Evaluation of CDX2 Protein Expression Pattern in Colorectal Adenocarcinoma and Exploring its Association with Prognostic Parameters

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Abstract

Background: The prognosis of colorectal carcinoma is poor despite the availability of multidisciplinary approaches. The histological type, grade, and stage are the classical prognostic parameters for colorectal cancer. Still, treatment response differs within the same grade or stage due to the molecular heterogeneity of the tumors. CDX2 is a marker unique to the intestinal epithelial cells andacts as a tumor suppressor in colorectal cancer. CDX2 expression decreases in high-gradeand advanced stages of CRCs, negatively affecting the patient's prognosis. This study was intended to evaluate the expression pattern of CDX2 in colorectal adenocarcinoma and assess its association with prognostic parameters.

Materials and methods: This cross-sectional observational study was done in the Department of Pathology of Chittagong Medical College in Chattogram, Bangladesh spanning from March 2019 to February 2021. Surgically resected specimens from forty-five patients with colorectal adenocarcinoma were included. CDX2 protein expression was analyzed immunohistochemically.

Results: CDX2 high expression was seen in 42.2% of cases and low in 57.8%. The proportion of low expression of CDX2 was significantly higher among the patients with a higher tumorgrade (P=0.021). There was no statistically significant association between CDX2 expression andother prognostic variables.

Conclusion: In this study, high-grade colorectal adenocarcinoma cases displayed diminished or absent CDX2 expression. Its expression pattern may be helpful in predicting the clinical outcome of the patients and also directing appropriate treatment strategies.

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Introduction

Worldwide one of the most prevalent malignancies is Colorectal Carcinoma (CRC). It is the third most common cancer globally.[1,2] The World Health Organization (WHO) global database, reported 1,931,590 new colorectal cancer cases and 935,173 CRC-related deaths in 2020 in the world.[3] Colorectal cancer incidence varies widely by geography around the world, with higher rates in North America, Australia and Northern and Western Europe. The rate of carcinoma of colon and rectum incidenceis relatively lower in developing countries in Africa and Asia. However, recent socioeconomic development in Southeast Asia has contributed to a rise in the occurrence of colorectal cancer.[4] The incidence of cancer of the colon and rectum in Bangladesh is 1.8% and 1.6% respectively. According to the Globocan estimates in 2020, there were approximately 5,283 new cases and 3,239 deaths from colorectal cancer in Bangladesh.[3]

Malignant neoplasms developing from the mucosal lining epithelium of the colon and rectum are termed colorectal carcinomas.[1] Adenocarcinomas make up more than 95% of colorectal carcinomas and 40% to 60% of them are conventional adenocarcinomas.[5,6]

According tothe World Health Organization (WHO) classification system 2010, there are several histologic sub-types of colorectal adenocarcinoma, such as mucinous adenocarcinoma, medullary carcinoma, signet ring cell carcinoma, serrated adenocarcinoma, micropapillary carcinoma, cribriform comedo-type adenocarcinoma. Colorectal carcinoma is the result of a multi-step intricate process that is influenced by environmental, genetic, and epigenetic variables. The presence of infla-mmatory bowel disease and adenomatous polyps elevates an individual's susceptibility to developing

colorectal cancer.[7] The sporadic form of colorectal cancer, which affects approximately 70% of the patients, is triggered by environmental factors, such as dietary habits, smoking and abuse of alcohol. Approximately 25% of CRC patients exhibit a genetic predisposition, with 5% of cases being inherited through the germline.[8]

The molecular features of CRC are heterogeneous. These molecular characteristics are different in different parts of the large bowel. While the American Joint Committee on Cancer (AJCC) staging guideline for colorectal cancer aids in prognosis assessment and treatment selection, the response to therapy can vary within a particular cancer stage due to the diverse molecular characteristics. Identifying certain molecular biomarkers involved in CRC development can help predict the disease prognosis, response to therapy, and disease outcome.[9] For the selection of treatment strategies for colorectal cancer, prognosis prediction is crucial. Various factors, including tumor size, histological type, tumor grade and stage, as well as the presence of lymphovascular invasion and nodal involvement, are acknowledged as prognostic factors influencing the outcome.These parameters have a limited ability to predict tumor progression or response to therapy or patient survival. So, different molecular and genetic markers are also important ininfluencing the prognosis of colorectal cancer.[10]

