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IMMUNOHISTOCHEMICAL EXPRESSION OF CD44 IN COLORECTAL CARCINOMA IN RELATION TO HISTOMORPHOLOGIC PARAMETERS AND CLINICO-PATHOLOGICAL FACTORS: A CROSS-SECTIONAL STUDY.

Dr Pragnya Paramita Mishra[a], Dr Madan K[b], Dr Anuradha Calicut Kini Rao[c], Dr Siddhartha Biswas[d], Dr Rohan Shetty[e], Dr Premanand panda[f]

[a]Associate Professor, Department of pathology, Hitech medical college, Rourkela, India.
[b]Assistant Professor Department of Oncopathology, Yenepoya Medical College, Mangalore, India.
[c]Professor, Department of Pathology, Kasturba Medical college, Mangalore, India.
[d]Professor & HOD, Department of Oncopathology, Yenepoya Medical College, Mangalore, India.
[e]Professor, Department of Oncology,Yenepoya Medical College, Mangalore, India.
[f]HOD, Department of Radiology, JP hospital, Rourkela, India.

ABSTRACT

Background

Cancer stem cells (CSC) have proven to play a vital role in cell invasion, metastasis, and treatment resistance in colorectal carcinoma (CRC), which subsequently led to poor outcomes. Cluster of differentiation 44 (CD44) is usually expressed in stem cells in CRCs and can be detected by immunohistochemistry (IHC).

Aims

The present study aimed to evaluate the role of immunohistochemical expression of CD44 in CRC cases of this region and its relationship with clinicopathological parameters and patient outcomes.

Methods

A cross-sectional study included 52 patients with primary CRC who were analyzed for CD44 expression by IHC on paraffin-embedded blocks. Data were collected, tabulated, and statistically analyzed by using SPSS Version 23.0.

Results

The study had a male predominance (70%) with most participants aged above 45 years (82%). Tumors were predominantly left-sided (69%) and larger than 5 cm (73%). CD44 membranous positivity was found in 78.8% of tumor cells and 59.6% of stromal cells. Signet ring cells showed weak CD44 positivity. CD44 expression correlated with higher tumor stages (T3, T4) and larger tumor sizes (>5 cm), but not with nodal stage, perineural, or lymphovascular invasion. Stromal CD44 positivity was found in 59.6% of cases and showed no significant correlation with tumor stage, size, lymphovascular invasion, perineural invasion, or nodal stage (e.g., T stage: T3 - 14 positive, 16 negative; N stage: N0 - 18 positive, 14 negative; tumor size >5 cm - 21 positive, 17 negative).

Conclusions

CRC prognosis is independently correlated with CD44 expression, a stem cell marker. They are linked to epithelial-mesenchymal transition (EMT) and tumor budding, with increased expression in high-burden instances.

Recommendation

Further research should be conducted on the role of CD44 expression in colorectal cancer, particularly focusing on post-neoadjuvant chemotherapy cases, to better understand its prognostic implications and potential as a therapeutic target.

***Keywords**: Colorectal carcinoma, Immunohistochemistry, Stromal cell, Stem Cell Marker*
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Corresponding Author
Dr Premanand panda,
HOD, Depatment of Radiology, JP hospital, Rourkela, India.
Email: panda.premanand@gmail.com

INTRODUCTION

GLOBOCAN 2018 data says that colorectal cancer (CRC) is the third most deadly and fourth most commonly diagnosed cancer in the world. It occurs when certain cells of the epithelium acquire a series of genetic or epigenetic mutations.[1]When diagnosed early, the prognosis is good, with a 5-year relative survival rate of 91% for localized carcinoma and 11% in metastatic cases. Several clinicopathological parameters and biological markers have been identified with an impact on prognosis. Although tremendous advancement has been achieved in the management of this progressive disease,[2] local invasion, distant metastasis, and therapy resistance hinder the survival of patients. A series of research explains the underlying molecular mechanisms responsible for this. A complex transmembrane adhesion glycoprotein called Cluster of Differentiation 44 (CD44) is expressed by cancer stem cells, differentiated cells, and embryonic stem cells.[3]

CD44-positive cells have CSC properties, meaning that a single cell could self-renew, differentiate, and form a tumor similar to the original lesion. There is evidence that CD44 is involved in the initiation and progression of intestinal tumors and the development of metastasis. Accordingly, it was demonstrated that CD44 is a part of the intestinal stem cell gene signature that predicts disease relapse in CRC patients.[4] Some studies also showed downregulation of CD44 inhibits tumor growth and metastasis in highly metastatic CRC cells.[5] It was observed that higher-grade CRCs had higher CD44 expression levels as compared with lower-grade tumors, and this overexpression affects the patient's survival. [5]

The present study aimed to evaluate the role of immunohistochemical expression of CD44 in CRC cases of this region and its relationship with clinicopathological parameters and patient outcomes.

