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Interplay between CD44 expression and acidic mucin histochemical alterations in colorectal cancer

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Abstract:

Colorectal cancer (CRC) represents a significant global health problem. Finding the biomarkers is crucial for the diagnosis and prognosis prediction. CD44 is a cancer stem cell (CSC) marker that plays an important role in tumor cell proliferation, adhesion, migration, and invasion. Altered mucin expression has been observed in cancer cells and also affects their biologic properties. This study aimed to analyze CD44 expression, assess acidic mucin histochemical alterations in CRC cases and to find the relation between both. A total of 40 colectomy specimens were included. Sections from formalin-fixed paraffin embedded blocks of the tumors were stained immunohistochemically with anti-CD44 antibodies and histochemically using Alcian Blue (AB) at pH 2.5. AB staining was positive in 26 (65%) cases including all mucinous carcinoma cases. It was significantly related to tumor histology (P= 0.000), lymph node status (P=0.000) and TNM staging groups (P= 0.000). CD44 expression was positive in 30 (75%) cases. Positive cases were scored as +1, +2 and +3. CD44 expression was significantly related to lymph node status (P= 0.001) and TNM staging groups (P= 0.006). There was a significant relationship between CD44 expression and AB staining (P = 0.006) with a moderate negative correlation (r= -0.424). Increased CD44 expression and decreased acidic mucin correspond with lymph node metastasis and higher stage in CRC cases, suggesting both as indicators of worse prognosis.

Keywords: Colorectal cancer; CD44 expression; mucin alterations, Alcian blue

1. Introduction:

Worldwide, colorectal cancer (CRC) represents the third most commonly diagnosed form of cancer, [1] and the second most commonly diagnosed malignancy in Egypt after breast cancer with high incidence rate in patients below the age of 40. [2, 3]

The cell surface glycoprotein CD44 is one of cancer stem cell (CSC) markers and has been widely investigated in CRC. [4] Given a

receptor for Hyaluronic acid, CD44 is essential for cell-extracellular matrix (ECM) adhesion. [5] .

Therefore, loss of CD44 leads to tumor cell detachment from the basement membrane, invasion and metastasis. [6, 7] Quantitative and qualitative alterations in mucin polysaccharides have been observed, in addition to multiple genetic mutations during colorectal tumorigenesis. [8].

These alterations or aberrant glycosylation in mucin affect the biologic properties of cancer cells and can be used as diagnostic and prognostic markers. [9]

This work aims to analyze CD44 expression, assess mucin alterations in CRC cases and to find the relation between both.

2. Materials and methods:

A total of 40 colectomy specimens of patients diagnosed as primary CRC were included in this study according to the following criteria: availability of clinical data and paraffin blocks, good processing, adequate viable tumor tissue and no preoperative chemotherapy or radiotherapy.

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks of each specimen were retrieved from the archives of Pathology Laboratory at Specialized Medical Centre, Faculty of Medicine, Beni-Suef University during the period from September 2017 to April 2018. Patients' names and hospital numbers were removed from each case for confidentiality of data. Names were replaced by numbers. This study was approved by the institutional ethical committee.

The available data including age, sex, tumour size, location, lymph node status and distant metastasis were obtained from patients' records.

2.1 Histopathological examination:

Hematoxylin and Eosin (H&E) slides were examined to confirm the diagnosis and to re-evaluate the tumor type and grade according to the 2019 World Health Organization classification of the digestive system tumors, 5^{th} edition. [10, 11] .

The depth of tumor invasion, presence of perineural invasion, lymphovascular invasion, lymph node metastasis, inflammatory reaction and dense desmoplastic stroma were also evaluated. Tumor staging was performed using the TNM system of the American Joint Committee on Cancer (AJCC), 8^{th} edition. [12].

2.2 Mucin histochemistry:

Sections from FFPE were stained with Alcian Blue (AB) at pH 2.5 to identify acidic mucin (blue). Nuclear fast red was used as a counter stain, it stains nuclei reddish pink. Normal colonic glandes were used as internal positive control. AB staining was divided into negative cases and positive cases. The positive cases were further categorized as positive focal and positive diffuse based on the distribution of staining.

