Original Article

Histopathological and Immunohistochemical Evaluation of CDX2 and Ki67 in Colorectal Lesions with their Expression Pattern in Different Histologic Variants, Grade, and Stage of Colorectal Carcinomas

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Abstract

Background: A variety of colorectal lesions are surgically treated encompassing both benign and malignant polyps and colorectal cancer (CRC). CRC is the third most common cause of death in developed countries. Over the last decade, CDX2 has been linked to CRC progression, with reduced expression of the protein associated with more advanced tumor stage, vessel invasion, and metastasis. **Aims and Objectives**: To analyze the histopathology and immunohistochemistry (IHC) of CDX2 and Ki67 with their expression pattern; in different lesions of colon and rectum with special reference to various grade/stage/histological variants of CRC and to find out whether they can be used as possible predictive marker. **Materials and Methods:** The study conducted was hospital based, both retrospective and perspective type comprising colorectal samples of total 367 cases (N) within a period of 2½ years. Surgical samples were collected, then grossed, processed, stained with routine hematoxylin and eosin stain in our department followed by IHC of CDX2 and Ki67 in only 60 randomly selected cases (n = 60). **Results:** Out of total 367 cases, 265 cases were prospective study and 102 cases were retrospective study (240 cases were colonic lesions, and 127 are rectal lesions). The samples included were both from colonoscopy biopsy (small) 319 cases and 48 colectomy specimen (large). Mean age of the study participants was 49.62 years with a standard deviation of 17.34 years and predominantly male, but the difference was not statistically significant ($P > 0.05$). Colon (238 cases, 64.9%) as a whole affected more than rectum and left sided tumors more than the right side. All 60 cases were found to be positive for CDX2 expression (i.e., 100%); majority (n = 38) being carcinoma cases possessing high score and was statistically significant ($P = 0.008$, using Chi-square test) indicating strong association, whereas Ki-67 showed an increased index from noneoplastic to neoplastic cases. **Conclusion:** These markers can be used as future predictive biomarkers which will precisely evaluate risk group, prognosis, and response to therapy hence can be used as target therapy reducing irrational treatment.

Keywords: Adenocarcinoma, adenomatous polyp, CDX2, colorectal carcinoma, transcription factor

Introduction

According to the World Health Organization, colorectal cancer (CRC) is the fourth common cancer, comprising 11% in the world and is steadily rising because of the western life style.[1,2] The molecular genesis of CRC involves four key mutations, including the oncogenes APC, KRAS, DDC, and the tumor-suppressor gene p53 that occurs only in 10% of tumors.[3,4] CDX2 is a homeobox protein responsible for the maintenance of the intestinal. Phenotype is over expressed in CRC.[5,6] Ki67 antigen is a proliferative marker and is used as predictive biomarker for many cancers that is yet to be proved in CRC.

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Materials and Methods

This was a hospital-based study undertaken over a period of 2½ years (June 2017–December 2019) in the department of pathology at a tertiary care teaching hospital. A total of 367 cases (N) of colorectal lesion samples were studied including both prospective (n = 265) and retrospective (n = 102) cases. Prospective cases included in the study were referred from the Department of General Surgery, Surgical oncology, Pediatric Surgery and gastrosurgery of our institute. Inclusion criteria set was, patient of either sex irrespective of age group presenting with gastrointestinal (GI) signs and symptoms with visible lower GI mucosal lesions by colonoscopy. Patients excluded were those refusing consent, uncooperative/with perforated viscus/recent myocardial infarction/inflammatory diseases of colon or rectum/Lymphoma/sarcoma/neuroendocrine tumors/known hereditary cancer syndromes. Both large colectomy samples and small four quadrant colonoscopic biopsy samples were collected in 10% buffered formalin for proper fixation after taking patient consent. Moreover, then processed, stained with hematoxylin and eosin (H and E) stain routinely. Retrospective cases were selected from the record having data similar to prospective ones and their paraffin embedded blocks were retrieved, sections made and stained with H and E. Eighth Edition, AJCC Staging Manual, Version: Colon Rectum 4.0.0.1, June 2017, College of American Pathologist (CAP) guideline was followed for histological reporting of grading and staging systems of CRC.[7] This was followed by immunohistochemistry (IHC). IHC is a commonly used staining method where selective antibodies are utilized to quantify and assess distribution of molecular markers in tumor tissue. Hence, only 60 randomly selected cases were submitted for IHC (PathnSitu) of CDX2 and Ki67 because of the financial constraint. IHC done on the paraffin-embedded tissue block by standard immunohistochemical method using horseradish peroxidase-linked antibody. Primary anti-CDX2 (EP25) and Ki-67 antibody were rabbit and mouse monoclonal antibody respectively supplied from PathnSitu and the dilution factor used was 1 in 100. CDX2, a nuclear transcription factor, is involved in the processes of differentiation, intestinal cell proliferation, adhesion and apoptosis, having organ-specific expression. Ki67 antigen (nuclear nonhistone protein), an important marker for cell proliferation, is expressed in all phases of cell cycle except for G0 phase. The cell growth fraction is directly correlated with tumor aggression. Positive tissue control taken for ki67: Reactive Lymph node, for CDX2: Normal colon. For Negative control, primary antibody was omitted in the procedure. While evaluating immunostaining, nuclear staining of tumor cells was taken into account for both CDX2, and Ki67. The Ki67 was calculated in the tumor hot spot area and percentage was given from 1000 cell count. Tumor cells that showed <5% for nuclear staining for Ki67, and CDX2, were considered score 0− (<5%), those that indicated 5%–25% staining were considered as Score 1+, those that showed 26%–75% staining were considered as Score 2++, and those that indicated more than 75% staining were considered as Score 3+++.[8] The intensity of staining was also scored on a categorical scale from 0 to 3: 0 indicated absent; 1+ very weak, dubious staining; 2+ definite, mild, or moderate staining; 3+ definite, strong staining.[9]

