Study of Immunohistochemical Expression of ALDH1A1 & CD44 in Colorectal Carcinoma

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Abstract

Background: Colon cancer is the fourth most common cancer in the world, while rectal cancer ranks the eighth among all cancers. Together (colorectal carcinomas; CRC) are the third most common cancer diagnoses all over the world and the second most deadly cancer in the world after lung cancer. In Egypt and according to WHO statistics, the colon cancer ranks the eighth most common cancer. It represents 2.7% of the total cancers and 2.4% of the total cases of death from cancer. Cancer stem cells (CSCs) play a critical role in the metastasis and relapse of colorectal cancer. Colorectal CSCs are defined with a group of cell surface markers, such as CD44, CD133, CD24, EpCAM, LGR5 and ALDH. They are highly tumorigenic, chemoresistant and radioresistant and thus are critical in the metastasis and recurrence of colorectal carcinoma and disease-free survival. The aim of current study is to investigate the relation between CD44 and ALDH1A1expression and the clinicopathological features of CRC.

Methods: Immunohistochemical staining for ALDH1A1 and CD44 was performed on 70 randomly selected tissue blocks of primary colorectal adenocarcinoma and their lymph node, including fifty-three (75.7%) of cases were conventional adenocarcinomas (NOS), 7 (10%) cases were mucinous carcinoma and 10 (14.3%) cases were Signet ring carcinoma.

Results: As regarding ALDH1A1, high expression was detected in 52 (74.3%) of cases. A statistically significant association was observed between ALDH1A1 high expression and higher tumor grade, poorly differentiated clusters (PDCs) grade, regional lymph node involvement, Lymphovascular invasion (LVI) advanced tumor stage, tumor necrosis and tumor infiltrating lymphocytes (P value> 0.001, <0.001, <0.001, <0.001, <0.001, P 0.038 and 0.002). As regarding CD44, high expression was detected in 43 (61.4%) of cases. A statistically significant association was observed between CD44 high expression and larger tumor size, higher tumor grade, poorly differentiated clusters (PDCs) grade, regional lymph node involvement, Lymphovascular invasion (LVI) advanced tumor stage, tumor necrosis and tumor infiltrating lymphocytes (P value 0.046, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001 and 0.021).

Association of ALDH1A1 and CD44 expression with different clinicopathological variables were further tested using univariate and multivariate regression analysis. The current study found that tumor grade, PDCs grade, modified Dukes staging, lymphovascular invasion and tumor necrosis were independently associated with ALDH1A1 expression (P value 0.034*, 0.022*, 0.047*,0.035*, 0.013 respectively).

The current study found that tumor grade, PDCs grade, modified Dukes staging and lymphovascular invasion were independently associated with CD 44 expression (P value 0.012*, 0.046*, 0.048*, 0.022* respectively).

A statistically significant association was found between both markers, ALDH1A1 and CD44 high expression in colorectal carcinoma (P value <0.001).

Conclusion: ALDH1A1 and CD 44 high expression could be considered as poor prognostic marker in the evaluation of patients with Colorectal Carcinoma. Both ALDH1 and CD 44 can play essential role in the pathogenesis, aggressiveness, invasion, and progression of CRC.

Keywords: Colorectal carcinoma, Clinicopathological Features, Immunohistochemistry, ALDH1A1, CD44.

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1. INTRODUCTION

Colon cancer is the fourth most common cancer in the world, while rectal cancer ranks the eighth among all cancers. Together (colorectal carcinomas; CRC) are the third most common cancer diagnoses all over the world and the second most deadly cancer in the world after lung cancer. They account for 10% of all cancer diagnoses and 9.4 % of cancer deaths [1]. In Egypt and according to WHO statistics, the colon cancer ranks the eighth most common cancer. It represents 2.7% of the total cancers and 2.4% of the

total cases of death from cancer. While, the rectal cancer is the seventeenth representing 1.2% of the total cancers and 0.98% of the total cases of death from cancer. Both (CRC) are the sixth most common cancer in Egypt. Colon cancer is more common than rectal cancers with high Age-standardized incidence rates (ASR) in females 3.3 than in males 3.2 [{2}.]

Cancer stem cells (CSCs) are defined as a group of self-renewal, unlimited proliferation, multidirectional differentiation potential, and driving tumor development. Two mechanisms have been proposed to induce the generation of CSCs: the first is the carcinogenic mutation of normal stem cells, which leads to the uncontrolled proliferation of cells and the second is the dedifferentiation of ordinary cancer cells and their transformation into stem cell-like cells [{3}.]

Colorectal CSCs are defined with a group of cell surface markers, such as CD44, CD133, CD24, EpCAM, LGR5 and ALDH. They are highly tumorigenic, chemoresistant and radioresistant and thus are critical in the metastasis and recurrence of colorectal carcinoma and disease-free survival [{4}].

ALDHs are generally categorized as detoxification enzymes. ALDH1A1 was found to offer cellular protection against cytotoxic drugs and implicated in drug-resistance in chemotherapy [{5}].

ALDH1A1 has been shown to be related to the stemness of both cancer stem cells and normal tissue stem cells. Recent reports reveal that ALDH1 and specifically ALDH1A1 is a useful cancer stem cell marker that can be used to enrich tumor-initiating subpopulations from various cell lines and primary tumors [{5}].

CD44 is significantly expressed in lymphocytes, smooth muscle, fibroblasts and various types of epithelia and is involved in lymphocyte homing, cell adhesion and aggregation, cell migration, leukocyte activation, lymphopoiesis and myelopoiesis, angiogenesis and cytokine release. CD44s was initially isolated from hematopoietic cells even though it is expressed in several other tissues including the liver, lung, pancreas, skin and central nervous system [{6}].

Knock-down of CD44 from colon cancer cells lead to reduced expression of anti-apoptotic molecules like Bcl-2, Bcl-xL and increased level of apoptotic molecules like Bax, caspase-3/8/9. AKT phosphorylation, p21, and pRb were downregulated in CD44-transfected cancer cells after anticancer reagent etoposide treatment. This suggests that expression of CD44 modulates cell cycle regulators pRb and p21, and the pro-survival protein AKT 2 [{7}].