MATERIALS & METHODS

Study Design

This is a cross-sectional study by simple random sampling.

Study Setting

The study was carried out at the Onco-pathology Department, Yenepoya Medical College, Mangalore, India, from January 2020 to June 2021.

Participants

The study included 52 cases of CRC. The clinicopathological data were archived from patient hospital records.

Inclusion criteria

- Patients with a confirmed diagnosis of colorectal carcinoma through histopathological examination.
- Patients aged 18 years and above.

Exclusion criteria

- Patients with other malignancies or benign colorectal conditions.
- Patients who have already received neoadjuvant chemotherapy.
- Cases with incomplete clinical records or insufficient tissue samples for analysis.
- Patients with severe comorbid conditions that could interfere with study outcomes, such as uncontrolled diabetes, severe cardiovascular diseases, or other life-threatening conditions.
- Pregnant women or those currently breastfeeding.

Histopathological evaluation

The hematoxylin and eosin-stained sections were evaluated for histological type. Tumor grade was determined based on gland formation, either low grade (≥50% gland formation) or high grade (< 50% gland formation). Lymphovascular invasion, perineural invasion, and tumor budding (low grade, high grade, absent) were assessed. Pathologic staging of the tumor was performed according to the TNM system.

Immunohistochemical staining

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections 3 μm thickness on positively charged slides. The primary antibody was a Rabbit monoclonal antibody against CD44 (anti-CD44 antibody with a dilution of 1: 200, overnight at 4°C) (Thermo Fisher Scientific, Fremont, California, USA). A negative control slide was included in each run by omitting the primary antibody. Normal tonsils were considered positive control and were run in each batch. The method used for immunostaining was a peroxidised- anti-peroxidase system.

Interpretation of immunohistochemical staining

Expression was evaluated according to the presence or absence of membranous expression of CD44 in both tumor cells and Stromal cells. The intensity (I) of staining was scored as follows: '0', no reaction; '1', weak reaction; '2', moderate reaction and '3', strong reaction. The proportion of signal was scored as a percentage of positive cells (P). Stromal expression of CD44 was positive if more than one-third of the stroma showed membranous CD44 expression and negative if the expression was less than one-third.

Survival analysis

All the patients were followed to date, with a median survival of 05 months. 15 patients were lost to follow up whereas 3 patients are dead and the rest are doing well with no recurrence.

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Statistical analysis

Data were collected, tabulated, and statistically analyzed. Considering the proportion and fixing the level of confidence to 90% with a margin error of 11%, the sample size was calculated as follows:

$n = Z - \alpha/D \ \beta(1-\beta)/E2$

P = 90%, z- $1-\alpha/D = 1.64$, E = 11%

Descriptive statistics were used to summarise the data. The Chi-square test is used to test the association between CD44 expressions and other selected variables. P values of 0.05 or less are considered statistically significant.

The correlation of CD44 expression (both in the stroma and tumor cells) with other prognostic histopathological parameters like tumor size, grade, size, laterality, lymphovascular invasion, perineural invasion, tumor stage, and lymph node involvement has been studied.

Ethical considerations

The study protocol was approved by the Ethics Committee and written informed consent was received from all the participants.

RESULTS

This study conducted on 52 cases of CRC showed male preponderance (70%) with 43 cases (82%) aged above 45 years. Predominant cases (69%) were located on the left side of the colon with size more than 5 cm (73%). Most of the cases (50%) were ulcerative type, grossly. Microscopic examination showed 11 (21%) mucinous adenocarcinoma cases. The Adenocarcinoma cases predominantly (70%) were of grade 2, with uninvolved margins. Vascular invasion was noted in a minority of patients (16 cases, 31%) with a slightly higher number (22 cases, 42%) depicting perineural invasion and nodal metastasis (20 cases, 39%). Tumor budding was predominantly of high grade. (Score 2)

CD44 expression: The current study revealed that 41 (78.8%) of the studied CRC cases showed membranous CD44 positivity in tumour cells (Fig 1 &2). Different staining patterns of CD44 like basolateral staining were also observed.CD44 expression was also noted in some lymphocytes and macrophages which also showed basal staining pattern and were used as internal controls. Nerve fibers also showed a similar pattern of staining. Signet ring appearing tumour cells showed weak membranous CD 44 positivity (Fig 3). A total of 31 (59.6%) cases showed CD44 positivity in stromal cells (Fig 4 & 5).