Statistical analysis

Qualitative data were expressed as proportion with percentages and quantitative data as mean with standard deviation. Statistical analysis of proportion was done on software version 20.0 licensed to the Institute.

Ethical approval was sought and obtained from the institutional ethical committee.

Results

A total of 367 cases of colorectal lesions were studied in our department, out of which 265 cases were prospective and 102 cases were retrospective ones. Of these 319 cases were small biopsy and 48 cases were large (colectomy) biopsy specimen. Out of total 48 cases of colectomy specimens; 13 cases were T2 N0 M0 (i.e., tumor invades muscularis propria), 25 cases were T3N0M0 (i.e., tumor invades muscularis propria into pericolorectal tissues) and 10 cases were T4a N1 M0 (i.e., tumor penetrates to the surface of visceral peritoneum with metastasis in 1–3 regional lymph nodes). From the total of 367 cases which were included in the study (n = 367), 60 randomly selected cases (n = 60) were subjected to IHC analysis for CDX2 and Ki67 and scoring was done as described with their expression patterns noted in different histologic types, grade and stage of carcinoma cases. No IHC was done on metastatic lymph node/organ but proved by radiography.

Mean age of the study participants was 49.62 years with a standard deviation of 17.34 years. It was seen that majority of the participants were from the age group of 40–60 years (165 cases, 45%) those reported with growth in the colon and rectum. Males were reported more among all the study participants (233 cases, 60.8%) in all age groups as compared to the females (144 cases, 39.2%). However, the difference was not statistically significant ($P > 0.05$, using Chi-square test) [Figure 1]. Three major complaints were found in these cases – pain abdomen, bleeding per rectum or constipation, or a combination of these. Bleeding per rectum was the most common presenting symptom both in male and female, with a total of 172 cases out of 367 (46.9%) presenting with this symptom. It was seen that left sided lesions were more compared to the right sided ones [Table 1] and rectum was the most common site of affection for both males and females (approximately 17% each) as compared to individual parts of colorectal region. But considering Rectum and the whole colon; colon was affected more, i.e., (238 cases, 64.9%). Again among colonic parts, descending colon was the most common site (23.2%) followed by ascending colon (19.3%), transverse colon (15.8%), and least common site was recto-sigmoid junction (1.4%). In the ascending and descending colon, males outnumbered females. Transverse

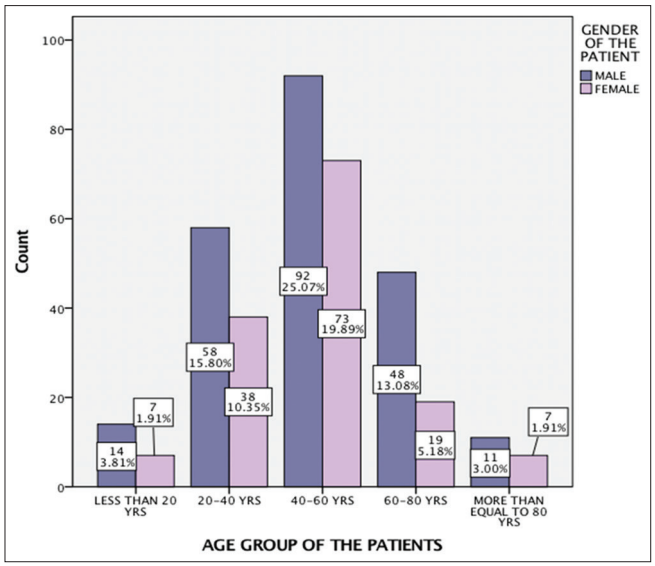

Figure 1: Age and sex distribution of the study participants (n = 367)

