted in 75 % of premalignant lesions with no statistically significant difference. **Conclusions:** This study emphasized the role of SOX2 and EpCAM in colorectal carcinogenesis and their implication in CRC progression, LN metastasis and distant metastasis.

Key Words: colorectal carcinoma; SOX2; EpCAM; prognostic factor

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second cause of mortality worldwide, with 1.9 million new cases and 930,000 deaths reported in 2020 [1]. In Egypt, it represents about 33.8% of all GIT tumors and 6.2% of total malignancies [2]. Cancer stem cells (CSCs) started to be a hot spot in the cancer research. CSCs are a subpopulation of the tumor cells that have self-renewal capacity and exhibits treatment resistance so promoting the cancer progression and recurrence [3]. Numerous transcriptional factors are involved in supporting the stemness phenotype of CSCs. Sex-determining region Y-box protein 2 (SOX2) [4] and epithelial cell adhesion molecule (EpCAM) [5] have been reported to have tumorigenic ability and as putative CSC markers in several malignancies [6].

The transcription factor SOX2 (sex-determining region Y-box 2) gene is situated on chromosome 3 at the position q26.3–27 and encodes for a protein of 317 amino acids and it is a master regulator of CSCs. SOX2 affects cancer cell behaviors as the capacity to proliferate, invade, and metastasize [4]. Moreover, SOX2 facilitates resistance to tumor therapies via regulation of stemness and self-renewal of CSCs [4]. However, further studies are needed to determine the molecular pathways associated with these biological functions [7]. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein that has also been recognized as a CSC marker. The EpCAM signaling pathway is involved in multiple cellular functions as cell adhesion, migration, and differentiation [5]. Overexpression of EpCAM enhances tumorigenesis via upregulation of reprogramming factors as Oct-4, Nanog, and SOX2 whereas its downregulation inhibited these factors, so suppressing tumor initiation, and progression [8]. Specific ablation of EpCAM expressing CSCs could be a novel cancer

therapeutic strategy [5 There is an actual need for novel prognostic biomarkers to improve CRC patient's outcome. Therefore, we evaluated SOX2 and EpCAM immunohistochemical expression in CRC and premalignant lesions

METHODS

Tissue specimens: This retrospective study included 60 paraffin blocks (40 CRC and 20 premalignant colorectal lesions) they were collected from the archive of Pathology Department during the period from 2018-2020. The clinicopathological data were obtained from patient files and all cases undergone histopathological evaluation and immunohistochemical staining. Twenty specimens of normal colonic mucosa adjacent to CRCs were taken as a control. They were obtained from the free safety margins of the submitted cases and were histologically examined for confirmation of neoplastic free state. Primary CRC (total colectomy), premalignant lesions, and only cases with complete clinicopathological data were included in this study. Cases that previously treated with chemotherapy or radiotherapy were excluded from the study as it changes the morphology of the cells and affects the diagnosis. The study was approved by the research ethical committee of Faculty of Medicine, Zagazig University. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Immunohistochemical staining: The immunohistochemical staining procedure was performed according to the polymer Envision detection system. The primary antibodies that used were: EpCAM (1:1000 dilution, ab124825; Abcam, UK), and SOX2 (1:400; rabbit polyclonal antibody, MA, USA), then diaminobenzidine substrate was used as the chromogen. Mayer's hematoxylin was used to counter stain the slides. In each cycle of doing IHC, positive controls [squamous cell carcinoma larynx for SOX2 and colon cancer tissue for EPCAM were included and negative controls were performed by omitting the primary antibodies.

Immunohistochemical evaluation

SOX2 scoring: Nuclear SOX2 expression was evaluated. Extension of nuclear staining was scored as: 0 (<5%) positive cells; 1 (5–25%) positive cells; 2 (26–75%) positive cells; and 3 (>76%) positive cells., whereas stain intensity was scored as: 0:no staining, 1: faint-yellow, 2: brown–yellow, and 3: dark brown. After summation scores ≥3+++ were defined as high-level expression and scores< 3+++ were defined as low-level expression (9).