CD44 expression and clinicopathological features:

The relationship between CD44 expression and a range of clinico-histopathological variables is summarized in Table 1. Tumour cells expressed CD44 irrespective of gross presentation with a higher predilection for ulcerative tumours. CD44 expression increases with higher tumour stage (T3, T4), highest in T4. Also, CD44 expression is more in tumour of size more than 5cm in comparision to tumour size less than 5cm. CD44 expression had no correlation with patient age, sex or laterality. It did not correlate with presence of perineural expression and lymphovascular invasion or likewise of nodal stage.

Stromal expression of CD44 was noted to be present and absent in almost equal /similar proportions in all cases, irrespective of the morphological (gross or microscopic) parameter assessed. When comparing tumour cell and stromal expression of CD44, we opine that tumour cell expression is of more utility and the stromal expression is not of any significant value in the study.

In addition, we included 8 post-NACT cases in our study, 6 of which had CD44 expression in tumour cells and 3 of which had it in stromal cells. The p value for the comparison of CD44 expression in stromal cells and cancer cells in primary resection versus NACT is 0.09 and 0.019, respectively.

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Table 1: Relationship between immunohistochemical expression of CD44 in tumour and clinicopathologic factors in studied CRC cases

Variables	Variables	Negative (N)	Positive (N)
Gross tumour type	Annular (11)	1	10
	Fungating (15)	5	10
	Ulcerative (26)	6	20
T Stage	T1 (1)	0	1
	T2 (10)	1	9
	T3 (30)	10	20
	T4 (11)	1	10
N Stage	N0 (32)	5	27
	N1 (11)	3	8
	N2(9)	4	5
Tumour size	< 5 cm (14)	3	11
	>5 cm (38)	9	29
Margin	Negative (2)	11	38
	Positive (50)	1	2
Lymphovascular invasion	Absent (36)	5	24
	Present (16)	7	16
Perineural invasion	Absent (30)	9	32
	Present (22)	3	8

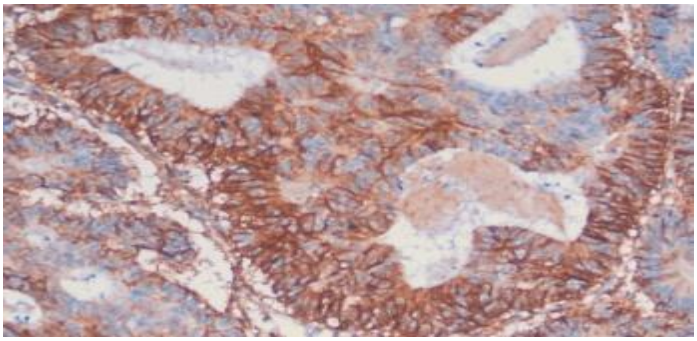

Fig 1: Photomicrograph: IHC; 40x: Intense complete membranous positivity of CD44 in tumour cells

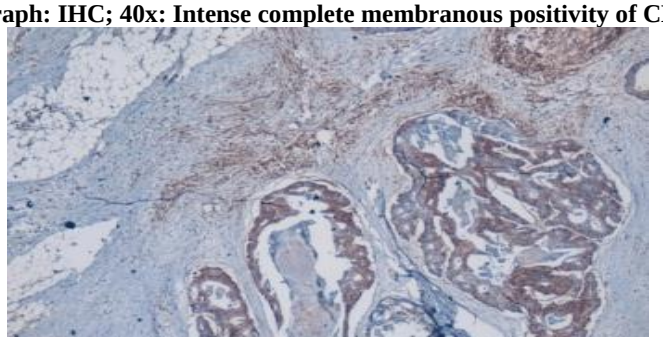

Fig 2: Photomicrograph: IHC; 20x: Weak membranous positivity of CD44 in tumour cells

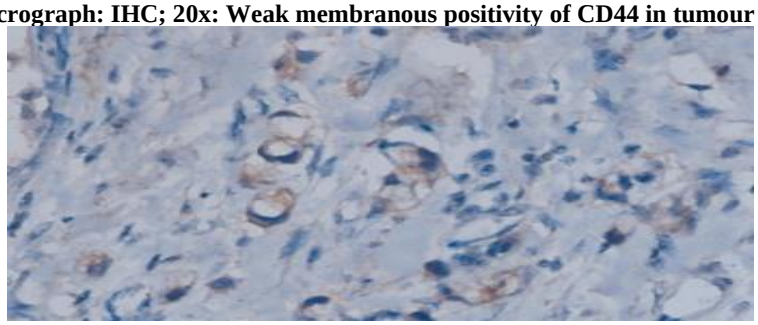

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Fig 3: Photomicrograph: IHC; 40x: Weak membranous positivity of CD44 in signet ring cells