High CD44 expression was also associated with poor differentiation, lymph node metastasis and distant metastasis in CRC [{8}].

2. MATERIAL AND METHODS

1. Tissue specimens

The present study comprised 70 randomly selected tissue blocks of primary colorectal adenocarcinoma and their lymph nodes, metastases if present. The available clinicopathological data included: patient age, sex, tumor size, tumor site, tumor grade, tumor histological subtypes, lymphovascular invasion, perineural invasion, poorly differentiated clusters (PDCs) grade, tumor necrosis, tumor infiltrating lymphocytes (TILs), lymph node status and Modified Dukes staging. Tumor type and grade were evaluated according to WHO criteria [{9}]. Central PDCs were graded by X 20 objective lens into 3 grades; Grade 1 has less than 5 PDC clusters; Grade 2 has from 5 to 9 PDCs; Grade 3 has more than 9 PDCs within tumor stroma [{10}]. Tumor stage was estimated by Modified Dukes Staging [{11}] (see table 1).

Table (1): Clinicopathological features for patients with CRC (n=70)

	CRC (N=70)	
	N	**%**
Age (y)		
≤ 45 y	18	25.7%
> 45 y	52	74.3%
Sex		
Male	35	50.0%
Female	35	50.0%
Tumor site		
Right Colon	45	64.3%
Left colon and Rectum	25	35.7%
Histological subtypes		
Conventional	53	75.7%
Signet ring cell carcinoma	10	14.3%
Mucinous carcinoma	7	10.0%
Tumor size		
<5 cm	31	44.3%

≥5 cm	39	55.7%
Nodal status		
Negative	23	32.9%
Positive	47	67.1%
Tumor's grade		
Grade I	12	17.1%
Grade II	30	42.9%
Grade III	28	40.0%
PDC Grade		
PDC Grade 1	13	18.6%
PDC Grade 2	23	32.9%
PDC Grade 3	34	48.6%
Modified Dukes Classification		
Stage A and B	23	32.9%
Stage C and D	47	67.1%
Tumor Necrosis		
Negative	32	45.7%
Positive	38	54.3%
Lymphovascular invasion		
Negative	28	40.0%
Positive	42	60.0%
Perineural Invasion (PNI)		
Absent	57	81.4%
Present	13	18.6%
Tumor infiltrating lymphocytes		
Absent	26	37.1%
Mild	19	27.1%
Moderate	13	18.6%
Marked	12	17.1%

The patients' age ranged from 18 to 85 years with the patient mean age was 52.2 ± 14.539 and the median was 45 years. Eighteen (25.7%) patients were ≥ 45 years and 52 (74.3%) patient were > 45 years. Thirty-five (50%) were males and 35 (50%) were females Concerning histological subtypes fifty-three (75.7%) of cases were conventional adenocarcinomas, 7 (10%) cases were MA, and 10 (14.3%) cases were SRCC. The grades of conventional adenocarcinoma cases were grade I in 12 (22%) of cases, grade II in 25 (47.5), and grade III in 16 (30.3%) of cases, MA and SRCC were considered poorly differentiated tumors (grade III). Forty-five (64.3%) of tumors were located primarily in the right colon, however 25 (35.7%) were located in the left colon and rectum.

Tumor size ranged between 2 and 9 cm, with a mean size 5.33 ± 2.15 and a median of 5 cm. Thirty-one (44.3%) tumors were > 5 cm while 39 (55.7%) tumors which were ≥ 5 cm. Twelve (17.7%) of tumors were grade I, 30 (42.9%) were grade II tumors were, and grade III tumors were 28 (40%). According to poorly differentiated clusters grading, 13 (18.6%) of cases were PDC grade 1, 23 (32.9%) cases were PDC grade 2 and 34 (48.5%) cases were PDC grade 3. At the time of primary diagnosis, 47 (67.1%) patients had positive lymph node metastases. lymphovascular invasion and Peri-neural invasion were present in 42 (60%) and 13 (18.6%) of cases respectively. Tumor necrosis was observed in (45.7%) of cases. Based on modified Dukes staging, twenty-two of cases (32.9%) were stage A and B while 47 cases (67.1%) were stage C and D. Lymphocytic infiltrate was absent in 26 (37.1%) of cases, 19 (27.1%) of them showed mild lymphocytic infiltrate, 13 (18.6%) tumors displayed moderate lymphocytic infiltrate and 12 tumors (17.1%) had marked lymphocytic infiltrate. The proximal and distal surgical resection margins were free in all cases (100%).

2. Immunohistochemical (IHC) procedure

Five µm sections were prepared on positive charged slides for immunohistochemistry of ALDH1A1 and CD44 primary antibodies utilising the avidin biotin-peroxidase complex method with diaminobenzidine (DAB) chromogen detection system. Initially tissue sections on the positive charged slides were deparaffinized and rehydrated. Then the endogenous peroxidase was blocked by immersion in a 3% solution of hydrogen peroxide and incubated for 30 minutes. Antigen retrieval was performed by immersing the slides in citrate buffer solution (pH 6) for 2 times (10 minutes each) at 750-W. In order to block nonspecific background staining, the slides were treated by UV block. Both Primary antibodies ALDH1A1 (Polyclonal mouse antibody (100 µ,

concentrated, Lab Vision Laboratories, USA) and CD44 (100 µ, concentrated, ABclonal laboratories, China) were then added and tissue sections were incubated for 1 hour at room temperature (dilution 1:100). Excess reagent was thrown off and the slides were then rinsed gently with buffer solution for 5 minutes. After that Secondary biotinylated antibody was added for each slide for 30 minutes. DAB substrate and chromagen solutions were added to each slide and following that tissue sections were counter stained by Mayer's haematoxylin.