EpCAM scoring: Cytoplasmic and membranous EpCAM expression was analyzed. The intensity (I) of EpCAM expression can vary between 0 (no expression), 1 (weak), 2 (moderate) and 3 (intense). The percentage (P) of cells showing EpCAM expression was: 1(<10%), 2 (10-50%), 3 (51- 80%), 4(>80%). Thus, total score (TS) can take the following values: 0, 1, 2, 3, 4, 6, 8, 9, 12. The results were afterwards grouped into 4 groups: TS 0 (no expression), TS 1-4 (low expression), TS 6-8 (moderate expression), and TS 9-12 (high expression) (10).

Statistics: The data were computerized and SPSS program version 18.0 was used for the statistical analysis. Qualitative data were presented in frequencies and relative percentages. The difference between qualitative variables was calculated by Chi-square test, and Fisher's exact test was used to calculate the difference when one or more of the studied cells were less than 5. Quantitative data were expressed as mean ± SD. P value of >0.05 is non- significant, while p value < 0.05 is significant, and < 0.001 was considered highly significant results.

RESULTS

Clinicopathological features of the studied cases

The premalignant lesions group included 20 cases; 15 cases were colorectal adenomas, and 5 cases were ulcerative colitis. Colorectal adenomas included 10 cases of tubular adenomas and 5 cases of tubulo-villous adenomas. Adenomas were histologically categorized as adenoma with low-grade dysplasia (5 cases) or high-grade dysplasia (10 cases).

The clinicopathological features of CRC patients (n=40) were summarized in (Table 1). The mean age was 51.7 ± 11.5 (range 28-79 years) while the age of patients in premalignant group was 50.1 ± 11.6 (range 35-79 years). Right colon was the commonest site of CRC in our study (75%); and the infiltrating border was noted in 75% of the cases. The predominant tumor size was 5 cm (67.5%). Most of CRC cases (57.5%) were low grade. Most of the patients (45%) were at stage III, while 70% had adenocarcinoma type (Fig. 1). Lymph node metastasis (LN) was noted in 62.5% of the patients.

SOX2 and EpCAM immunoexpression in the studied premalignant group

Normal colonic mucosa and 14 (70%) of premalignant group revealed a negative SOX2 expression. No significant relation was found between SOX2 expression and the adenoma type or grade. Fifteen (75%) cases of premalignant group

showed low EpCAM expression without significant relation between EpCAM expression and the grading of adenomas (Fig. 2) & (Table 2).

SOX2 and EpCAM immunoexpression in the studied CRC cases : The high nuclear SOX2 expression was detected in (57.5%) of the studied CRC cases (Fig. 3). There was a significant upregulation of SOX2 expression with tumor size (p=0.04), high grade (p<0.00), lymphovascular invasion (LVI) (p= 0.02), LN metastasis (p= 0.001), advanced tumor stage (p=0.001), and tumor budding (p=0.001). On the other hand, there was a non-significant association of SOX2 expression with the histological type.

Regarding EpCAM, all the cases of CRC were positive with varying degrees of intensity. Among CRC, only 9 (22.5%) showed a low expression, 14 (35%) showed moderate expression, and 21(52.5%) revealed EpCAM expression (Fig. 4). There was a significant upregulation of EpCAM expression with the tumor size (p=0.03) high grade (p=0.005), LVI (p = 0.002), LN metastasis (p<0.001), and advanced tumor stage (p<0.001), and tumor budding (p=0.005) (Table S3, 4). However, there was a non-significant association of EpCAM expression with the histopathological type. A highly statistically significant difference was found between CRC and premalignant group regarding SOX2 and EpCAM expression p= (<0.001 for each). A significant association between SOX2 and EpCAM expression in the studied CRC patients was detected (p<0.001) (Table S5, 6).