2.3 CD44 immunohistochemical staining:

Immunostaining was performed by standard autostaining protocols using the Ventana Benchmark XT autostainer (Ventana Medical Systems, Inc. Tucson, AZ) as following:

Four µm-thick tissue sections were cut from paraffin blocks, placed on positively charged slides. Deparaffinization and antigen retrieval (i-view detection system; Ventana) were carried out as an automated program of the Ventana autostainer. The slides were incubated with ready to use monoclonal primary anti-CD44 antibodies (CELL MARQUE, Catalogue No. 144M-98, Rocklin, CA, USA) before the application of 3, 30-diaminobenzidine tetra hydrochloride (DAB) as chromogen and then counterstaining with Hematoxylin. Positively stained lymphocytes were used as internal positive control in the current study while negative controls were done by replacing the primary antibody with phosphate buffered saline.

2.4 Interpretation of CD44 immunostaining:

CD44 staining was detected as brownish staining mainly in the cell membrane and the degree of positivity was scored according to the percentage of immune-positive cells in relation to the total number of tumor cells as follows:

- Negative: Positive cells were ≤ 1%
- +1: Positive cells were >1% and ≤ 10%
- +2: Positive cells were >10 and ≤ 60%
- +3: Positive cells were > 60%. [13]

2.5 Slides examination and imaging

All slides were examined by light microscopy (Olympus model BX53) while all included photos were taken by Leica digital pathology slide scanner (APERIO LV1) at Pathology lab, Beni-Suef University hospital.

Statistical analysis:

The collected data were coded then entered and was analyzed using the statistical package for social science (SPSS) for Windows, version 22.0 (IBM Corp., Armonk, N.Y., USA) Descriptive statistics for the demographic and pathological characteristics of cases were first analyzed. Frequency and percentage were used to describe qualitative variables, whereas, quantitative variables were represented by the values of mean ± standard deviation. Suitable statistical tests were used (Chi-square (χ^2), way ANOVA test, non-parametric Spearman correlation and independent sample t test). P-values equal to or less than 0.05 were considered statistically significant.

3. Results:

Clinicopathological details of the studied CRC cases are summarized in Table (1) and Table (2). The mean age of patients was 55.52±12.1 years; (range 30-84). Twenty three males and 17 females with a ratio of 1.4:1 were included in this study.

Seventy five percent of tumors (n=30/40) were located in the colon. Tumor size ranged between 2.5 and11 cm with a mean of 5.87± 2.1 cm. Adenocarcinoma, NOS was the commonest histologic type (n= 36) including five showed focal mucin production and one with signet ring cell component. The majority of patients (n= 33, 82.5%) had moderately differentiated tumors.

Regarding the extent of primary tumor, pT3 was common among the cases (n=24/40). Half the cases were (pN0) and only two (5%) were known to have distant metastasis (M1a). Forty five percent patients (n=18) presented with TNM stage III at diagnosis and 37.5% (n=15) were stage II.

AB staining was positive in 26 cases, Figure (1).

The relation between the histologic type of the tumor and AB staining was statistically significant (P= 0.000); Table (1) where all mucinous carcinoma cases showed diffuse staining (4/4) and all adenocarcinoma cases with positive diffuse staining had histologic evidence of mucin production. Significant differences were also found between lymph node status (P= 0.000), TNM staging groups (P= 0.000) and AB staining, Table (2). No other clinicopathological feature statistically related to AB staining.

Of the analyzed tumors, 30 (75%) cases showed positive membranous CD44 expression, Figure (2). No significant relation was found between CD44 expression and patients' age (P= 0.701), gender (P= 0.853), tumor location (P= 0.443), tumor size (P= 0.432), histologic type (P= 0.931), tumor grade (P= 0.472), perineural invasion (P= 0.558), lymphovascular invasion (P= 0.089), heavy inflammatory reaction (P= 0.423) or dense stromal desmoplasia (P= 0.492).

Although more than 3/4 locally advanced tumors (pT3 and pT4) showed positive CD44 expression, the difference did not reach statistical significance (P= 0.917). Meanwhile, significant relations were found between nodal status (P= 0.001), TNM staging groups (P= 0.006) and CD44 expression scores as shown in Table (3).

Table (4) illustrates a statistically significant relationship between CD44 expression and AB staining (P= 0.006) with a moderate negative correlation, where increasing CD44 expression corresponds with decreasing AB staining.