The Positive control for ALDH1A1 was normal human liver tissue and the positive control for CD44 was normal rat kidney tissue while the negative control tissue sections was obtained by omitting the specific primary antibody from the staining procedure and replaced with PBS

3. Scoring of Immunostaining

3.1. Scoring of ALDH1A1:

ALDH1A1 was expressed mainly in the cytoplasm. The immunohistochemical scores were obtained by light microscopy (Olympus, Tokyo, Japan) as the staining intensity (scored from 0–3) multiplied by the percentage of positive cells within 5 high power fields (in hot areas) (scored from 0–4). The intensity of ALDH1A1 protein expression was scored as: 0 (no staining); 1 (weak staining, light yellow color); 2 (moderate staining, pale brown); or 3 (strong staining, chocolate brown). The percentage of positive cells was scored as: 0 (<5%); 1 (5–25%); 2 (26–50%); 3 (51–75%); or 4 (>75%). Final score ranged from 0-12. The cut-off value for high versus low expression of the ALDH1A1 protein was determined using receiver-operating characteristic (ROC) curve analysis and SPSS statistical software, defining a final immunostaining score of > 6 as high ALDH1A1 protein expression {12}.

3.2. Scoring of CD44:

CD44 positive staining was detected in the cell membrane and/ or the cytoplasm of the tumor cells. Scoring of CD44 was based on both the intensity and the percentage of immunoreactive tumor cells. the staining intensity (scored from 0–3) multiplied by the percentage of positive cells within 5 high power fields (scored from 0–4). The intensity of CD44 protein expression was scored as: 0 (no staining); 1 (weak staining); 2 (moderate staining); or 3 (strong staining). The percentage of positive cells was scored as: 0 (absence of immunoreactivity); 1 (< 10 % immunoreactive tumor cells); 2 (10–50% immunoreactive tumor cells); 3 (> 50 immunoreactive tumor cells %). Final score ranges from 0-12. Total score ≤ 3 considered as negative expression, while score > 3 was considered positive expression {13}.

STATISTICAL ANALYSIS:

The analysis of the data was carried out using the IBM SPSS 28.0 statistical package software (IBM; Armonk, New York, USA). Data were expressed both number and percentage for qualitative data and were analyzed by the Chi‑square test or Fisher's exact test. A binary logistic regression model was used to evaluate the predictive value of the different variables, using high expression of ALDH1 and CD44 as dependent variables. A p-value less than 0.05 was considered significant.

3. RESULTS

ALDH1A1 immunoexpression was detected in the cytoplasm, with expression of ALDH1A1 was negative to weak cytoplasmic reaction in normal colorectal epithelium **Figure (1).** ALDH1A1 exhibited low cytoplasmic expression in 18 cases (25.7%), whereas 52 (74.3%) revealed high ALDH1A1 expression.

No statistically significant association was found between ALDH1A1 expression and patient's age, sex, and tumor site, tumor size, perineural invasion (P= 0.694, P= 0.274, and P= 0.166, P= 0.095, P= 0.809 respectively). However, A statistically significant association was observed between ALDH1A1 high expression and higher tumor grade **Figure (2).,** poorly differentiated clusters (PDCs) grade **Figure (3)**., regional lymph node involvement **Figure (4)**., Lymphovascular invasion (LVI) **Figure (5).** advanced tumor stage, tumor necrosis **Figure (6).** and tumor infiltrating lymphocytes **Figure (7).** (P value> 0.001, <0.001, <0.001, <0.001, <0.001, P 0.038 and 0.002) see **table (2).**

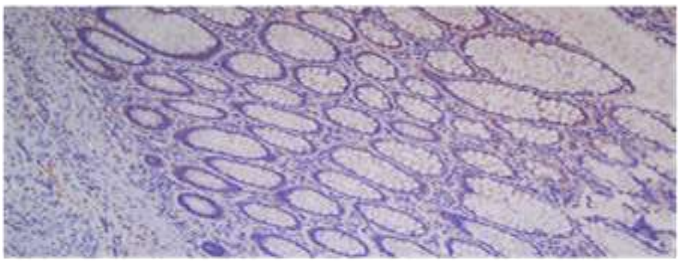

Figure 1: Weak cytoplasmic ALDH1A1 expression in the base of the normal crypts (IHC, X100)

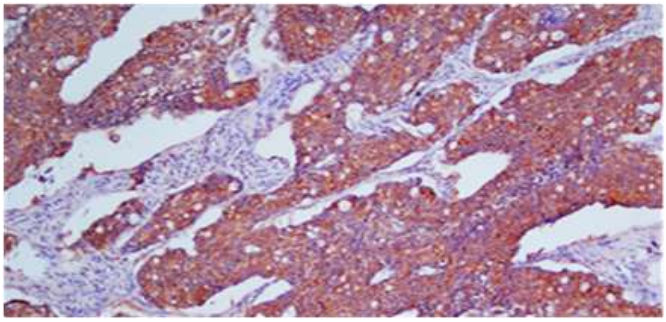

Figure 2: High cytoplasmic ALDH1A1 expression in grade III conventional adenocarcinoma (IHC, X200).

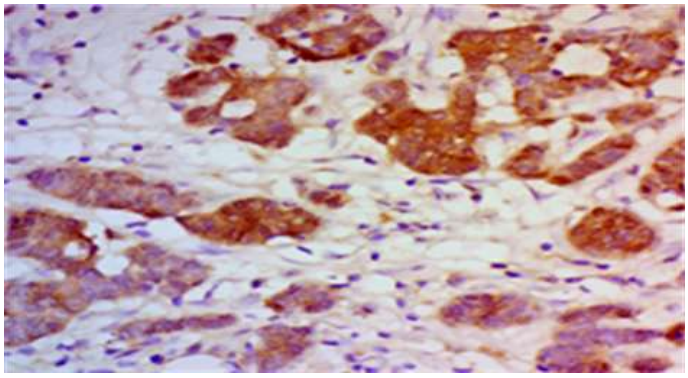

Figure 3: High cytoplasmic ALDH1A1 expression high grade PDCs grade III colorectal adenocarcinoma (IHC, X200)

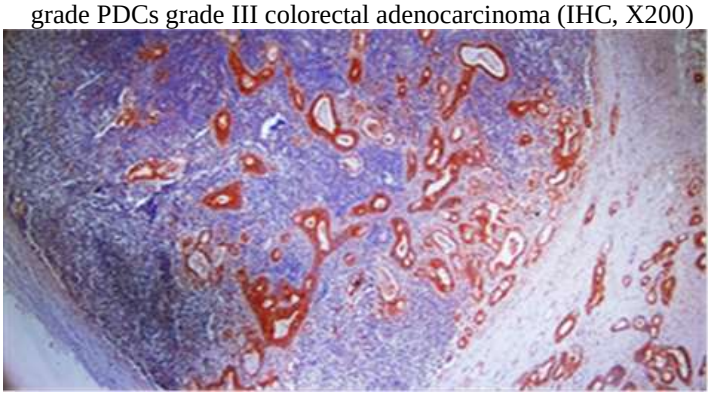

Figure 4: High expression of ALDH1 in lymph node infiltrated by conventional adenocarcinoma (IHC, X100).

