

Evaluation and Clinicopathological Correlation of CD44 in Colorectal Adenoma with Low/High-Grade Dysplasia and Carcinoma

Abstract

Colorectal carcinoma (CRC) is one of the most common malignant neoplasms with significant morbidity and mortality worldwide. Cancer stem cell hypotheses have gathered significant attention to their molecular progression. CD44, a stem cell marker, is reported to be more concentrated in the malignant and premalignant cells. We aimed to study the prognostic and therapeutic utility of CD 44 in colorectal adenoma with low/high-grade dysplasia and carcinoma and its correlation with clinicopathological parameters. Histological examination was performed according to the criteria outlined in the World Health Organization Classification of tumors 2019 and AJCC 8th edition. RAmBispective analysis of colorectal neoplasms yielded 50 cases, of which 30 were malignant. Positive staining and high expression of CD44 were more frequent in carcinoma than in adenoma. Further staining and expression were higher in adenoma with high-grade dysplasia than in adenoma with low-grade dysplasia (p value=0.021). A significant statistical correlation was noted in CRC cases with younger age (0.0006), increased mitosis (0.038), and higher AJCC stage (0.014). Our study suggests that CD 44 expression perhaps is higher in adenomas with high-grade dysplasia and CRC with higher pathological stage, and thus could predict a worse prognosis. Larger multi-institutional studies might help study its role as a prognostic and therapeutic stem cell marker.

Keywords: Colorectal, Carcinoma, Adenoma, Dysplasia, Stem cell, CD44.

Introduction

Colorectal cancer (CRC) is one of the world's most prevalent malignant neoplasms. It accounts for 1.9 million new cases and 935,000 deaths, as estimated in the year 2020. It represents about one in 10 cancer cases and deaths worldwide, ranking third in terms of incidence and second in mortality. CRC is responsible for a significant burden of disease in the Indian population. Colon carcinoma is reported to develop in 1 in 298 individuals and rectal carcinoma in 1 in 295 individuals.^[1]

Many hypotheses have been postulated for its development. Among all, the cancer stem cell hypothesis has garnered a lot of attention in recent years and has been the subject of much ongoing research. Cancer stem cells are a small population of cells in cancer that are immortal.^[2] They have selectively acquired tumor-related features like uncontrolled growth and the ability to metastasize while maintaining their property of self-renewal and so are responsible for tumor initiation, growth, and recurrence.^[3-5] They were discovered as

early as the mid-19th century by Rudolf Virchow^[3, 6] but were first isolated from Acute myeloid leukemia by Bonnet and Dick in the late 20th century.^[7] The colorectal stem cells were first identified in the year 2007 by Brien *et al.*^[6, 8]

Several stem cells, such as CD44, CD133, EpCAM, and ALDH1, have been studied in colorectal carcinomas. CD44, also known as Hermes antigen,^[9] is a membrane receptor for hyaluronic acid, found to be more concentrated in the malignant and premalignant cells.^[10] After getting activated by binding to hyaluronic acid, it regulates stem cell homing and attracts tumor-associated macrophages in the niches. These macrophages produce growth factors like platelet-derived growth factor, which helps to maintain tumor cells in a continuous proliferative phase. The important functions of CD44 are angiogenesis, cell adhesion, cell migration, cell-to-cell interaction,^[11, 12] epithelial-mesenchymal transition, tumor invasion, and metastasis.^[11] In normal colonic mucosa and hyperplastic colonic epithelium, the CD44 cells are expressed at the base of crypts,^[13] their over-expression is

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reported in inflammation, autoimmune diseases, and in neoplastic conditions.^[10] A Pubmed search of English literature revealed human and animal studies of CD44 staining and expression in colorectal carcinoma and adenoma. However, to the best of the authors' knowledge, there are only a few human studies that studied its expression in adenoma with varying grades of dysplasia and compared the degree of expression with carcinoma, and there was no published study on CD44 expression in colorectal adenoma from Indian population. The role of CD44 has also been studied in many other neoplasms like carcinoma of the breast, pancreas, stomach, and lungs.^[14-17]