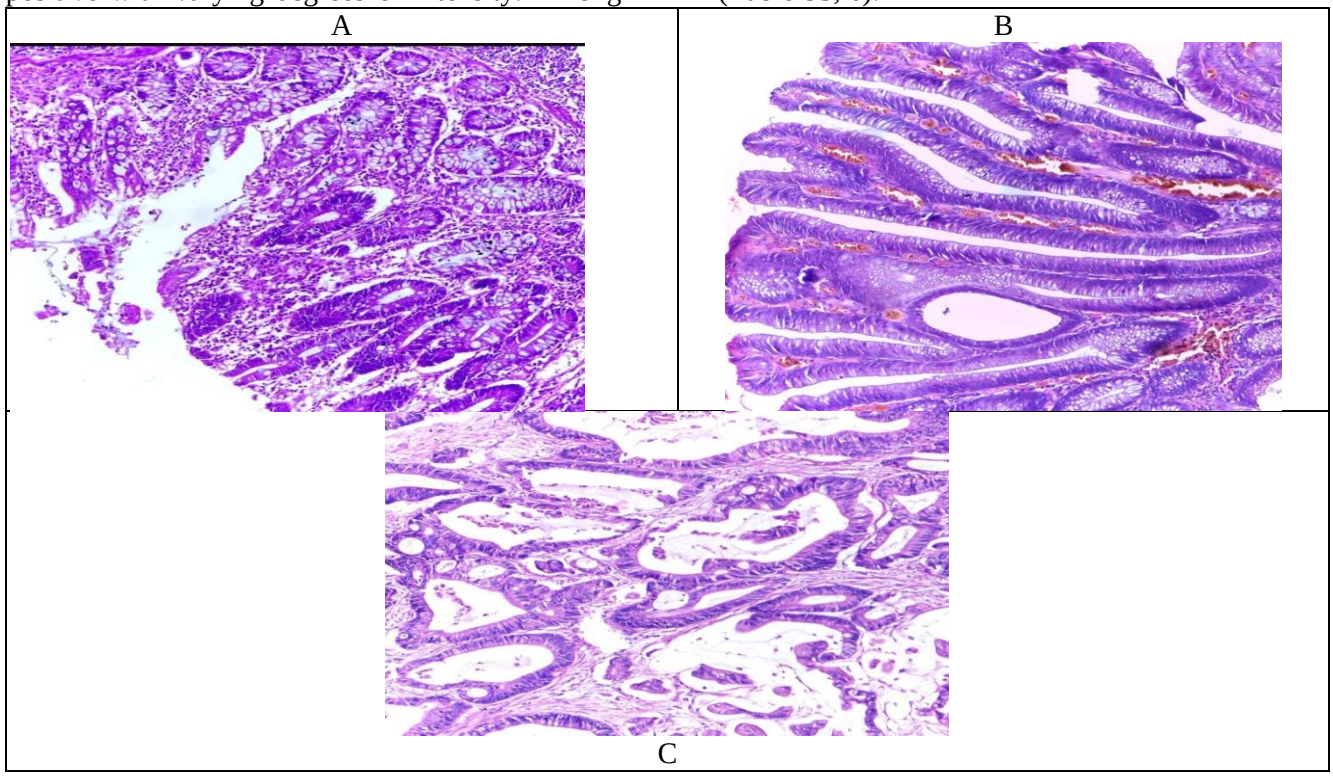

Figure 1: a) Ulcerative colitis showing partially ulcerated mucosal surface, moderate dysplastic changes in the intestinal crypts (H&E x 100), b) Tubulovillous adenoma showing low-grade dysplasia (H&E x 400), c) Low-grade adenocarcinoma with mucoid change (H&E x 200).

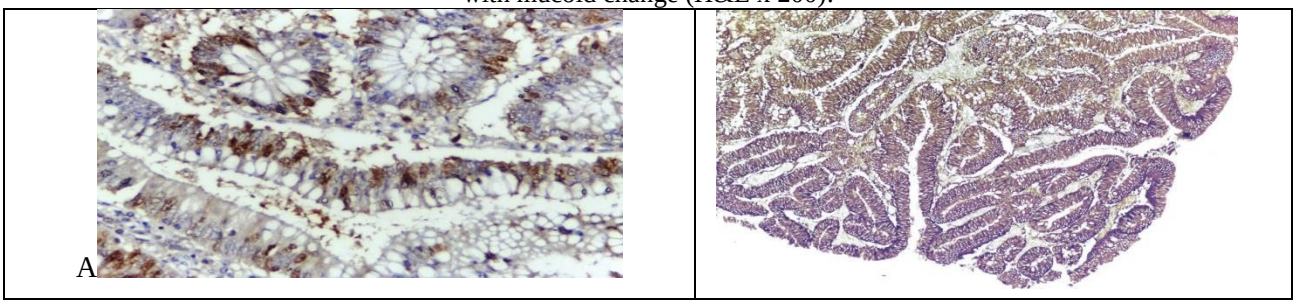

Figure 2: a) Tubular adenoma with high grade dysplasia showing low nuclear SOX2 expression (IHC x 400), b) Tubulo-villous adenoma showing low membranous and EpCAM expression (IHC x 100).