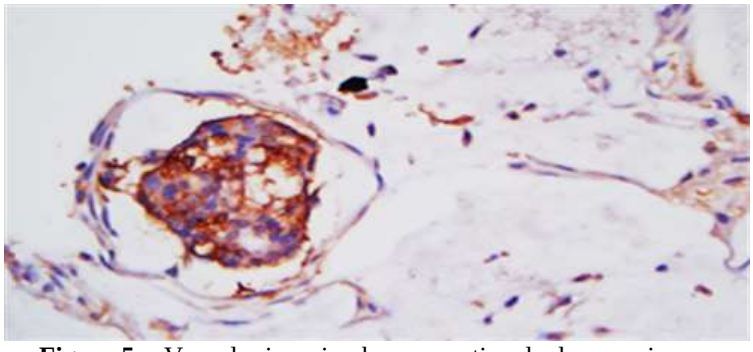

Figure 5: Vascular invasion by conventional adenocarcinoma showing high ALDH1A1 expression (IHC, X400).

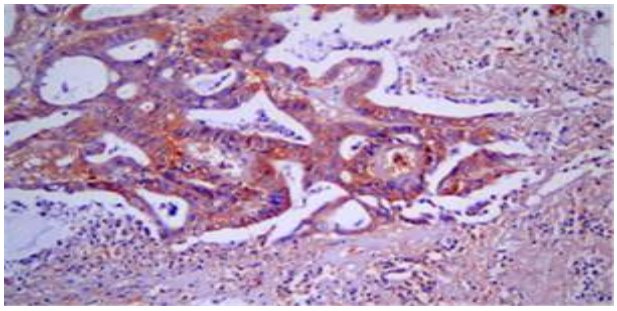

Figure 6: colorectal adenocarcinoma with tumor necrosis showing high ALDH1A1 expression (IHC, X400).

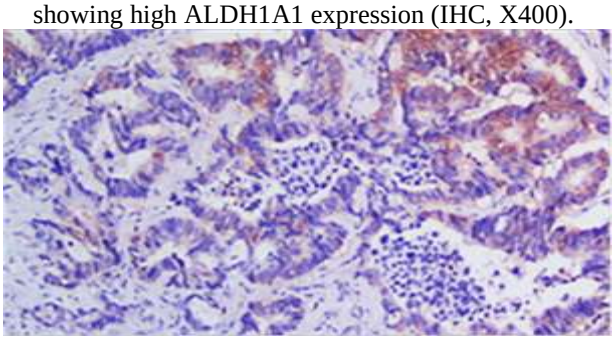

Figure 7: Low ALDH1A1 expression in conventional adenocarcinoma associated with tumor infiltrating lymphocytes (IHC, X400).

Table (2): Association between cytoplasmic ALDH1A1 expression and clinicopathological features for patients with CRC (n=70)

	ALDH1A1		**p value**
	Low expression (N=18)	**High expression (N=52)**	
Age (y)			
≤ 45 y	4 (22.2%)	14 (77.8%)	0.694
> 45 y	14 (26.9%)	38 (73.1%)	
Sex			
Male	11 (31.4%)	24 (68.6%)	0.274
Female	7 (20.0%)	28 (80.0%)	
Tumor site			
Right Colon	14 (31.1%)	31 (68.9%)	0.166
Left colon and Rectum	4 (16.0%)	21 (84.0%)	
Histological subtypes			
Conventional	11 (20.8%)	42 (79.2%)	0.243
Signet ring cell carcinoma	4 (40.0%)	6 (60.0%)	
Mucinous carcinoma	3 (42.9%)	4 (57.1%)	
Tumor size			
<5 cm	11 (35.5%)	20 (64.5%)	0.095
≥5 cm	7 (17.9%)	32 (82.1%)	
Nodal status			
Negative	16 (69.6%)	7 (30.4%)	<0.001*
Positive	2 (4.3%)	45 (95.7%)	
Tumor's grade			
Grade I	10 (83.3%)	2 (16.7%)	<0.001*
Grade II	5 (16.7%)	25 (83.3%)	
Grade III	3 (10.7%)	25 (89.3%)	
PDC Grade			
PDC Grade 1	11 (84.6%)	2 (15.4%)	<0.001*
PDC Grade 2	4 (17.4%)	19 (82.6%)	
PDC Grade 3	3 (8.8%)	31 (91.2%)	
Modified Dukes Classification			
Stage A and B	14 (60.9%)	9 (39.1%)	<0.001*

Stage C and D	4 (8.5%)	43 (91.5%)	
Tumor Necrosis			
Negative	12 (37.5%)	20 (62.5%)	0.038*
Positive	6 (15.8%)	32 (84.2%)	
Lymphovascular invasion			
Negative	14 (50.0%)	14 (50.0%)	<0.001*
Positive	4 (9.5%)	38 (90.5%)	
Perineural Invasion (PNI)			
Absent	15 (26.3%)	42 (73.7%)	0.809
Present	3 (23.1%)	10 (76.9%)	
Tumor infiltrating lymphocytes			
Absent	2 (7.7%)	24 (92.3%)	0.002*
Mild	3 (15.8%)	16 (84.2%)	
Moderate	6 (46.2%)	7 (53.8%)	
Marked	7 (58.3%)	5 (41.7%)	

* P - value > 0.05 are considered statistically significant according to Chi-Square test and Fisher's exact test.

Regarding expression of CD44 in CRC, the present study, 27 cases (38.6%) exhibited low cytoplasmic CD44 expression, whereas 43 (61.4%) revealed high expression.