Table 1: Clinicopathological characteristics and relation with Alcian blue staining (N=40)

Characteristics		Alcian Blue Staining			p-value*
		Negative N=14	Positive Focal N=18	Positive Diffuse N= 8	
Age; (years)	**≤ 40**	3 (60%)	2(40%)	0 (0%)	0.121
	>40-<60	6 (28.6%)	10(47.6%)	5 (23.8%)	
	≥60	5 (35.7%)	6(42.9%)	3(21.4%)	
Sex; N (%)	**Males**	10(43.5%)	10(43.5%)	3 (13%)	0.294
	Females	4 (23.5%)	8(47.1%)	5 (29.4%)	
Tumor Site; N (%)	**Right Side**	7(43.8%)	5(31.2%)	4 (25%)	0.346
	Left Side	5(35.7%)	7 (50%)	2 (14.3%)	
	Rectum	2(20%)	6 (60%)	2 (20%)	
Tumor Size	**≤ 5cm**	7 (46.7)	5 (33.3)	3 (20%)	0.436
	> 5 cm	7 (28%)	13 (52%)	5 (20%)	
Histological Type; N (%)	Mucinous carcinoma	0 (0%)	0 (0%)	4 (100%)	0.000†
	Adenocarcinoma, NOS	14(38.9%)	18(50%)	4(11.1%)	
Grade; N (%)	**G I**	0 (0%)	1(33.3%)	2(66.7)	0.271
	G II	13(39.4%)	15(45.5%)	5(15.2%)	
	G III	1 (25%)	2 (50%)	1(25%)	
Perineural invasion	**Absent**	13(34.2%)	17(44.7%)	8(21.1%)	0.621
	Present	1 (50%)	1 (50%)	0 (0%)	

Lympho-	**Absent**	13(40.1%)	14(43.8%)	5(15.6%)	0.498
vascular emboli	**Present**	1(12.5%)	4(50%)	3(37.5%)	
Heavy	**Absent**	1(12.5%)	4(50%)	3(37.5%)	0.811
inflammation	**Present**	1(12.5%)	4(50%)	3(37.5%)	
Dense	**Absent**	9(34.6%)	13(50%)	4(15.4%)	0.553
desmoplasia	**Present**	5(35.7%)	5(35.7%)	4(28.6%)	

**** Analysis done by Chi-square test***

† Statistically significant

Table 2: TNM staging and relation with Alcian Blue staining Distribution in CRC (N=40)

		Alcian Blue Staining			
		Negative	**Positive Focal**	**Positive Diffuse**	
Characteristics		**N= 14**	**N= 18**	**N= 8**	**P-value***
	T2	1 (14.3%)	4 (57.1)	2 (28.6%)	0.170
T (extent of primary tumor)	**T3**	8 (33.3%)	10 (41.7)	6 (25%)	
	T4	5 (55.6%)	4 (44.4)	0 (0%)	
	N0	1 (5%)	15 (75%)	4(20)%	**0.000** †
N (LN status)	**N1**	8(72.7%)	2 (18.2%)	1 (9.1%)	
	N2	5(55.6%)	1 (11.1%)	3(33.3%	
	Stage I	0 (0%)	3 (60%)	2 (40%)	**0.000** †
	Stage II	1 (6.7%)	12 (80%)	2 (13.3%)	
TNM groups	**Stage III**	11(61.1%)	3(16.7%)	4 (22.2%)	
	Stage IV	2 (100%)	0 (0%)	0 (0%)	