Materials and Methods

Participants and procedures

The present study was an ambispective study conducted from September 2019 to August 2021. Our study included histopathologically proven CRC cases and adenomas diagnosed during the study period and archived blocks of cases from January 2018 to August 2019. A total of 30 cases of CRC and 20 cases of colorectal adenoma were included in the study. All cases of CRC with a history of previous treatment or those with only incisional biopsy were excluded. Informed consent was taken from all the patients, and the study adhered to the Declarations of Helsinki. The Institutional Ethics Committee approved the study.

Histopathological evaluation

Histological examinations of tumor type and grade were performed according to the criteria outlined in the World Health Organization Classification of tumors, 2019. The parameters evaluated were tumor depth, lymphovascular invasion (LVI), perineural invasion (PNI), and lymph node status. The pathological stage was assessed according to AJCC 8th edition. The presence of mitoses and necrosis was also recorded. The demographic and clinical data included age, gender, and location of the tumor. Adenomas included in the study were classified as adenomas with low-grade dysplasia and high-grade dysplasia.

Immunohistochemical evaluation

The immunohistochemical marker CD44 was evaluated by secondary labeling method on formalin-fixed paraffin-embedded tissue sections (4-5micron thick) mounted on poly L-lysine coated slides. The monoclonal antibody used was CD44 (clone- BSB-12). Tonsillar tissue was taken as a positive control. Negative control was performed by skipping the step of adding a primary antibody.

The assessment of the staining of CD44 was done by two observers independently. When there was discordance between the observers, the slides were reviewed again by the two observers to reach a consensus opinion.

Score	Interpretation	Score	% of positive cells
0	None	1	<5%
1	Mild	2	5-20%
2	Moderate	3	21-50%
3	Strong	4	>50%

Membranous expression of CD44 was recorded in adenoma, carcinoma, and adjacent uninvolved epithelium. As tabulated in **Table 1**, the intensity of staining was graded into four groups: 0- none, 1- mild, 2- moderate, and 3- strong staining. The percentage of staining was also graded into four groups: 1: expression in <5%; 2: 5-20%; 3: 21-50%; and 4: in >50% of cells.

For evaluation of staining, the cases were divided into two groups based on the addition of intensity and percentage score, a cumulative score of 0-2: negative staining and 3-7: positive staining. The expression was evaluated based on multiplying the intensity and percentage of cells stained positive. A score of <100 was assigned as a low expression and >100 as a high expression (**Figures 1a-1c**).

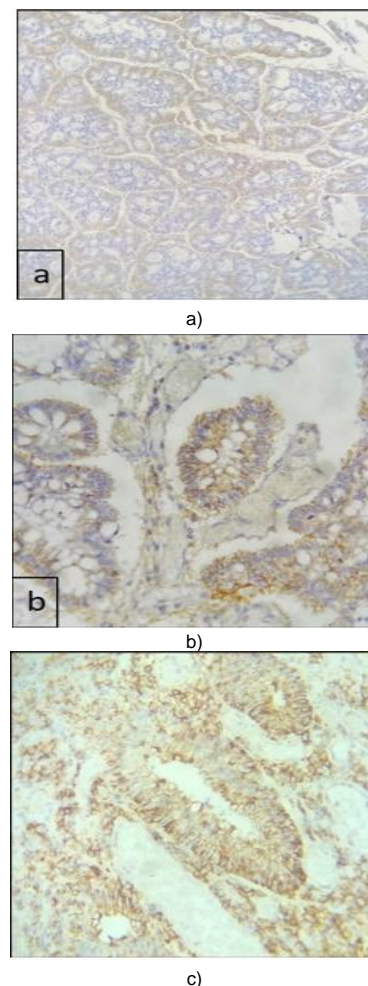


Figure 1. CD44 staining in colorectal carcinoma. a) Mild intensity of staining of CD44 in less than 50% of tumor cells- Low Expression (IHC, X100); b) Moderate intensity of staining in less than 50% of tumor cells- Low Expression (IHC, X400); c) Strong intensity of staining in almost 100% of tumor cells- High Expression (IHC, X400).