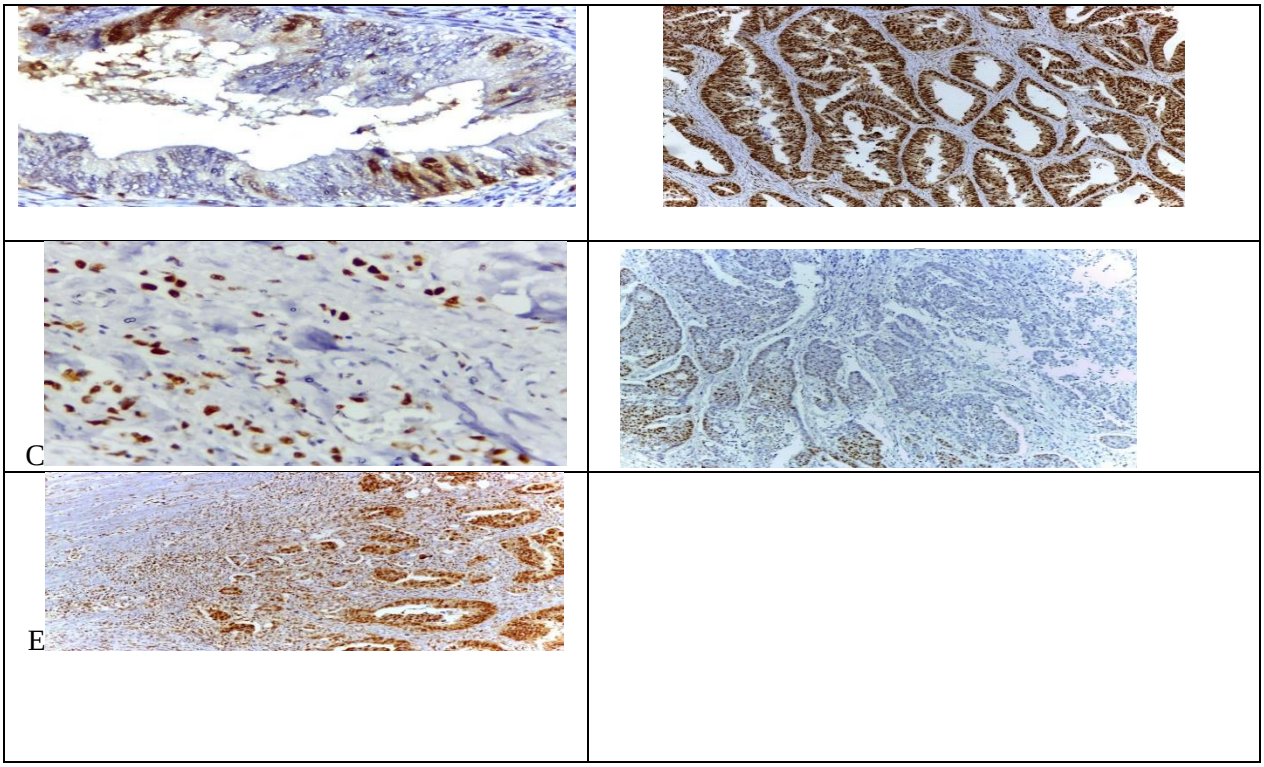

Figure 3: a) Well differentiated adenocarcinoma showing low SOX2 expression (IHC x 400) b) Well differentiated adenocarcinoma showing high nuclear SOX2 expression (IHC x 100), c) Signet ring carcinoma showing high nuclear SOX2 expression (IHC x 400), d) Poor differentiated adenocarcinoma showing low SOX2 expression (IHC x 100), e) Well differentiated adenocarcinoma with the invasive border showing high SOX2 expression in tumor buds (IHC, original magnification x 100).