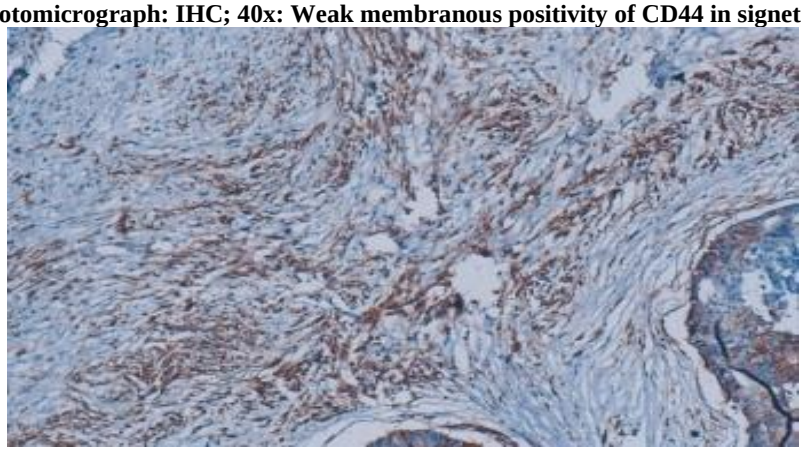

Fig 4: Photomicrograph: IHC; 40x: Strong CD 44 positivity of stromal cells

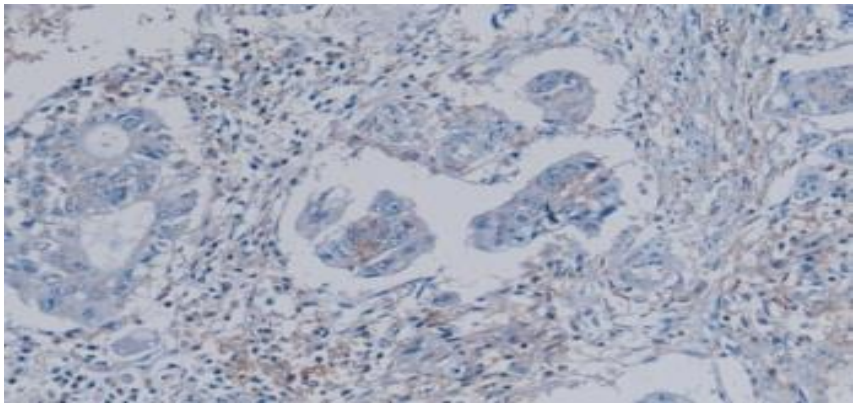

Fig 5: Photomicrograph: IHC; 20x: Weak CD 44 positivity of stromal cells, but tumour cells are negative

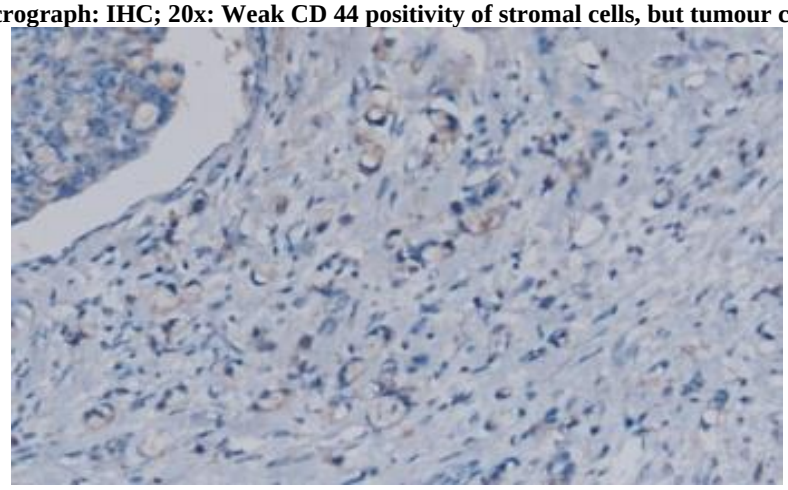

Fig 6: Photomicrograph: IHC; 20x: Weak CD44 positive in budding of tumour cell

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Table 2: Relationship between stromal expression of CD44 and clinicopathologic factors in studied CRC cases