CDX2 is among the numerous genetic changes implicated in the onset of sporadic colorectal carcinoma. CDX2, belonging to the caudal-related family of the CDX homeobox genes, functions as a nuclear transcription factor. Its encoding occurs through a non-clustered hexapeptide gene situated on chromosome 13q.[12-13] CDX2 is specific to the intestinal epithelium. It performs a crucial function in embryonic development by contributing significantly to the axial mapping of the gut. By regulating intestinal epithelial cell proliferation, differentiation and apoptosis, it also aids in maintaining intestinal epithelium homeostasis in adults.[11,12] CDX2 has been known to have tumor suppressor properties in colorectal carcinoma. Tumor progression and spread of colorectal cancer are prevented by CDX2.[9,13] There is a suggestion that CDX2 controls the expression of numerous genes related to intestinal cell proliferation, adhesion, migration and tumorigenesis.[14]

As per several previous studies, the percentage of Colorectal Carcinomas (CRCs) exhibiting CDX2 positivity varies between 71% and 100%, while only 10% to 30% of cases have been documented to experience a loss of expression.[15,16] Colorectal cancer susceptibility is increased by low CDX2 expression, whereas increasing CDX2 expression limits proliferation and encourages differentiation of colorectal cancer cells.[17] A decrease or absence of expression is correlated with high-grade and advanced-stage tumors, resulting in an unfavorable prognosis. This association is particularly evident in specific subtypes of colorectal cancer, including mucinous adenocarcinoma and signet ring cell carcinoma. The absence of CDX2 expression signifies aggressive tumor behavior and serves as a predictor for unfavorable clinical outcomes in individuals diagnosed with colorectal carcinoma.[13,18]

For individuals at a heightened risk of relapse, CDX2 can serve as a predictive factor for the effectiveness of adjuvant therapy. Notably, adjuvant chemotherapy markedly enhances the 5-year Disease-Free Survival (DFS) for those with stage II with an average-risk (T3N0) colon cancer lacking CDX2 expression, underscoring its substantial predictive utility.[19]

Deletion of CDX2 increases APC-initiated colonic tumorigenesis, indicating that this transcription factor has a tumor-suppressive function. Mutation of the CDX2 gene is a rare event in colorectal carcinoma. Epigenetic modifications are believed to play a significant role in the reduced expression of CDX2. In primary colorectal cancer tumors, the loss of expression of the intestine-specific transcription factor CDX2 is commonly associated with promoter methylation and histone deacetylation. Several studies have demonstrated that the expression of CDX2 in colorectal cancer cells can be successfully reinstated through global demethylation and histone acetylation. This is achieved by utilizing a DNA Methyltransferase inhibitor (DNMTi) like Decitabine for demethylation and a Histone Deacetylase inhibitor (HDACi) such as Trichostatin A for histone acetylation.[20,21] The effectiveness of chemotherapy in treating colon cancer in humans has improved recently, although it is still not sufficient. Because colorectal cancer exhibits significant epigenetic dysregulation, some studies have suggested that combining traditional chemotherapy with epigenetic modifiers may be

beneficial. Cancer cells may be reprogrammed by epigenetic modifiers, making them susceptible to cytotoxic anti-cancer medications and reversing chemo-resistance.[22,23] So, CDX2 can also be a potential therapeutic target in CRC patients.

Despite the frequent use of CDX2 as an immunohistochemical marker inroutine diagnostic procedures, there is, to our knowledge, no prior study examining its expression profile in human colorectal tissue in our country. Conversely, there is an ongoing debate regarding the correlation between the expression of CDX2 and the prognosis, disease outcome and clinicopathological characteristics in patients with colorectal cancer.[24]

Due to these considerations, the objective of this study was to examine the patterns of CDX2 expression in colorectal adenocarcinoma and its association with crucial prognostic factors, including age, gender, size of the tumor, histological type, histopathological grade, stage, and lymphovascular invasion in a tertiary care hospital in our country.