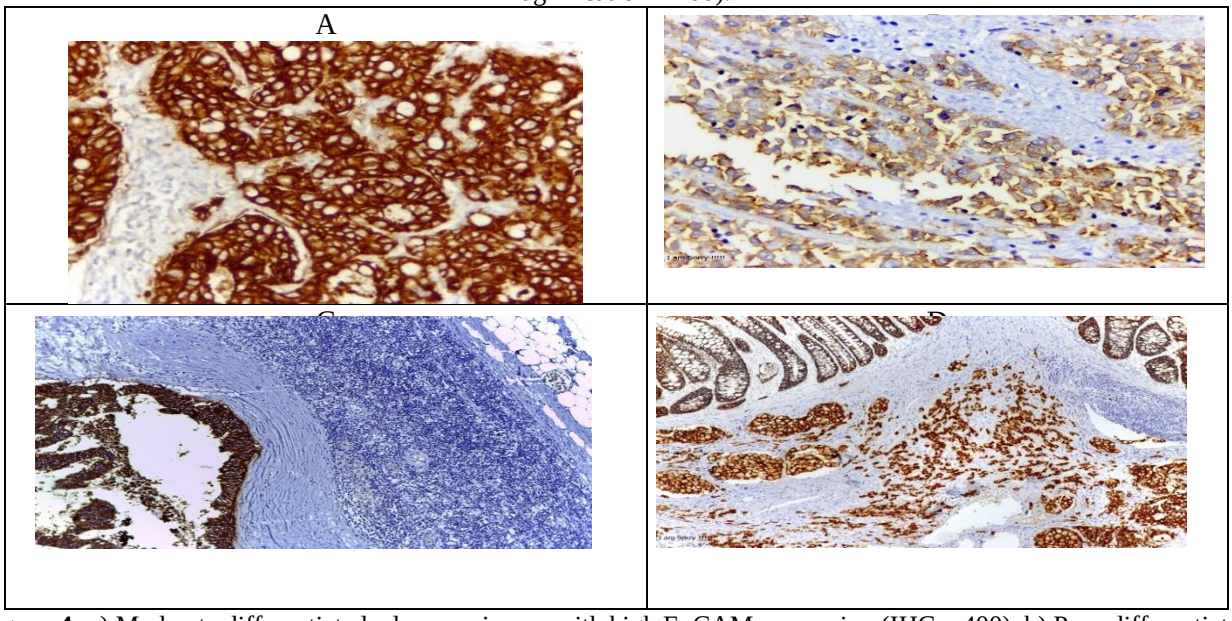

Figure 4: a) Moderate differentiated adenocarcinoma with high EpCAM expression (IHC x 400), b) Poor differentiated adenocarcinoma showing moderate membranous EpCAM expression (IHC x 400), c) High membranous EpCAM expression in malignant acini infiltrating lymph node (IHC x 100), d) Poor differentiated adenocarcinoma showing high membranous EpCAM expression with lower expression in the overlying mucosa (IHC x 100).

Table 1: Clinicopathological features of CRC patients (n= 40)

Characteristics	All patients (N=40)		Characteristics	All patients (N=40)	
	No.	%		No.	%
Age (years)			**N stage**		
Mean±SD	51.7± 11.5		N0	15	37.5%
Sex			N1	18	45.5%
Male	27	67.5%	N2	7	17.5%
Female	13	32.5%			
Size			**M stage**		
< 5 cm	13	32.5%	M0	30	75%
> 5 cm	27	67.5%	M1	10	25%
Site			**AJCC stage**		
Right colon	20	75%	I	6	15%
Left colon	8	20%	II	6	15%
Rectum	2	5%	III	18	45%
			IV	10	25%
Histological type:					
Adenocarcinoma	28	70%	**Lympho-vascular invasion**	17	42.5%
Mucinous	7	17.5%	Present	23	57.5%
Signet ring	5	12.5%	Absent		
Tumor border			**Tumor Grade:**		
Pushing	10	25%	Low grade	23	57.5%
Infiltrating	40	75%	High grade	17	42.5%
T stage			**Tumor budding**		
T1	4	20.0	Present	18	45%
T2	8	10.0	Absent	22	55%
T3	17	42.5%			
T4	11	27.5%			