****Analysis done by Chi-Square test***

†Statistically significant

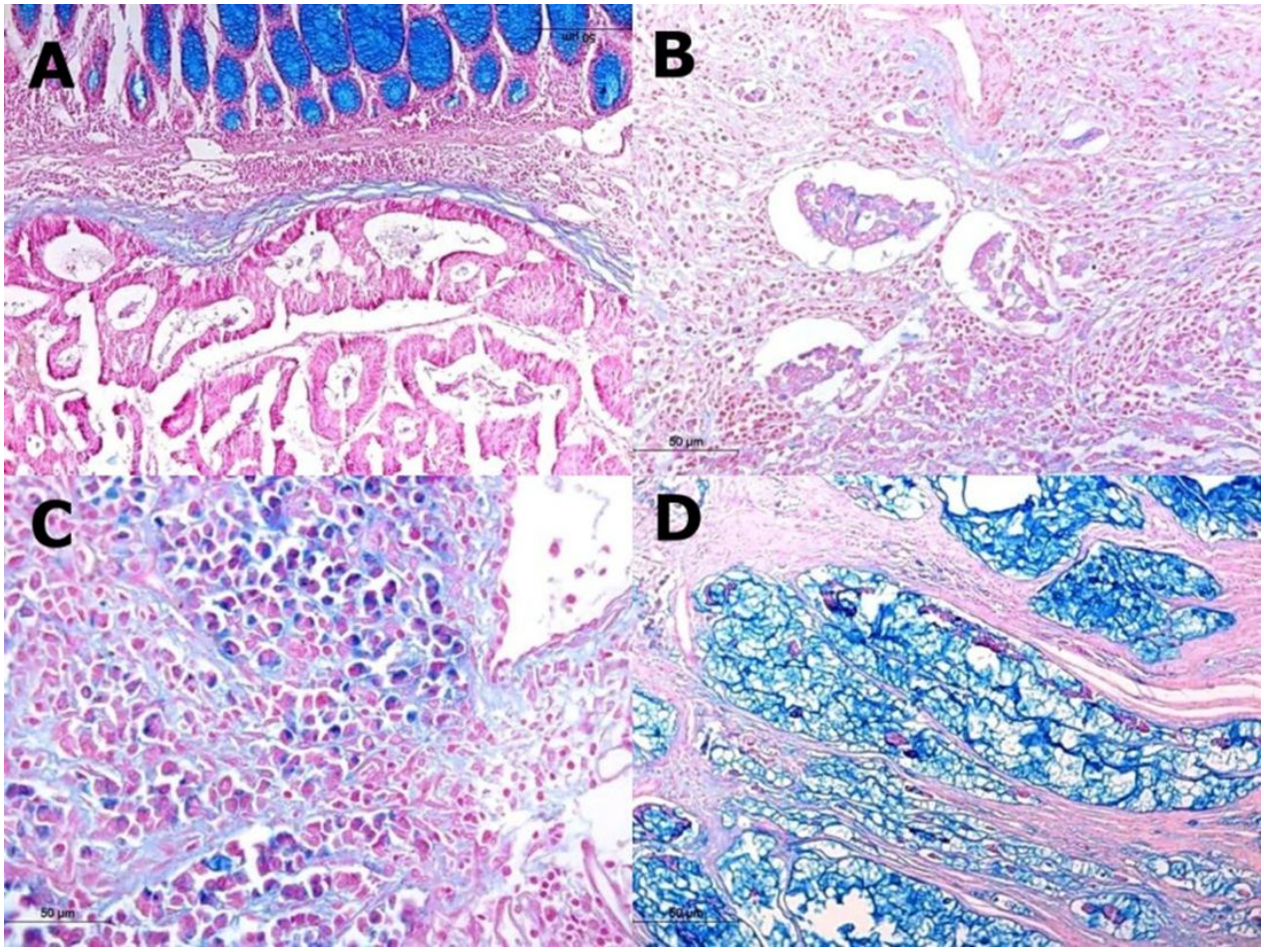

Figure 1: Alcian Blue staining distribution in CRC cases. A) Negative staining in adenocarcinoma (red), overlying positive normal colonic glands (blue) *(AB staining, original magnification x10)*. B) Focal positive staining in lymphovascular tumor emboli *(AB staining, original magnification x20)*. C) Positive staining in the signet ring cells *(AB staining, original magnification x20)*. D) Diffuse positive staining *(AB staining, original magnification x20*

Table 3: The relation between CD44 expression score and TNM staging of the cases:

Variables		CD44 Expression score				
		Negative	**Score +1**	**Score +2**	**Score +3**	
		N=10	**N=9**	**N=13**	**N=8**	**p-value***
T ***(Extent of Primary Tumor)***	**T2**	2(28.6%)	2(28.6%)	2(28.6%)	1(14.3%)	0.917
	T3	7(29.2%)	4(16.7%)	8(33.3%)	5(20.8%)	
	T4	1(11.1%)	3(33.3%)	3(33.3%)	2 (22.2%)	
N ***(Lymph Nodes Status)***	**N0**	9(45%)	8(40%)	2(10%)	1(5%)	**0.001** †

	N1	0(0%)	0(0%)	6(54.5%)	5(45.5%)	
	N2	1(11.1%)	1(11.1%)	5(55.6%)	2 (22.2%)	
	M1a	0(0%)	0(0%)	1(50%)	1(50%)	
TNM groups	**Stage I**	2(40%)	2(40%)	1(20%)	0(0%)	**0.006 †**
	Stage II	7(46.7%)	6(40%)	1(6.7%)	1(6.7%)	
	Stage III	1(5.6%)	1(5.6%)	10(55.6%)	6(33.2%)	
	Stage IV	0(0%)	0(0%)	1(50%)	1(50%)	