No statistically significant association was found between CD44 expression and patient's age, sex, and tumor site, perineural invasion (P= 0.974, P= 0.806, and P= 0.176, P= 0.522 respectively).

A statistically significant association was observed between CD44 high expression and larger tumor size, higher tumor grade **Figure (8)**., poorly differentiated clusters (PDCs) grade **Figure (9)**., regional lymph node involvement **Figure (10)**, Lymphovascular invasion (LVI) **Figure (11)**. advanced tumor stage, tumor necrosis **Figure (12)** and tumor infiltrating lymphocytes **Figure (13)** (P value 0.046, <0.001, <0.001, <0.001, <0.001, <0.001, <0.001 and 0.021). see **table (3)**.

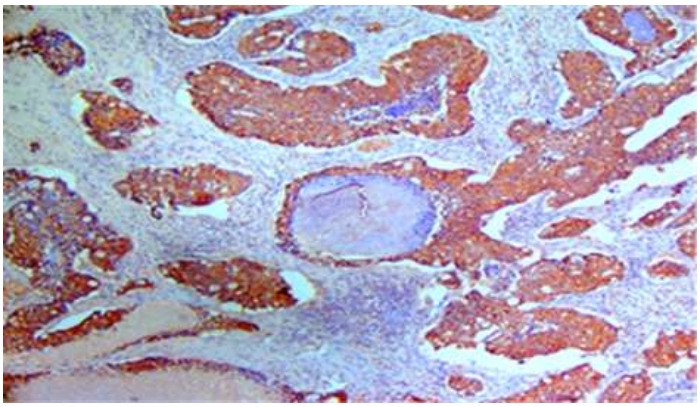

Figure 8: High membranous expression of CD44 in grade III conventional adenocarcinoma (IHC, X200).

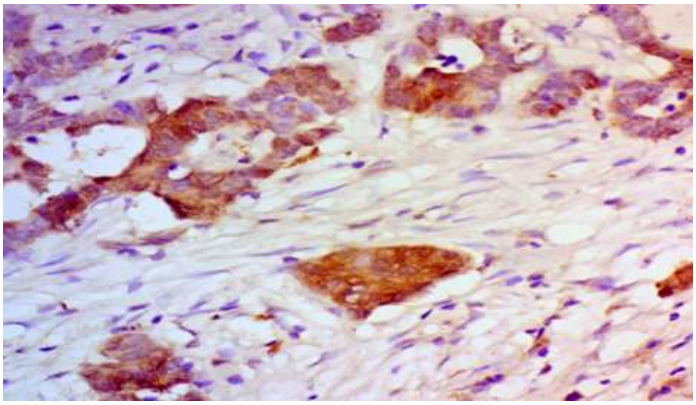

Figure 9: High membranous/cytoplasmic CD44 expression high grade PDCs grade III colorectal adenocarcinoma (IHC, X200)

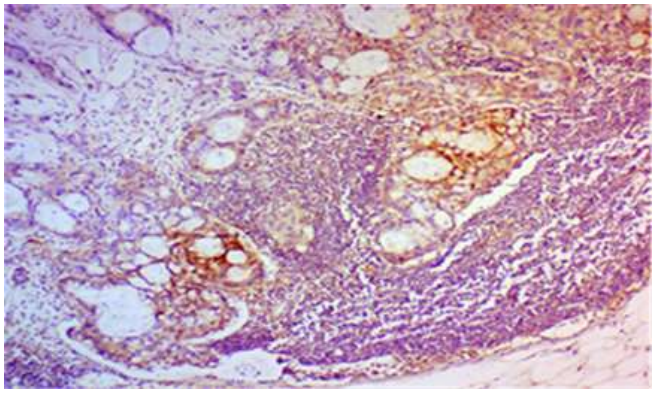

Figure 10: High membranous expression of CD44 in lymph node infiltrated by conventional adenocarcinoma (IHC, X100).

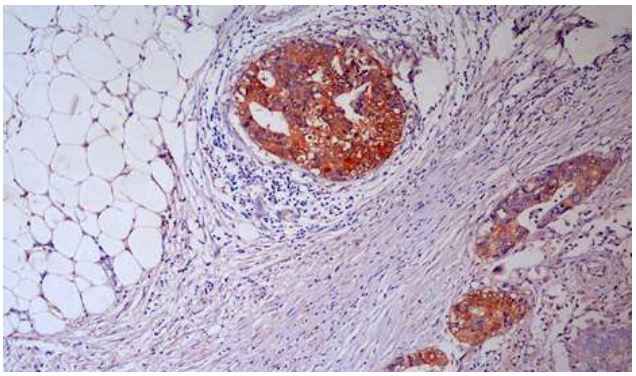

Figure 11: Vascular invasion by conventional adenocarcinoma showing high membranous expression of CD44 (IHC, X400).

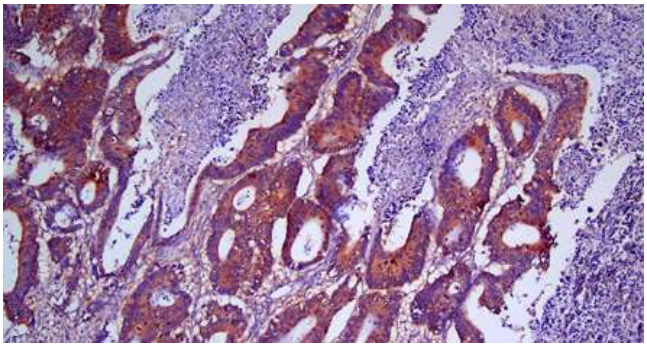

Figure 12: colorectal adenocarcinoma with tumor necrosis showing high membranous expression of CD44 (IHC, X400).