Table 2: SOX2, EpCAM expression in premalignant group

Premalignant lesions	SOX2				P	Premalignant lesions	EpCAM						P
	Negative		LOW				NEGATIVE		LOW		MODERATE		
	No.	%	No	%			No	%	No	%	No	%	
Diagnosis:						**Diagnosis:**							0.4
Adenoma (n=15)	10	66.7			0.9	Adenoma (n=15)	2	13.3	12	80	1	6.7	
Ulcerative colitis (n=5)	5	33.3				Ulcerative colitis (n=5)	2	40.0	3	60	0	0.0	
	4	80	1	20									
Adenoma type:						**Adenoma type**:							0.2
Tubular adenoma (n=10)	8	80			0.3	Tubular adenoma (n=10)	2	20	8	80	0	0.0	
	2	20					0	0.0	4	80	1		
	2	40											

Tubulo-villous adenoma(n=5)	3 60		Tubulo-villous adenoma (n=5)	20	
Adenoma grade: **Low (n=5)** **High (n=10)**	5 100 0 0 5 50 5 50	0.1	**Adenoma grade**: Low (n=5) High (n=10)	0 0.0 5 100 0 0.0 2 20 7 70 1 10	0.4

DISCUSSION

Colorectal cancer (CRC) is among the commonest reasons of morbidity and mortality representing a major public health challenge [1]. Growing evidence indicates that cancer-related lethality is principally caused by therapy-resistant CSCs [3]. SOX2 and EpCAM have been identified to drive CSCs properties and contribute to tumor aggressiveness. They were recognized as a potential target for cancer therapy [4,5]. Lin et al., reported that EpCAM downregulation suppressed tumor initiation and progression of cancer colon by inhibition of SOX2 expression [8]. Previous studies have reported considerably SOX2 upregulation in cancer cells as compared to normal tissue [11,12]. In the current study, a highly statistically significant increase in SOX2 expression in CRC is noted when compared to normal colonic mucosa or premalignant group. High SOX2 expression was found in (57.5%) of CRC cases and none of the premalignant cases showed high SOX2.

SOX2 induced cancer stemness in CRC cells and control many receptors mediating signaling pathways that participate in CRC progress such as epidermal growth factor receptor, which is one of the most important therapeutic targets of CRCs [12].

In our CRC cases high SOX2 expression showed no significant relationship with histological subtypes, age, site, and sex as previously reported [13]. However, we noticed a positive association between SOX2 expression with larger tumor size, poor differentiation, lymph metastasis as well as advanced stage of CRC in agreement with previous studies [6,11,13]; contrarily to what has been described previously [12,14,15]. This discrepancy can be explained by different scoring methods and diverse geographic distribution. Our results confirm the crucial role of SOX2 in stemness and malignant progression in CRC [11]. SOX2 enhances and facilitates the dissemination process via EMT in CRC [11]. In our study, a significant relation has been noted between high SOX2 expression and distant metastasis. Similar results were published by Javaeed and Ghauri who conducted a meta-analysis that compared the association of SOX2 expression with LN metastasis and distant metastasis and reported a significant relation between high SOX2 expression and distant metastasis in hepatic ($P = 0.006$), head and neck ($P < 0.001$), and CRC cancers ($P = 0.03$) [14].

Prognostic value of EpCAM expression varies depending on the tumor entity. Previously, high expression of EpCAM was associated with better prognosis in many tumors as esophageal, renal, and gastric cancers. In contrast, EpCAM high expression was associated with poor prognosis in breast, and bladder cancer [16]. Multiple cellular functions of EpCAM might differently affect single cells within tumors. The discrepancy of the prognostic value of EpCAM expression remains poorly understood and requires further investigations [16]. In the present study, high EpCAM expression is associated with poor prognostic factors in CRC which is in agree with Seeber et al study [17]. However, high EpCAM expression was associated with better prognosis in CRC in other investigations [18,19]. The association of EpCAM with different clinical outcome is complex and may vary depending on the origin of the tumor or even the stage of tumor progression [16]. In the current study, a significant increase of EpCAM expression in CRC is noted when compared to normal colonic mucosa or premalignant lesions, in agreement with Zhou et al., who reported that EpCAM was highly expressed in tumor tissue (92%) but was poorly or not expressed in benign lesions (6%) or para-carcinoma tissue (10%) [20]. This confirm that EpCAM expression is associated with the carcinogenesis of CRC. However, our results were against what was published by Mokhtari et al. 2017 who reported that EpCAM expression in the tumoral tissue was significantly less than that in the normal tissue [21]. No correlation in EpCAM expression was found between CRC and benign colonic lesions in Han et

al study [19]. There was no significant association between EpCAM expression and either age or sex of the submitted cases in our study. In agreement with Seeber et al. who found non-significant association between EpCAM expression either with age or sex of their CRC cases [17]. Kim et al. found no significant association existed between EpCAM expression and histologic subtypes of CRC which is in line with our results [22].