Table 1. Scoring of intensity and percentage of staining CD44 evaluation

Intensity (I)	Percentage (P)
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Statistical analysis

Analysis was conducted with SPSS 20 software. To check the association *between* two categorical variables, chi-square and Fisher exact tests were used. For all statistical tests, a p-value less than 0.05 was considered significant.

Results and Discussion

Clinicopathological parameters

Our study comprised 30 cases of CRC. The average age of patients was 62.7±12.5 years. The study included 17 males and 13 females, with an M: F ratio of 1.3:1; most cases had a tumor size of ≤5 cm. The most common site of carcinoma was the left colon (53%). The maximum number of cases were well differentiated (70%), and only one case of poorly differentiated carcinoma was observed. Most of the cases belonged to Stage III (60%), with none in Stage IV. The presence and absence of lymphovascular invasion (LVI) were almost similar, with 53% of cases that showed LVI. However, most cases showed the absence of perineural invasion (70%). Lymph node metastasis was observed in 60% of the cases. All the clinicopathological observations are tabulated in **Table 2**.

Histological Grade		
I	21	70%
II	8	27%
III	1	3%
AJCC stage		
I	4	13%
II	8	27%
III	18	60%
LVI		
Present	16	53%
Absent	14	47%
PNI		
Present	9	30%
Absent	21	70%
LN metastasis		
Present	18	60%
Absent	12	40%
Mitosis(/10HPF)		
≤30	16	53%
>30	14	47%
Necrosis		
Present	20	66.7%
Absent	10	33.3%

Abbreviations: SD- Standard deviation; AJCC- American Joint Committee on Cancer; LVI- Lymphovascular Invasion; PNI- Perineural Invasion; LN- Lymph node; HPF- High power field.

Table 2. Clinicopathological Data of Colorectal carcinoma cases

Variable	Number (N=30)	Percentage (%)
Age		
Mean±SD	62.7±12.5 years	
≤60 years	13	43.3%
>60 years	17	56.7%
Gender		
Male (M)	17	56.7%
Female (F)	13	43.3%
M: F	1.3:1	
Size		
≤5 cm	19	63.3%
>5 cm	11	36.7%
Tumor site		
Left colon	16	53%
Right colon	11	37%
Rectum	3	10%

Staining of adjacent uninvolved epithelium

Out of the 30 carcinoma cases, adjacent uninvolved mucosa was included in 18 cases. Out of these 18 tissue samples, 77.8% showed positive staining of the uninvolved epithelium. The staining was noted in the crypts and was moderate in intensity in 28.5% and strong in 71.5% of cases.

Correlation of CD44 with clinicopathological parameters

The correlation of CD44 staining and expression with clinicopathological parameters is tabulated in **Table 3**. Amongst the cases of CRC, 66.7% cases showed positive staining, and 43.3% showed high expression with CD44.

Table 3. Results and Statistical significance of CD44 staining and expression and its correlation with various clinicopathological parameters.

Variables	Staining		p-value	Expression		p-value
	Negative N (%)	Positive N (%)		<100 [Low] N (%)	>100 [High] N (%)	
Age						
≤60 years (N=13)	2 (15.4)	11 (84.6)	0.0006	9 (69.3)	4 (30.7)	0.1216
>60 years (N=17)	13(76.5)	4 (23.5)		15(88.3)	2(11.7)	
Gender						
M (N=17)	7 (41.2)	10 (58.8)	0.1668	10 (58.8)	7 (41.2)	0.3991
F (N=13)	3 (23.1)	10 (76.9)		7 (53.9)	6 (46.1)	
Tumor size						