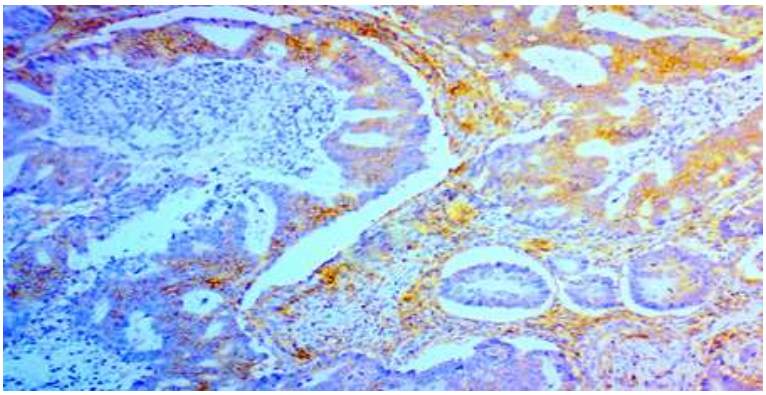

Figure 13: Low membranous expression of CD44 in conventional adenocarcinoma associated with tumor infiltrating lymphocytes (IHC, X400).

Table (3): Association between cytoplasmic CD 44 expression and clinicopathological features for patients with CRC (n=70)

	CD44		**p value**
	Low expression (N=27)	**High expression (N=43)**	
Age (y)			
≤ 45 y	7 (38.9%)	11 (61.1%)	0.974
> 45 y	20 (38.5%)	32 (61.5%)	
Sex			
Male	14 (40.0%)	21 (60.0%)	0.806
Female	13 (37.1%)	22 (62.9%)	
Tumor site			
Right Colon	20 (44.4%)	25 (55.6%)	0.176
Left colon and Rectum	7 (28.0%)	18 (72.0%)	
Histological subtypes			
Conventional	18 (34.0%)	35 (66.0%)	0.36
Signet ring cell carcinoma	5 (50.0%)	5 (50.0%)	
Mucinous carcinoma	4 (57.1%)	3 (42.9%)	
Tumor size			
<5 cm	16 (51.6%)	15 (48.4%)	0.046*
≥5 cm	11 (28.2%)	28 (71.8%)	
Nodal status			
Negative	15 (65.2%)	8 (34.8%)	<0.001*
Positive	12 (25.5%)	35 (74.5%)	
Tumor's grade			
Grade I	11 (91.7%)	1 (8.3%)	<0.001*
Grade II	9 (30.0%)	21 (70.0%)	
Grade III	7 (25.0%)	21 (75.0%)	
PDC Grade			
PDC Grade 1	11 (84.6%)	2 (15.4%)	<0.001*
PDC Grade 2	5 (21.7%)	18 (78.3%)	
PDC Grade 3	11 (32.4%)	23 (67.6%)	
Modified Dukes Classification			
Stage A and B	16 (69.6%)	7 (30.4%)	<0.001*
Stage C and D	11 (23.4%)	36 (76.6%)	
Tumor Necrosis			
Negative	16 (50.0%)	16 (50.0%)	0.071
Positive	11 (28.9%)	27 (71.1%)	
Lymphovascular invasion			
Negative	18 (64.3%)	10 (35.7%)	<0.001*
Positive	9 (21.4%)	33 (78.6%)	
Perineural Invasion (PNI)			
Absent	23 (40.4%)	34 (59.6%)	0.522
Present	4 (30.8%)	9 (69.2%)	
Tumor infiltrating lymphocytes			
Absent	7 (26.9%)	19 (73.1%)	0.021*
Mild	5 (26.3%)	14 (73.7%)	
Moderate	6 (46.2%)	7 (53.8%)	
Marked	9 (75.0%)	3 (25.0%)	

* P - value > 0.05 are considered statistically significant according to Chi-Square test and Fisher's exact test.

Association of ALDH1A1 and CD 44 expression with different clinicopathological variables were further tested using univariate and multivariate analysis.

The current study found that tumor grade, PDCs grade, modified Dukes staging, lymphovascular invasion and tumor necrosis were independently associated with ALDH1A1 expression (p= 0.034*, 0.022*, 0.047*,0.035*, 0.013 respectively). See table 4.

Table (4): Univariate and Multivariate regression analysis of factors predicting high ALDH1A1 expression

	ALDH1A1			
	crude OR (95% CI)	**p value**	**a OR (95% CI)**	**p value**
Age (y)				
≤ 45 y	1 (reference)			
> 45 y	0.78 (0.22-2.76)	0.695		
Sex				
Male	1 (reference)			
Female	1.83 (0.61-5.47)	0.277		
Tumor site				
Colon	1 (reference)			
Rectum	2.37 (0.69-8.21)	0.173		
Histological subtypes				
Conventional	1 (reference)			
Signet ring cell carcinoma	0.39 (0.09-1.64)	0.2		
Mucinous carcinoma	0.35 (0.07-1.80)	0.208		
Tumor size				
<5 cm	1 (reference)			
≥5 cm	2.51 (0.84-7.55)	0.1		
Nodal status				
Negative	1 (reference)			
Positive	51.43 (9.66-273.69)	<0.001*		
Tumor's grade				
Grade I	1 (reference)		1 (reference)	
Grade II	25.00 (4.15-150.69)	<0.001*	9.97 (0.28-350.35)	0.206
Grade III	41.67 (6.03-288.11)	<0.001*	42.76 (1.33-1376.97)	0.034*
PDC Grade				
PDC Grade 1	1 (reference)		1 (reference)	
PDC Grade 2	26.13 (4.10-166.60)	<0.001*	36.76 (0.44-3041.79)	0.11
PDC Grade 3	56.83 (8.36-386.44)	<0.001*	280.86 (2.26-34925.56)	0.022*
Modified Dukes Classification				
Stage A and B	1 (reference)		1 (reference)	
Stage C and D	16.72 (4.45-62.80)	<0.001*	29.16 (1.05-808.35)	0.047*
Tumor Necrosis				
Negative	1 (reference)		1 (reference)	
Positive	3.20 (1.04-9.89)	0.043*	45.54 (1.31-1578.64)	0.035*
Lymphovascular invasion				
Negative	1 (reference)		1 (reference)	
Positive	9.50 (2.67-33.79)	<0.001*	414.49 (3.61-47541.46)	0.013*
Perineural Invasion (PNI)				
Absent	1 (reference)			
Present	1.19 (0.29-4.92)	0.81		
Tumor infiltrating lymphocytes				
Marked	1 (reference)			
Absent	16.80 (2.66-106.14)	0.003*		
Mild	7.47 (1.39-40.25)	0.019*		
Moderate	1.63 (0.34-7.95)	0.544		