EpCAM expression stimulates cell differentiation and cell proliferation via up-regulation of the proto-oncogene c-myc, which causes carcinogenic effects (19). Consistently, we noted a positive association between EpCAM expression and larger tumor size, tumor grade and LN metastasis in harmony with the previous studies [17, 23]. Moreover, these results came in line with Abd Elmaqsoud et al. who found a significant relation between high EpCAM expression and tumor grade in breast cancer [24], but against to that reported by Hong et al. who found negative EpCAM staining in poorly differentiated carcinomas [25] and this difference may be related to different grades of tumor used in their study. The current study showed a statistically significant relation between high EpCAM expression and tumor budding. In agreement with our results, De Smedt et al. noticed a significant relation between high EpCAM expression and tumor budding in cancer colon [26], contrary to Hong et al. who found that the frequency of EpCAM expression was significantly decreased in the tumor budding and in poorly differentiated clusters [25]. Han et al explained the correlation between high EpCAM expression and tumor budding by the role of EpCAM in epithelial mesenchymal transition where it is thought to facilitate the dissemination process. Strong expression of EpCAM promotes EMT and a cancer stem cell phenotype with increased migration and invasion, via activation of AKT, mTOR, p70S6K and 4EBP1 [19]. Our study revealed a statistically significant relation between EpCAM expression and LVI in agreement with Kim et al. [22] and against results of Wang et al. who found a significant association between loss of EpCAM expression and LVI [27].

The AJCC staging system considered an independent prognostic factor in CRC. The current study showed that there was a significant relation between EpCAM expression and tumor stages in CRC cases, like previous findings [17]. In colon cancer cell lines, EpCAM enhanced the transcription of reprogramming factor genes as c-Myc, Oct3/4, SOX2, and Nanog, besides the EMT regulators Snail and Slug through EpICD signaling [17]. A positive relation was found between SOX2 and EpCAM expression in our studied CRC that assumed that both EpCAM and SOX2 drive the malignant progression in CRC.

CONCLUSIONS

This study emphasized the role of SOX2 and EpCAM in colorectal carcinogenesis and their implication in CRC progression, LN metastasis and distant metastasis. Targeted therapy of SOX2 and EpCAM can open new channel for treating CRC.

Conflict(s) of interest: None

Financial Disclosures: None

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domain of epithelial cell adhesion molecule correlates with poor patient outcome in primary colorectal adenocarcinoma.Curr Oncol. 2016;23(3):e171-178. doi:10.1371/journal.pone.0099594