Materials and methods

From March 2019 to February 2021, the cross-sectional study was conducted at Chittagong Medical College's Department of Pathology in Chattogram, Bangladesh. Prior to commencing this study, approval for the research protocol was obtained from the Institutional Review Board (IRB) and ethical clearance was granted by the ethical review committee.

Surgically resected specimens of colon and rectum embedded in 10% formalin were received from the Department of Surgery, Chittagong Medical College and Hospital, Chattogram. The gross features of all the received specimens were recorded. Representative tissue was taken and routine processing was done to prepare Hematoxylin and Eosin-stained slides. Using a consecutive sampling technique, a total of 57 cases diagnosed with colorectal carcinoma were examined histopathologically. Patients who had previously had chemotherapy or radiation therapy for colorectal carcinoma were excluded, as were those with metastatic colorectal cancer and primary tumor other than colorectal adenocarcinoma. Finally, 45 cases of colorectal adenocarcinoma were prepared for the immunohistochemical examination. After obtaining proper informed written consent from the patients, data on variables of interest were recorded by interview and using a structured questionnaire.

Grading of colorectal adenocarcinoma was done following the 2010 World Health Organization (WHO) Classification of Tumors of the Digestive System. The staging was done based on the Tumor-Node-Metastasis (TNM) classification of colorectal carcinoma by the American Joint Committee on Cancer (AJCC).[25,26,27]

Immunohistochemical Evaluation

Tumor tissue sections fixed in formalin and embedded in paraffin were sliced to a thickness of 3-5 µm. Subsequently, the sections were placed on slides coated with poly-L lysine and subjected to drying at 60°C for a duration of 30 minutes. After deparaffinization and rehydration, antigen retrieval on a hotplate was performed using 1 mmol/L of EDTA (pH 8) for a duration of 10 minutes. Following a 20-minute cooling period, a 10-minute incubation with avidin and a 10-minute incubation with biotin was done and tris (Hydroxymethyl) aminomethane (Tris) buffer was used in the interim. As the primary antibody, the FLEX Monoclonal Mouse Anti-Human CDX2 Clone DAK-CDX2 Ready-to-Use (Dako Autostainer/Autostainer Plus) was utilizedIt was left at room temperature for 30 minutes, followed by three washes of the slides with wash buffer, each lasting 5 minutes. DAKO REAL^{TM} EnVision^{TM} (HRP RABBIT/MOUSE)(ENV) was used as a secondaryantibody. Further incubation of the slides with streptavid in peroxidase was carried out at room temperature for a duration of 30 minutes. Sections were then incubated in DAKO 3-amino-9-ethyl carbazole followed by counter staining with Mayer’s hematoxylin and lastly, dehydration and mounting by DPX were done. Positive control was taken from a section of the vermiform appendix.

Positive staining was exclusively assigned to nuclear staining and the final scoring took into consideration the percentage of stained cells, along with the staining intensity. By multiplying the proportion score by the intensity score, the final staining score was determined. The low-expression group consisted of samples with staining scores ≤4 and the high-expression group consisted of samples with staining scores >4.[28] The scoring was done by using the 40x objective lens and counting at least 100 cells for immunoreactivity in 10 fields.[11]

Statistical analysis was performed using Statistical Package for the Social Sciences, version 25 (SPSS Inc., Chicago, IL). Qualitative variables were expressed in frequency and percentages. Continuous variables were expressed as frequency, percentage, mean ±SD, median, mode and range. An unpaired t-test was done to compare the continuous variables. The chi-square test and Fisher exact test were utilized to explore potential associations between CDX2 expression and qualitative variables. Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$) and 95% confidence interval level was set.