≤5 cm (N=19)	8 (42.2)	11 (57.8)	0.180	12 (63.2)	7 (36.8)	0.346
>5 cm (N=11)	2 (18.2)	9 (81.8)		5 (45.5)	6 (54.5)	
Tumor site						
Left colon (N=16)	6 (37.5)	10 (62.5)	0.857	9 (56.3)	7 (43.7)	0.642
Right colon (N=11)	3 (27.3)	8 (72.7)		7 (63.6)	4 (36.4)	
Rectum (N=3)	1 (33.3)	2 (66.7)		1 (33.3)	2 (66.7)	
Histological Grade						
I (N=21)	8 (38.1)	13 (61.9)	0.179	12 (57.2)	9 (42.8)	0.634
II (N=8)	1 (12.5)	7 (87.5)		4 (50)	4 (50)	
III (N=1)	1 (100)	0 (0)		1 (100)	0 (0)	
AJCC stage						
I (N=4)	4 (100)	0 (0)	0.014*	4 (100)	0 (0)	0.119
II (N=8)	2 (25)	6 (75)		5 (62.5)	3 (37.5)	
III (N=18)	4 (22.2)	14 (77.8)		8 (44.4)	10 (55.6)	
LVI						
Present (N=16)	4 (25)	12 (75)	0.301	9 (56.2)	7 (43.8)	0.961
Absent (N=14)	6 (42.9)	8 (57.1)		8 (57.1)	6 (42.9)	
PNI						
Present (N=9)	2 (22.2)	7 (77.8)	0.398	3 (33.3)	6 (66.7)	0.091
Absent (N=21)	8 (38.1)	13 (61.9)		14 (66.7)	7 (33.3)	
LN Metastasis						
Present (N=18)	4 (22.2)	14 (77.8)	0.114	8 (44.4)	10 (55.6)	0.098
Absent (N=12)	6 (50)	6 (50)		9 (75)	3 (25)	
Mitosis (/10hpf)						
≤30 (N=16)	8 (50)	8 (50)	0.038	11 (68.8)	5 (31.2)	0.153
>30 (N=14)	2 (14.3)	12 (85.7)		6 (42.9)	8 (57.1)	
Necrosis						
Present (N=20)	6 (30)	14 (70)	0.584	11 (55)	9 (45)	0.794
Absent (N=10)	4 (40)	6 (60)		6 (60)	4 (40)	

*Fisher's exact test

Abbreviations: N- Number of cases; AJCC- American Joint Committee on Cancer; LVI- Lymphovascular Invasion; PNI- Perineural Invasion; LN- Lymph node; HPPF- High power field.

Most cases (84.6%) in the age group of ≤60 years showed positive staining, as compared to 23.5% cases in the >60 years age group, and this difference was statistically significant (p-value=0.0006). High expression was noted in 30.7% of cases in the age group of ≤60 years, as compared to 11.7% of cases in the age group of >60 years. Positive staining and high expression were noted in females (76.9%; 46.1%) with carcinoma as compared to males (58.8%; 41.2%), but these results did not show statistical significance. Cases with a size >5 cm showed positive staining in 81.8%, and cases with a tumor size ≤5 cm showed positive staining in 57.8% of cases. Similarly, high expression was seen in 54.5% of tumors with a size >5 cm and 36.8% with a size ≤5 cm. More cases in the right colon (72.7%) showed positive staining as compared to cases in the left colon (62.5%) and rectum (66.7%). This difference was not statistically significant. However, higher expression was seen predominantly in tumors located in the rectum (66.7%). All positive cases located in the rectum also showed high expression. 87.5% of cases of Grade II and 61.9% of cases of Grade I showed positive staining. Similarly,