N.B. Dependent variable high ALDH1 expression, a OR adjusted odds ratio, CI confidence interval, NE not estimate R2= 0.580

As regarding CD44, the current study found that tumor grade, PDCs grade, modified Dukes staging and lymphovascular invasion were independently associated with CD 44 expression (p= 0.012*, 0.046*, 0.048*, 0.022* respectively). See **table (5)**

Table (5): Univariate and Multivariate regression analysis of factors predicting high CD44 expression

	CD44			
	crude OR (95% CI)	**p value**	**a OR (95% CI)**	**p value**
Age (y)				
≤ 45 y	1 (reference)			
> 45 y	0.74 (0.24-2.28)	0.597		
Sex				
Male	1 (reference)			
Female	1.44 (0.55-3.78)	0.462		
Tumor site				
Colon	1 (reference)			
Rectum	2.06 (0.72-5.89)	0.179		
Histological subtypes				
Conventional	1 (reference)			
Signet ring cell carcinoma	0.71 (0.18-2.85)	0.627		
Mucinous carcinoma	0.08 (0.01-0.71)	0.023*		
Tumor size				
<5 cm	1 (reference)			
≥5 cm	1.65 (0.62-4.35)	0.314		
Nodal status				
Negative	1 (reference)			
Positive	NE	0.998		
Tumor's grade				
Grade I	1 (reference)			
Grade II	36.14 (3.95-331.14)	0.002*	36.66 (2.22-606.69)	0.012*
Grade III	23.22 (2.59-208.62)	0.005*	13.54 (0.97-188.50)	0.053
PDC Grade				
PDC Grade 1	1 (reference)			
PDC Grade 2	22.50 (2.46-205.74)	0.006*	13.93 (1.04-185.77)	0.046*
PDC Grade 3	46.29 (5.11-418.93)	<0.001*	1.76 (0.14-21.81)	0.659
Modified Dukes Classification				
Stage A and B	1 (reference)			
Stage C and D	7.48 (2.45-22.83)	<0.001*	6.58 (1.01-42.70)	0.048*
Tumor Necrosis				
Negative	1 (reference)			
Positive	2.46 (0.92-6.58)	0.074		
Lymphovascular invasion				
Negative	1 (reference)			
Positive	6.60 (2.27-19.21)	<0.001*	5.86 (1.30-26.51)	0.022*
Perineural Invasion (PNI)				
Absent	1 (reference)			
Present	0.68 (0.20-2.30)	0.535		
Tumor infiltrating lymphocytes				
Marked	1 (reference)			
Absent	13.57 (2.36-77.95)	0.003*		
Mild	26.67 (3.77-188.54)	0.001*		
Moderate	4.29 (0.66-27.79)	0.127		

N.B. Dependent variable high CD44 expression, a OR adjusted odds ratio, CI confidence interval, NE not estimated R2= 0.431

On studying the presence of possible association between ALDH1A1 and CD 44 expression, a statistically significant association was found between both markers (p= <0.001*). See **table (6)**

Table (6): Association of ALDH1A1 and CD44 expression in CRC

CD44	**ALDH1A1**		**p value**
	Low (n=18)	**High (n=52)**	
Low (n=27)	17 (63%)	10 (37%)	<0.001*
High (n=43)	1 (2.3%)	42 (97.7%)	

P - value ≤ 0.05 are considered statistically significant according to Chi-Square and Fisher's exact tests.

4. DISCUSSION

The current study included 70 cases of CRC with patients mean age 52.2 years ± SD 14.539 and the median was 45 years. This was in a line with several previous studies performed by Holah et al., 2017; **Mohamed et al., 2019** and **Sharaf El Din et al., 2022** {14.15.16}. on the other hand, other studies reported an older mean of age ranging from 60 to 93 years {17.18.19}. In this series 55.7% of tumours were ≥ 5 cm (median level) in size while 44.3% were < 5cm. Also, **Zhu et al.,2022** and **Sugiyama et al,2022** {20.21} reported that 68.7% and 53.2% of case were ≥ 5cm respectively on the other hand **Mohamed et al., 2019** {15} reported that 68.7 of cases were less than 5cm. As regarding this study 64.3% of the tumors were located at right side of colon while 35.7% were located at left side of the colon and rectum. This was close to finding reported by **Said et al., 2022** {22} who reported that 68.5% of the tumors were located at right side of colon while 32.5% were located at left side of colon and rectum however Mohamed et al., 2019 and **Sharaf El Din et al., 2022** {15.16} reported more percentage of tumors in left side of colon and rectum. With respect to tumor grades 17.7% %of cases were low grade while 82.9 % were high grade. This was in line with **Said et al.,2022** {22} who reported 10.5% of their cases were low grade. On the other side, **Rezaee et al.,2021** {19} reported 42.1 % of their cases were low grade. On current study PDC grade 18.6%% of cases PDC grade 1, 32.9 % of cases were grade 2 and 48.5% cases were grade 3. PDC grade has an important prognostic impact on CRC prognosis {23}. Histological subtypes were adenocarcinoma, NOS (75.7%), mucinous adenocarcinoma (10%) and (14.3%) were signet ring carcinoma. This was in line with **Said et al., 2022; Mohamed et al.,2022** and **Sharaf El Din et al., 2022** {22.15.16} who reported the predominance of adenocarcinoma, NOS subtype over the other two subtypes in their studies. Regarding regional lymph node involvement 67.1% of cases had positive lymph node metastasis while 32.9 % were without lymph node metastasis. This was in accordance with **Rezaee et al.,2021** {19} who reported a slight lower percentage of cases with lymph node involvement. The present study included 32.9 % of cases modified Dukes stage A and B and 67.1% modified Dukes stage C and D. this was in line with **Sharaf El Din et al., 2022 and Said et al., 2022** {16.22} who reported advanced tumor stage at 70.6 % and 63.6 of their study cases. On the other hand, **Ji et al., 2014 and Rezaee et al.,2021**{24.19} reported that 55 % and 60 % of their cases were early stage respectively. This may be attributed to widely used screening programs that led to early detection of CRC.