Results

In this study, there were a total of 45 colorectal adenocarcinomacases, consisting of 21 (46.7%) males and 24 (53.3%) females. The ratio of males to females was1:1.14. The patients' mean age (±SD) was 48.89±17.71 years, ranging from 13 to 80 years. The highest number of patients (10 cases, 22.2%) fell within the age range of 41 to 50 years. The demographic variables of the patients are shown in Table I.

Table I Patient distribution according to demographic variables (n=45)

variables	Frequency	Per cent
Age (Years)		
o ≤50	25	55.56
o >50	20	44.44
Mean ± SD (Min-Max)	48.89 ± 17.71 (13-80)	
Median	50	
Sex		
o Male	21	46.7
o Female	24	53.3

Only 11 (24.4%) patients were diagnosed having mucinous adenocarcinoma and the remaining 34 (75.6%) patients had conventional adenocarcinoma. The majority of the tumors were moderately differentiated (Grade II) (26 cases, 57.8%). Most of the tumors were in stages T2 and T3 (48.9% and 46.7% respectively). Positive lymph nodes were present in 20 (44.44%) cases. In 2 (4.4%) cases regional lymph node metastasis could not be assessed, as no specimen of lymph node was found with the supplied colectomy specimens. Distant metastasis (In the ovary) could be identified in only one patient. The prognostic variables of the patients are shown in Table II.

Table II Patient distribution according to prognostic variables (n=45)

variables	Frequency	Per cent
Tumor Size (cm)		
o ≤5 27	60.0	
o >5 18	40.0	
Histological type		
o Conventional adenocarcinoma	34	75.6
o Mucinousadenocarcinoma	11	24.4
Histopathological grade		
o Well-differentiated	11	24.4
o Moderately differentiated	26	57.8
o Poorly differentiated	8	17.8
Lymphovascular invasion		
o Present	27	60.0
o Absent	18	40.0
T staging		
o T1 1	2.2	
o T2 22	48.9	
o T3 21	46.7	
o T4 1	2.2	
N staging		
o Nx 2	4.4	
o N0 23	51.1	
o N1 13	28.9	
o N2 7	15.6	
M staging		
o Mx 44	97.8	
o M0 0	0.0	
o M1 1	2.2	

Table III illustrates the patient distribution based on CDX2 expression.

Table III Patient distribution according to CDX2 expression (n=45)

CDX2 Expression pattern	Frequency	Percent
Low expression	26	57.8
High expression	19	42.2
Total	45	100.0

Tables IV and V present the association between CDX2 expression and various demographic and prognostic parameters.

Table IV Association of CDX2 expression with different demographic variables (n=45)

Variables	CDX2 expression pattern		p-value*
	Low expression	High expression	
Age (Years)			
o ≤50	16 (64.0)	9 (36.0)	
o >50	10 (50.0)	10 (50.0)	0.252[a]
Mean ± SD	46.27 ± 19.10	52.47 ± 15.55	
Sex			
o Male	11 (52.4)	10 (47.6)	0.493[b]
o Female	15 (62.5)	9 (37.5)	

[a]Unpairedt-test was done to measure the level of significance.

[b]Chi-square test was done to measure the level of significance.

Statistically, a significant inverse association was found between tumor grade and CDX2 expression. Nosignificant association was found between the expression of CDX2 and other prognostic factors, Including the patients' age and gender, tumorsize, histological type of colorectal adenocarcinoma, tumor stage, nodal involvement, lymphovascular invasion, etc.