50% of cases of Grade II and 42.8% of cases of Grade I showed high expression. Our study had only 4 cases of stage I, and none of the cases (15.3%) in Stage I showed positive staining or expression. Our study had maximum cases of Stage III, and 77.8% of stage III cases showed positive staining as compared to 75% of cases in Stage II. This difference in staining with increasing stage from I to III was statistically significant (p-value= 0.014). Similarly, 55.6% of cases in Stage III showed higher expression as compared to 37.5% of cases in Stage II (**Figures 2a-2d**). Positive staining with LVI was noted in 75% of cases and 57.1% without LVI. The presence of staining and high expression in patients with LN metastasis was noted in 77.8% and 55.6% of cases, respectively. Positive staining with the presence of PNI was more (77.8%) than in cases without PNI (61.9%). Similarly, more (66.7%) cases with PNI showed high expression as compared to cases (33.3%) without PNI.

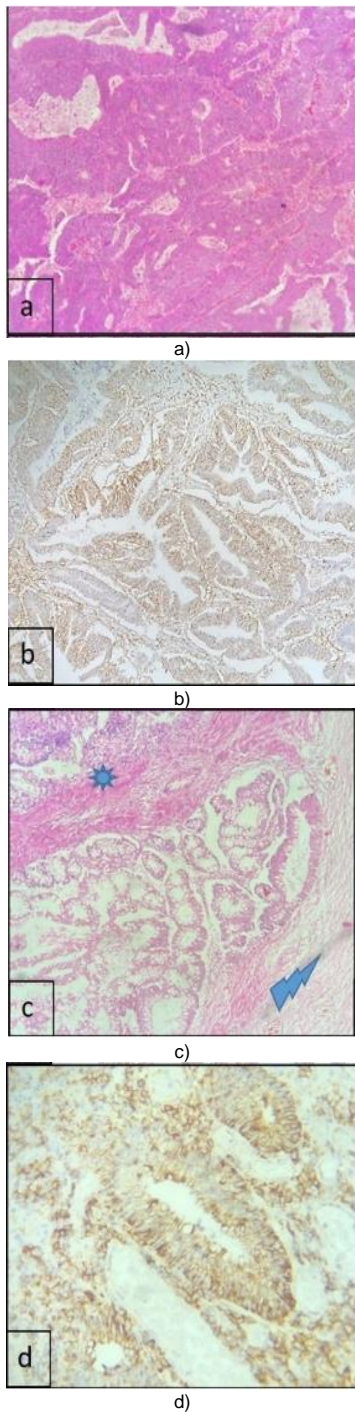


Figure 2. CD44 expression in colorectal carcinoma. a) Photomicrograph shows stage II adenocarcinoma (H&E X400); b) Moderate intensity and high expression of CD44 is noted in stage II carcinoma (IHC X100); c) Photomicrograph shows stage III adenocarcinoma (blue asterisk-muscularis propria; blue arrow- sub-serosal connective tissue (H&E X100); d) Strong intensity and high expression of CD44 in stage III carcinoma (IHC X400).

Positive staining was seen in 85.7% of cases with higher mitotic activity as compared to 50% of cases with lower mitotic activity, and this difference was statistically significant (p-value= 0.038). More cases with necrosis showed positive staining and high expression (70% and 45%) as compared to cases without necrosis (60% and 40%).

A statistically significant correlation was found between staining of CD44 with age, tumor stage, and mitosis by Fisher Exact test and Chi-square test.

Staining and expression of CD44 in adenoma and its correlation with carcinoma

Adenoma showed positive staining in 45% of cases and high expression only in 15% of cases.

Table 4. Comparison of results of expression and statistical significance of CD44 between adenoma and carcinoma.

Diagnosis	High Expr N (%)		Low Expr N (%)		p-value
	3 (15%)		17 (85%)		
Adenoma (N=20)	HGD 3	LGD 0	HGD 4	LGD 13	0.021
Carcinoma (N=30)	13 (43.3%)		17 (56.7%)		
Total	16		34		

Abbreviations: N: Number of cases; Expr: expression; HGD: High-grade dysplasia; LGD: Low-grade dysplasia.