Analysis done by Chi-Square test

†Statistically significant

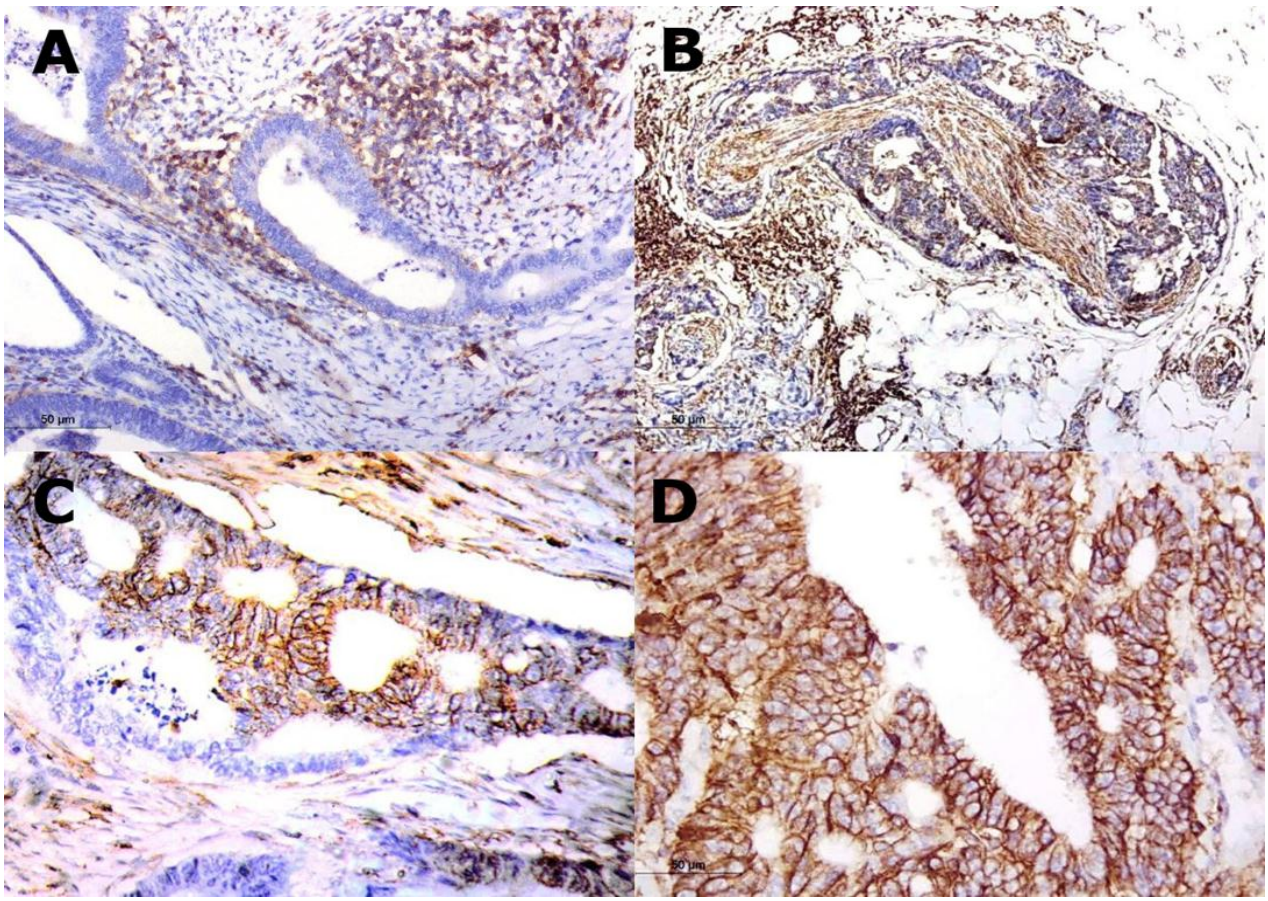

Figure 2: Immunohistochemical expression of CD44 in CRC cases: A) Negative CD44 expression in malignant glands, surrounded by positively stained lymphocytes as internal control *(CD44, original magnification x20)*. B) Perineural invasion by malignant glands showing membranous expression (score+1) with adjacent stained lymphocytes *(CD44, original magnification x20)*. C) Positive membranous CD44 expression (score+2) *(CD44, original magnification x40)*. D) Strong and diffuse membranous staining (score+3) *(CD44, original magnification x40)*.