Table V Association of CDX2 expression with different prognostic variables (n=45)

Variables	CDX2 expression pattern		p-value*
	Low expression	High expression	
Size of tumor			
o ≤5 cm	15 (55.6)	12 (44.4)	
o >5 cm	11 (61.1)	7 (38.9)	0.580[a]
Mean ± SD	5.13 ± 1.57	4.87 ± 1.61	
Histological type			
o Conventional adenocarcinoma	18 (52.9)	16 (47.1)	0.309[c]
o Mucinous adenocarcinoma	8 (72.7)	3 (27.3)	
Grade			
o Well-differentiated	3 (27.3)	8 (72.7)	
o Moderately differentiated	16 (61.5)	10 (38.5)	0.021[b]
o Poorly differentiated	7 (87.5)	1 (12.5)	
T stage			
o T1	0 (.0)	1 (100.0)	
o T2	13 (59.1)	9 (40.9)	0.548[b]
o T3	12 (57.1)	9 (42.9)	
o T4	1 (100.0)	0 (.0)	
N stage			
o Nx	2 (100.0)	0 (0.0)	
o N0	12 (52.2)	11 (47.8)	0.688[b]
o N1	7 (53.8)	6 (46.2)	
o N2	5 (71.4)	2 (28.6)	
M stage			
o Mx	25 (56.8)	19 (43.2)	
o M0	0 (.0)	0 (.0)	0.999[c]
o M1	1 (100.0)	0 (0.0)	
Lymphovascular invasion			
o Present	18 (66.7)	9 (33.3)	0.139[b]
o Absent	8 (44.4)	10 (55.6)	

[a]Unpaired t-test was done to measure the level of significance.

[b]Chi-square test was done to measure the level of significance.

[c]Fisher's Exact test was done to measure the level of significance.

In comparison to well and moderately-differentiated tumors, patients with poorly differentiated tumors exhibited a significantly higher proportion of low CDX2 expression (Figure-1).

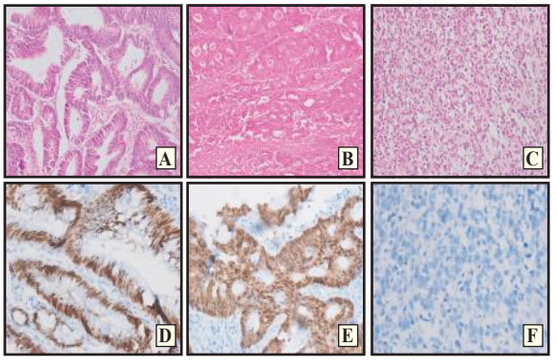

Figure 1: A) Well-differentiated adenocarcinoma, H & E (x20) **B)** Moderately differentiated adenocarcinoma, H & E (x20) **C)** Poorly differentiated adenocarcinoma, H & E (x20) **D)** High expression of CDX2 in well-differentiated adenocarcinoma (x40), **E)** High expression of CDX2 in moderately differentiated adenocarcinoma (x40) **F)** Loss of expression of CDX2 in poorly differentiated adenocarcinoma (x40).

The majority of the mucinous adenocarcinoma showed low expression of CDX2 protein, although the difference between the CDX2 expression in conventional adenocarcinoma and mucinous adenocarcinoma was not statistically significant. Expression of CDX2 in mucinous adenocarcinoma is shown in Figure 2.

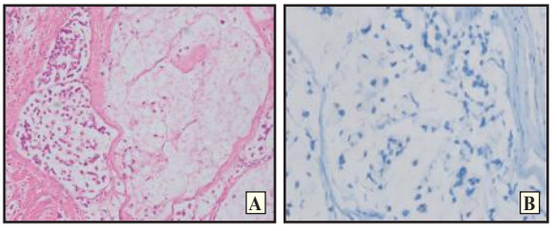

Figure 2 A) Photomicrograph showing mucinous adenocarcinoma, H & E (x20) **B)** Low expression of CDX2 in mucinous adenocarcinoma (x40) (CDX2 Score: 0)

Discussion

Many studies have been performed to discover biomarkers capable of predicting the response to therapy and disease outcomes. The identification of important molecular markers also leads to the evolution of appropriate treatment options. Intestinal epithelial cell growth and differentiation are both significantly influenced by the caudal-type homeobox transcription gene-2 (CDX2). The significance of the CDX2 protein in CRC development is still debated, with studies indicating both negative and positive tumorigenesis modulation.[2,9,29]

In this study, CDX2 protein expression in colorectal adenocarcinoma tissue was assessed using the IHC method, and its relationships to prognostic factors like patient age, gender, tumor size, histological type, lymphovascular invasion, histopathological grade, and tumor stage were examined.