Variables	Variables	Negative (N)	Positive (N)
Gross tumour type	Annular (11)	6	5
	Fungating (15)	7	8
	Ulcerative (26)	11	15
T Stage	T1 (1)	0	1
	T2 (10)	3	7
	T3 (30)	16	14
	T4 (11)	5	6
N Stage	N0 (32)	14	18
	N1 (11)	5	6
	N2(9)	5	4
Tumour size	< 5 cm (14)	7	7
	>5 cm (38)	17	21
Margin	Negative (2)	23	26
	Positive (50)	1	2
Lymphovascular invasion	Absent (36)	12	17
	Present (16)	12	11
Perineural invasion	Absent (30)	18	23
	Present (22)	6	5

Table 3: Relationship between expression of CD44 and tumour budding studied CRC cases

Tumor budding	**CD 44 IN TUMOUR**		**CD44 in stroma**	
	Positive	Negative	Positive	Negative
Absent budding(BD 0)	10	2	8	4
Low grade BD 1	10	3	8	5
High grade(BD2+BD3)	20	7	12	15

DISCUSSION

Cancer stem cells constitute a small percentage of the tumor population. Several markers for CSCs have been investigated and CD44 is said to be the most likely marker in CRC.[6]CD44 is described as a putative colorectal CSC marker and is thought to be a marker of tumor invasiveness and metastasis.[7] Upregulation is also considered as the earliest marker of neoplastic transformation of colonic epithelium [8] whereas downregulation has been demonstrated in the metastatic phase of CRC. [9] The study had one case of colorectal carcinoma with metastasis disease to the Liver, where CD44 expression was not seen in tumor cells and also in Stromal cells which corroborates with the above theory.

The study showed that 78.8 % of CRC cases had positive CD44 expression compared with 51% reported by [10] and 70% reported by [11]. The current study doesn't show a statistically significant association between the positive expression of CD44 and left-sided tumors similar to [10]. However, a study [12] showed a statistically significant association between CD44 expression and left-sided CRCs. Another study [13] showed a statistically significant association of CD44 expression with older age, which is not found in the present study. Some studies did not establish a significant relationship between CD44 expression and the stage of studied CRC patients [14, 15]. One Indian study [16] showed a significant correlation between CD44 expression and tumor stage.

It is also noted that tumor size >5cm had a high CD44 expression. Similar findings have not been seen in any other study.

CD44 expression showed no significant relationship, neither with grade nor with stage; this result is in agreement with [15]. In contrast, a study [17] found a significant association between CD44 expression and stage II and III tumors. This difference might be due to the low number of CRC cases (52 cases) compared with 187 cases in the study by Zhao.

There was no statistical association between stromal expression of CD44 and lymph node invasion as well as T and N stage, in the study. However, several previous studies

showed that CD44 expression was associated with a good prognosis of CRC.[8]

Comparison of the grade of tumor budding with CD44 in tumor cells (table 3) showed that irrespective of the presence/absence of tumor budding CD44 was expressed in the tumor. This could mean that the two were independent of each other and the tumor was independent of stem cell factor acquisition responsible for invasiveness in tumor cells.

In addition, the study included 8 post-NACT cases in the study, 6 of which had CD44 expression in tumor cells and 3 of which had it in stromal cells. The p-value for the comparison of CD44 expression in stromal cells and cancer cells in primary resection versus NACT is 0.09 and 0.019, respectively.

Survival data

Patients were followed up to date, with a median follow-up of 5 months. 15 (29%) patients were lost to follow-up, 3 (19%) patients succumbed to disease, rest are doing well with no recurrence. When compared with tumor budding and dead with live patients, the P value is 0.515.

CONCLUSION

Increased expression of CD44 in tumor cells may indicate the involvement of CD44 in the pathogenesis of CRC with a consensus on the relationship with tumor size, stage, and presence of tumor budding. Various studies suggest different data about the association of CD44 expression with known clinicopathologic parameters, the biological behavior of the tumor, and survival outcomes. Tumor cell expression rather than stromal expression could be studied. However, its role in tumor invasiveness remains questionable.

LIMITATION OF STUDY

The study is of 18 months duration with a smaller sample size. If the sample size had been larger, a better correlation would have been achieved. Also, evaluation of other stem cell markers and a longer follow-up would provide a better picture.

RECOMMENDATION

Further studies regarding the relationship of CD44 expression in tumor cells in post-NACT cases would help provide sufficient data regards its role in prognostication.

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LIST OF ABBREVIATIONS

CRC: Colorectal Carcinoma
CD44: Cluster of Differentiation 44
IHC: Immunohistochemistry
CSC: Cancer Stem Cells
TNM: Tumor, Node, Metastasis
EMT: Epithelial-Mesenchymal Transition
NACT: Neoadjuvant Chemotherapy

SOURCE OF FUNDING

No funding received.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

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