When compared with carcinoma, 66.7% of carcinomas cases showed positive staining compared to 45% of adenoma cases. This difference was close to significance (p-value=0.072). As shown in **Table 4**, 43.3% of carcinomas showed high expression as compared to 15% of cases of adenoma, and this difference was statistically significant (p-value=0.021).

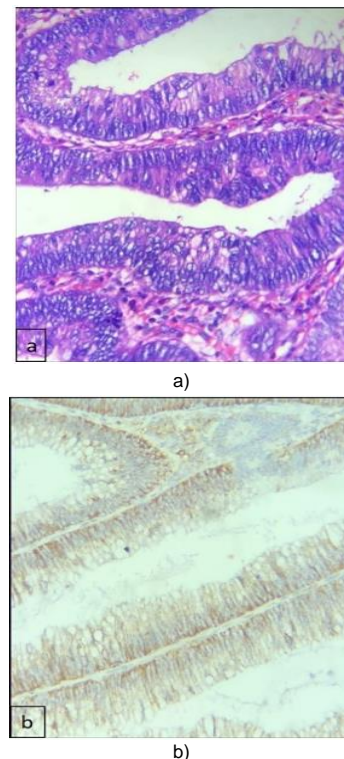


Figure 3. CD44: Adenoma with high-grade dysplasia. a) Photomicrograph shows adenoma with high-grade dysplasia (H&E X400); b) High expression of CD44 in adenoma with high-grade dysplasia (IHC X100).

Most adenomas with HGD (71.4%) showed positive staining with CD44 compared to those with LGD (30.8%). This difference was also close to significance ($p = 0.057$). High expression was noted in 42.9% of cases of adenoma with high-grade dysplasia. A striking observation was the absence of high expression in those with LGD, with a statistical significance of 0.015 (**Figures 3a and 3b**).

Our study showed positive staining of CD44 in 66.7% of CRC cases. Our observations were similar to the studies conducted by Holah *et al.*, Mohamed *et al.*, and Bo Zhu *et al.*^[18-20] As described by Hong *et al.*, high expression was noted in more than 40% of cases in our study also.^[21]

Positive staining in younger age group patients showed statistical significance (p -value=0.0006), further with high expression in younger age. Higher expression of CD44 stem cell marker in younger age and decrease in stem cell pool in aging is plausible as aging is known to be associated with a decline in stem cells and reduction in regeneration potential. However, animal models have described an age-related increase in cancer stem cells,^[22] and aging is also one of the most studied known risk factors for carcinogenesis. Our study also showed a greater number of cases in older age. Observation of lower expression of cancer stem cell marker CD44 in older age, with more cases in older age, is enigmatic.^[23] Although, the role of additional mutations, altered metabolism and epigenetic modifications in carcinogenesis may explain it. Studies by Bo Zhu *et al.* and Holah *et al.* also showed similar results.^[18, 20] However, their results did not reach statistical significance.

In our study, CD44 staining and expression were seen more in CRC in females than in males and in tumors with increased tumor size. The higher percentage of staining and expression of CD44 in CRC in females could be attributed to their younger age but the higher stage at presentation, both of which were also associated with positive staining and high expression in our study. Similar results have also been reported in the literature.^[19, 21]

A significant observation was an increased percentage of cases with positive staining (p -value=0.14) and high expression in higher-stage tumors. Our study had only 4 cases in stage I as compared to 18 cases in Stage III, but none of the cases in stage I, and in contrast, 77.8% of cases with stage III showed positive staining with CD44. Comparable results have been published by Mohamed *et al.* in their study.^[19] Although low numbers of stage I tumors could have skewed our results, it is still possible that tumors that present as lower stage might have different oncogenesis. In the colorectal cancer cell line model, CD133+/CD44+ tumors had maximum tumorigenic potential compared to CD44 negative cell lines.^[24]