SUPPLEMENTARY FILE

Variables	SOX2						P	EpCAM						P
	Negative		Low		High			Low		Moderate		High		
	No.	%	No.	%	No	%		No.	%	No.	%	No	%	
Tumor Size: **< 5cm (n=13)** **>5cm(n=27)**	1 2	7.7 7.4	8 6	61.5 22.2	4 19	30.8 70.4	**0.04 S**	4 1	30.8 3.7	5 9	38.5 33.3	4 17	30.8 63.0	**0.03 S**
Initial site: **Right colon (n=30)** **Left colon (n=8)** **Rectum (n=2)**	2 1 0	6.7 12.5 0.0	12 1 1	40.0 12.5 50.0	.16 6 1	53.3 75.0 50.0	0.6	4 1 0	13.3 12.5 0.0	11 3 0	36.7 37.5 0.0	15 4 2	50.0 50.0 100	0.8
Histological type: **Adenocarcinoma (n=28** **Mucinous (n=7)** **Signet ring (n=5)**	3 0 0	10.7 0.0 0.0	13 1 0	46.4 14.3 0.0	12 6 5	42.9 85.7 100	0.07	4 1 0	14.3 14.3 0.0	12 1 1	42.9 14.3 20.0	12 5 4	42.9 71.4 80.0	0.4
Grade: **Low grade (n=23)** **High grade (n=17)**	3 0	13.0 0.0	13 1	56.5 5.9	7 16	30.4 94.1	**<0.001 HS**	4 1	17.4 5.9	12 2	52.2 14.3	7 14	30.4 85.4	**0.005 S**
Tumor budding **Present(n=18)** **Absent(n=22)**	0 3	0.0 13.6	2 12	11.1 54.6	16 7	88.9 31.8	**0.001 S**	1 4	5.6 18.1	3 11	16.7 50.0	14 7	77.8 31.8	**0.005 S**
Lympho-vascular invasion **Present(n=17)** **absent (n=23)**	0 3	0.0 13	3 11	17.6 47.8	14 9	82.4 39.1	**0.02 S**	0 5	0.0 21.7	3 11	17.6 47.8	14 7	82.3 30.4	**0.002 S**
LN metastasis **Present(n=25)** **absent (n=15)**	0 3	0.0 20	5 9	20.0 60.0	20 3	80.0 20.0	**0.001 S**	1 4	4.0 26.7	5 9	20.0 60.0	19 2	76.0 13.3	**<0.001 HS**

Table 3: The association between SOX2, EpCAM expression and clinicopathological parameters.

Variables	SOX2						χ^2	P	EpCAM						χ^2	P
	Negative		Low		High				Low		Moderate		High			
	No.	%	No.	%	No	%			No.	%	No.	%	No	%		
T stage: **T1 (n=4)** **T2 (n=8)** **T3(n=17)** **T4(n=11)**	1 0 1 1	25. 0 0.0 5.9 9.1	3 2 7 2	75.0 25.0 41.2 18.2	0 6 9 8	0.0 75.0 52.9 72.7	8.7	0.2	3 1 1 0	75.0 12.5 5.9 0.0	1 2 8 3	25.0 25.0 47.1 27.3	0 5 8 8	0.0 62.5 47.1 72.7	18. 9	**0.004 S**
N stage: **N0 (n=15)** **N1-2 (n=25)**	3 0	20. 0 0.0	9 5	60.0 20.0	3 2 0	20.0 80.0	15. 2	**0.001 S**	4 1	26.7 4.0	9 5	60.0 20.0	2 19	13.3 76.0	15. 2	**0.001 S**
M stage **M0(n=30)** **M1(n=10)**	3 0	10 0.0	1 4 0	46.7 0.0	1 3 1 0	43.3 43.5	9.8 6	**0.007 s**	5 0	16.7 0.0	1 4 0	46.7 0.0	11 10	36.6 100. 0		**0.001 S**

AJCC Stage **I (n=6)** **II (n=6)** **III (n=18)** **IV (n=10)**	1 2 0 0	16.7 33.3 0.0 0.0	5 4 5 0	83.3 66.7 27.8 0.0	0 0 1 3 1 0	0.0 0.0 72.2 100	28.3	**<0.001 HS**	4 0 1 0	66.7 0.0 5.6 0.0	2 6 6 0	33.3 100 33.3 0.0	0 0 11 10	0.0 0.0 61.1 100	38.4	**<0.001 HS**

Table 4: The association between SOX2, EpCAM expression and staging of colorectal carcinoma

Study group	SOX2						χ^2	P
	Negative		Low		High			
	No.	%	No.	%	No.	%		
Control group	20	100	14	70.0	3	7.5	34.6	<0.001 HS
Premalignant group	0	0	6	30.0	14	35.0		
Malignant group	0	0	0	0.0	23	57.5		

HS: P-value<0.001 is high significant

Table (5): Comparison between control, premalignant and malignant groups in SOX2 immunoexpression:

EpCAM	Control group (n=20)		premalignant group (n=20)		Malignant group (n=40)		χ^2	P
	No.	%	No.	%	No.	%		
Negative	8	40	4	20.0	0	0.0	42.9	<0.001 HS
Low	12	60	16	80.0	5	12.5		
Moderate	0	0	0	0.0	14	35.0		
High	0	0	0	0.0	21	52.5		

HS: P-value<0.001 is high significant

Table 6: Comparison between control, premalignant and malignant groups in EpCAM expression:

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