In this investigation, CDX2 immunostaining was semi-quantitatively evaluated based on the intensity and the proportion of immune-reactive cells. It was observed that 26 (57.8%) cases showed low expression of CDX2 and 19 (42.2%) cases showed high expression. Similarly, Sakamoto et al. observed that 58% of the cases of CRCs showed low expression of CDX2.[30] On the contrary, in studies conducted by Bedeer et al. and Calik et al. the majority of the cases were positive or showed high expression of CDX2.[2,16] The percentage of loss of expression is higher in our study.

In this current study, there was no statistically significant difference in CDX2 expression between the two histological types, conventional and mucinous adenocarcinoma (p=0.309). Similarly, in the research conducted by Bedeer et al. and Rajarajan et al. no statistically significant difference was found in the CDX2 expression between the two types of colorectal adenocarcinoma.[2.31] In contrast, Baba et al. identified a statistically significant distinction between these two histological types.[32]

In this research, a statistically significant association was observed in the expression of CDX2 among the three grades of colorectal adenocarcinoma (p=0.021). This suggests an inverse association between CDX2 expression and tumor grade. High-grade tumorsshowed low expression of CDX2. Similarly, the negative association between the expression of CDX2 and tumor grade was found in different studies by Olsen et al. (p <.001) Dawson et al. (p<0.0001) and Asgari-Karchekani et al. (p=0.031).[14,24,33] In their respective studies, Bedeer et al. and Altintas et al. observed that there was no statistically significant difference between tumor grade and expression of CDX2 (p=0.405 and p=0.209, respectively).[2,17]

The association between CDX2 expression and the TNM stage of Colorectal adenocarcinoma did not reach statistical significance in this study (p>0.05). Likewise, Olsen et al. did not find a significant association between CDX2 expression and the stage of Colorectal Cancer (CRC) in their study.[34] In both the studies conducted by Calik et al. and Asgari-Karchekani et al. no significant association was reported between the T stage of the tumor and CDX2 expression. However, they did report a significant association between nodal involvement and CDX2 expression.[16,24] Contrarily, Bedeer et al. identified a significant negative association between the stage of colorectal carcinoma and CDX2 expression in their study.[2] Despite numerous research describing aninverse relationship between the stage of CRC and CDX2 expression, we were unable to verify this correlation, which may be due to a smaller sample size.[16,21,35] In this study, there was no statistically significant association detected between CDX2 expression and patient age, gender, tumor size or lymphovascular invasion (p>0.05). These variations in CDX2 expression in different studies may be related to genetic heterogeneity, quality of antibody, method of detection, different standards for CDX2 scoring and difference in sample size.[35]

Limitations

Due to resource and time limitations, the samples in this study were taken from a single institute which may not reflect the exact scenario of the whole country. Since no follow-up was conducted, there was no opportunity to examine the prognosis of these cases based on the results of the current study.

Conclusion

According to the findings of this study, we can infer that reduced or absent CDX2 protein expression in colorectal adenocarcinoma is linked to high-grade tumors. This indicates that CDX2 expression depletionleads to tumor progression. However, the expression of CDX2 did not exhibit a significant association with the stage of CRC, histological type or other prognostic parameters in this study. The CDX2 expression profile could be valuable forpredicting the clinical outcome of the patients and also guiding treatment plans and it can help to get a therapeutic target.

Recommendations

Further studies with a multicenter approach, the use of other biomarkers, more logistic supports, a larger sample size and proper follow-up are necessary.

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Authors contribution

SAR-Conception, acquisition of data, drafting & final approval.

ADJ-Data analysis, critical revision & final approval.

TF-Acquisition of data, drafting & final approval.

SNIJ-Interpretation of data, critical revision & final approval.

NNM-Data analysis, drafting & final approval.

MZR-Design, interpretation of data, critical revision & final approval.

Disclosure

All the authors declared no conflict of interest.

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