Table 4: Relation and correlation between CD44 expression and Alcian Blue staining in the studied cases:

CD44 Expression	Alcian Blue Staining		p-value*	Correlation (r)†
	Negative (N=14)	Positive (N=26)		
Negative (N = 10)	0 (0%)	10 (38.5%)	**0.006 ‡**	**- 0.424**
Positive: (N = 30)	14 (100%)	16 (61.5%)		

Analysis done by Chi-Square test
†Non-Parametric Spearman's Correlation
‡ Statistically significant

4. Discussion:

CRC represents a significant global health problem with estimated 147,950 newly diagnosed cases in the United States in 2020. The majority of these occur in individuals aged 50 years and older. [14] This study showed that CRC is common in patients between 40 and 60 years old with male predominance and quite right colonic predilection. These findings were close to those reported by Jain et al, [15] and Sakr et al, [16] however; the latter found that 72.9% of cases had left colonic location.

Acidic mucins represent the main component of the normal colonic mucus layer. These can be divided into weakly acidic sialomucin (containing hexosamine and sialic acid) and highly acidic sulfomucin (containing hexosamine, immersion acid and sulfate group). [9, 17] Colon cancer mucins have shown decreased carbohydrate content compared to those in non-neoplastic colonic diseases. [18] Changes in mucin carbohydrate content can be analyzed with modified histochemical techniques, using periodic-acid Schiff reaction, and AB staining. [8]

In our study, AB staining was positive in 65% cases, and absent in 35% cases indicating that acidic mucin is increased in CRC. There was significant association between histological variants of CRCs and AB staining. Similar results were shown in studies by Kasprzak et al, [8] and Jain et al, [15] where most of their cases were positive with significantly higher expression in the mucinous subtype, compared to non-mucinous subtypes.

Hadi et al, [19] found no relationship between mucin secretion and the degree of differentiation of tumor in one study. Similarly, we found that the relation between tumor grade and AB staining was statistically insignificant, although all well differentiated tumors were AB positive. In contrast, the findings from Danquah et al, [20] showed significantly general decline in the acid mucin as cases progress from low-grade to high grade

adenocarcinomas. There was significant relation between lymph node status, TNM staging groups and AB staining distribution in the current work. Also, Lugli et al, [21] reported that loss of mucin was associated with the presence of nodal metastasis and worse survival. However, Kasprzak et al, [8] found no significant differences of AB polysaccharide expression in different TNM clinical stages. Different histochemical techniques as well as lack of standard methods of evaluation of mucin expression may explain heterogeneity in results.

CD44 plays a role in tumorigenesis through activating various signaling pathways involved in cell adhesion and migration. [22] This may suggest CD44 as a potential target for cancer therapy. [23]

In this study, expression of CD44 was positive in 75% of CRC cases. This result was comparable with Negan et al, [24] who found positive CD44 expression in 80% of their cases. However, in many previous studies CD44 was expressed in much lower rates. [7, 25, 26]

We found a significant relationship between lymph node metastasis, TNM stage groups and CD44 expression. Similar findings were demonstrated by many studies including meta-analysis. [27-29] It is worth to mention that several studies in literature had reported the association of CD44 expression in CRC with clinicopathological features but, controversies still exist regarding the definite role of CD44 in cancer development and progression. For example, Mohamed et al, [7] reported significant associations between CD44 expression and all clinicopathological features except the histological type, another study by Rohani et al, [30] found no association with any clinicopathological parameter except the age of patients. These discrepancies may be due to variable patient material, antibodies, and immunohistochemical techniques used in different studies.

This study revealed a significant relation between the histochemical expression of acidic mucin and immunohistochemical expression of CD44 with a moderate negative correlation. To our knowledge, there are no other researches discussing this point to compare with our results.

The main limitations of this work include the relative small sample size and the lack of follow-up and outcome data of patients.

5. Conclusion and Recommendations

We concluded that loss of Alcian blue staining and increased CD44 expression were strongly related to lymph node metastasis and higher TNM stage group, suggesting both as potential bad prognostic markers. Therefore, mucin histochemistry is still valuable and targeting CD44 may hopefully have therapeutic implication in CRC. Our study also demonstrated a significant association between CD44 expression and Alcian blue staining among the cases, further studies with larger

samples are recommended to prove this relation.

6.References:

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