Regarding lymphovascular invasion, 60 % of cases showed lymphovascular invasion this was in agreement with **Said et al., 2022**{22} detect lymphovascular invasion in 78 %. However, **Sharaf El Din et al.,2022** {16} reported lymphovascular invasion in 40.2 of cases. Lymphocytic infiltration was high in 17.1% of the tumors. This was in agreement with **Sharaf El Din et al.,2022** {16} who reported that 12.5 % of tumors showed high lymphocytic infiltration. In the current study, perineural invasion was present in about 18.6 % of cases. This was in a line with **Ko and Pyo, 2019** {25} and **Rezaee et al.,2021**{19} who noted presence of perineural invasion in about 16.7% and 20.1 of their cases respectively. On the other hand, **Bassam et al., 2021**{26} reported presence of perineural invasion in 41 % of cases.

Tumor necrosis occur often in human solid cancers and is associated with unfavourable prognosis **Väyrynen et al., 2016**{27}. In this study, tumor necrosis was present in 45.7 % of tumors. This finding is close to {28} Richards et al., 2012 who reported 42.3 % of tumors had necrosis respectively. On the opposite side **Väyrynen et al., 2016{27}** reported tumor necrosis in 95.9 % in their studies.

Regarding ALDH1A1 expression, the current study reported positive ALDH1A1 in 74.3% of tumors. This finding was in a line with **Holah et al., 2017**{14} who reported positive ALDH1A1 expression in 75% of cases. In the current study, a statistically significant positive association between ALDH1A1 positive expression and high tumor grade. This finding was similar to that reported by **Mohamed et al.,2019 and Said et al.,2022**{15.22}. This finding was inconsistent with previous studies who reported no significant association between ALDH1A1 and tumor grade {14.19}.

Concerning tumor stage, there was a significant association between ALDH1A1 and advanced tumor stage, ALDH1A1 was more expressed in modified Dukes stage C and D than modified dukes stage A and B. This result was in analogy with previous studies **van der Waals et al., 2018**; **Mohamed et al., 2018 and Said et al., 2022** {29.15.22}. However, **Yang et al., 2018 and Rezaee et al., 2021**{30.19} showed no association between ALDH1A1 expression and tumors stage.

Regarding lymphovascular invasion, ALDH1A1 expression was significantly associated with presence of lymphovascular invasion. This finding was compatible with **Mohamed et al.,2018 and Said et al., 2022**{15.22} who concluded the same association. However, these finding were not in accordance with **Holah et al., 2017 and Rezaee et al.,2021**{14.19} who found no significant association between ALDH1A1 and lymphovascular invasion. Concerning tumor necrosis, ALDH1A1 expression was significantly associated with tumors with necrosis than tumors without necrosis, this was compatible with **Kozovska et al.,2018 and Liao et al.,2022**{31.32}.

However, **Holah et al.,2017**{14} reported no significant association between ALDH1A1 expression and tumor necrosis. A statistically significant association between ALDH1 expression and degree of lymphocytic infiltration. Finding also detected by **Mohamed et al.,2018**{15}.

This is the first study that evaluated the association between ALDH1A1 expression and PDC grade. The present study demonstrated a statistically significant association between ALDH1A1 and PDC grade. The possible explanation for this association is that ALDH1A1 expression was associated with poor prognostic features in CRC as high grade, advanced stage and poor tumor differentiation {22}.

Multivariate analysis confirmed the independent association between positive ALDH1A1 expression and poor prognostic factors including high tumor grade, lymphovascular invasion, advanced tumor stage, PDCs grade and tumor necrosis suggesting the role of ALDH1A1 expression in tumor aggressive behavior.

The current study revealed that CD44 was positively expressed in 61.4 % of tumors. This finding was in agreement with **Khelwatty et al.,2019 and Mohamed et al.,2019** {33.15} who reported positive CD44 expression in 58% and 64.5 % of cases respectively. However, **Sadeghi et al., 2019**{34} revealed a lower positivity 24 % of cases

In the current study CD44 positive expression was significantly associated with tumor size being more expressed in tumor size ≥5 cm. this finding was in agreement with **Zhu et al.,2018; Mohamed et al., 2019 and Wang et al., 2019**{14.15.4}. On the other hand, {34} **Sadeghi et al., 2019** reported no significant association between CD44 expression and large tumor size.

In the present study CD44 positive expression was significantly associated with high tumor grade than lower tumor grade. This finding was similar to that reported by **Zhu et al.,2018**; **Mohamed et al.,2019; Han et al., 2019 and Wang et al.,2019**{20.15.35.4}. This finding was inconsistent with previous studies who reported no significant association between CD44 and tumor grade {34.33}. The possible explanation for this difference is the use of different scoring systems and different study sample size.

Concerning tumor stage, there was a significant association between CD44 and advanced tumor stage, CD44 was more expressed in modified Dukes stage C and D than modified dukes stage A and B. This result was in analogy with several previous studies {20.15.35.33.36}. However, **Wang et al.,2019**{4} showed no association between CD44 expression and tumors stage. This may be due to different staging system and the use of different clones of antibodies.

Regarding lymphovascular invasion, CD44 expression was significantly associated with presence of lymphovascular invasion. This finding was compatible with **Bhavikatti et al.,2023**{36} who concluded the same association. However, these finding were not in accordance with **Sadeghi et al., 2019** {34} who found no significant association between CD44 and lymphovascular invasion. This difference may be due to the use of different clones of antibodies and lower percentage of cases who had lymphovascular invasion in their studies.

A statistically significant association p between CD44 expression and tumor necrosis. Finding also detected by **Muys et al., 2021**{37}. A statistically significant association between CD44 expression and degree of lymphocytic infiltration. Finding also detected by **Mohamed et al.,2018**{15}. This is the first study that evaluated the association between CD44 expression and PDCs grade, the present study demonstrated a statistically significant association between CD44 and PDCs grade.

Multivariate regression analysis confirmed the independent association between positive CD44 expression and poor prognostic factors including high tumor grade, lymphovascular invasion, advanced tumor stage and PDCs grade suggesting the role of CD44 expression in tumor aggressive behavior. On studying the presence of possible association between ALDH1A1 and CD 44 expression, a statistically significant association was found between both markers' high expression and clinicopathological variables.

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