Our study demonstrated increased staining and expression in cases with a higher mitotic count, with statistical significance. An extensive search of English literature did not reveal any human study demonstrating a correlation between CD44 staining and expression with mitotic activity. However,

CD133+/CD44+ cell line models have been reported to have larger tumors with higher mitoses and large areas of necrosis.^[24] In our study, positive staining and a higher degree of expression were also associated with necrosis cases. Holah *et al.*, in their study, got similar results where more cases with the presence of necrosis showed positive staining.^[18]

In 1995, a study in Amsterdam showed shown role of CD44 in colorectal carcinogenesis, with the absence of CD44 in low-grade adenomas and expression in 40% of cases of high-grade adenomas and increased expression in colorectal carcinoma with increasing Dukes stage.^[25] At the same time, work on a rat model of colorectal oncogenesis had also shown expression of CD44 in adenomas, with increased hybridization signals with increasing dysplasia.^[26, 27] In our study, positive staining with CD44 was seen in 45% of cases of adenomas, and amongst the adenomas, high expression was only noted in adenomas with high-grade dysplasia (p -value=0.021). Negative staining or low expression in adenomas with low-grade dysplasia or carcinomas with lower stage and grade suggest CD44 negative colorectal neoplasms might behave less aggressively and have a lower proliferative index. Thus, our study suggests that CD44-positive tumors, specific patients with adenoma, must be managed more cautiously and judiciously with closer follow-up for better patient outcomes.

Our study included only a single case of Grade III tumor; thus, we could not study the staining and expression results of Grade III tumors. In addition, our study included only 4 cases (13%) of Stage I, compared to 61% cases of Stage III tumors. Therefore, the authors recommend larger and multi-institutional studies to validate their study results.

To conclude, there is very limited literature on the study of CD44 expression in colorectal carcinoma from the Indian subcontinent. Furthermore, there is very limited data on the study of CD44 in colorectal adenoma, and to the best of the authors' knowledge, this is the first study from the Indian subcontinent on CD44 expression in adenomas with low-grade and high-grade dysplasia.

The salient feature of our study is the significant statistical correlation between staining of CD44 and younger age group, increased mitosis, and AJCC stage. We understand that these are very significant observations, and as we all know, younger age at presentation, increased rate of proliferation, and higher AJCC stage portend a poor prognosis in neoplasia. To the best of the authors' knowledge, a Pubmed search of English literature yielded only a few studies that have highlighted the expression of CD44 in adenoma with varying grades of dysplasia and its comparison with CRC. Higher staining and expression of CD44 in colorectal carcinoma as compared to adenoma or absence of high expression in low-grade adenomas as compared to high expression in high-grade adenoma. Higher staining and expression in larger tumors with high mitoses, necrosis, lymphovascular invasion, perineural invasion, and lymph node metastasis importantly suggests poor prognosis in CD44 positive tumors and thus highlight the role of close follow-up of CD44 positive cases

and their plausible role in planning targeted or personalized therapy.

Conclusion

- Our study demonstrated a significant statistical correlation of CD44 expression in adenomas with high-grade dysplasia and in colorectal carcinoma, suggesting its significance in oncogenesis.
- The absence of high CD 44 expression in low-grade adenomas compared to high expression in high-grade adenomas and carcinoma suggests its role in the adenoma-carcinoma sequence.
- We also found a significant correlation of CD44 in CRC with the younger age group, increased mitosis, and AJCC stage, suggesting the role of CD44 in assessing tumor prognosis.
- Our results suggest the need for close follow-up of CD44-positive neoplasms and the possible role of targeted/personalized therapy in chemo-resistant and recurrent tumors.

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Conflict of interest

None.

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None.

Ethics statement

Informed consent was taken from all the patients. The study adhered to the Declarations of Helsinki. The study was approved by the Institutional Ethics Committee (KIIT/KIMS/IEC/98/2019).

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