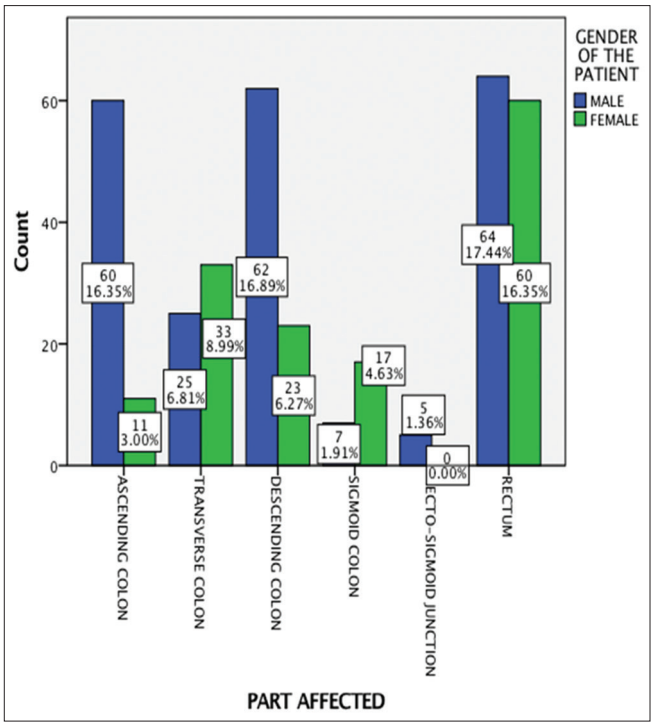

Figure 2: Site distribution by gender of the patient (n = 367)

colon, sigmoid colon, and rectum were found to have more association with the female. This association was statistically significant (P = 0.000, Chi-square value was 47.296) [Figure 2].

In histopathology, the nonneoplastic lesions comprised of total 38 cases, out of which juvenile polyp followed by inflammatory polyps were in order of 15 and 13 cases, respectively. It was seen that left-sided distribution was more among cases of nonneoplastic polyp with more than 50% of the cases being limited to transverse and descending colon (20 cases, 52.6%).

Rectum was the single most important site that was affected among all cases of adenoma (neoplastic polyp) resulting in more predominant left-sided cases like nonneoplastic polyps. Within total neoplastic lesion; the adenoma (neoplastic polyp) evident was 218 cases with different grades of dysplasia. The tubulovillous adenoma with low-grade dysplasia was the highest in number with a total of 58 cases (26.6%). Moreover, majority of neoplastic polyps were found in the rectum followed by descending colon, whereas sigmoid colon was the least common site for neoplastic polyp (n = 16,7.34%) [Table 2].

Among all the carcinoma, adenocarcinoma number was the highest and adenosquamous carcinoma was only 6 cases (5.4%) and was lowest in number. The ascending and descending colon and rectum were the usual sites that are affected by carcinoma, and these cases were almost equal in numbers (25-28% each). Hence, carcinomas found to be more common in the left side.

IHC of both CDX2 and Ki67 was performed in total 60 cases from both colectomy and colonoscopic biopsy sections which included both neoplastic (carcinoma 38 cases, adenoma 20 cases) and nonneoplastic cases (inflammatory polyp 2 cases) and was interpreted as per the scoring system elaborated in material and method [Table 3 and Figure 3]. The expression pattern of both CDX2 and Ki67 was also correlated with the different histologic variant, grade and stage of the tumor as well as in polyps [Figure 4]. While seeing the pattern of expression

Table 1: Age and gender distribution of the cases (n=367) by the tumor sides

	Right sided cancer, n (%)		Left sided cancer, n (%)	
	Male	Female	Male	Female
<20 years	5 (5.9)	2 (4.5)	9 (6.5)	5 (5.0)
20–40 years	27 (31.8)	8 (18.2)	31 (22.5)	30 (30.0)
40–60 years	45 (52.9)	28 (63.6)	47 (34.1)	45 (45.0)
60–80 years	8 (9.4)	2 (4.5)	40 (29.0)	17 (17.0)
≥80 years	0	4 (9.1)	11 (8.0)	3 (3.0)
Total	85 (100.0)	44 (100.0)	138 (100.0)	100 (100.0)

of both CDX2 and Ki67 in the right side versus left side and in different age/sex group, no significant difference of pattern was discovered in our study. While considering nonneoplastic versus neoplastic polyp, CDX2 score 2++ was found in the majority of neoplastic polyp (adenoma) cases, whereas in all the nonneopastic polyps (inflammatory polyp, n = 2) score 2++ were expressed. A strong intensity and high score (2++, 3+++) of CDX2 immunoexpression was seen in colorectal carcinoma patient in stage pT2 and pT3 stages without lymphovasular invasion/lymph node/distant metastasis, and the positive index was decreased with score (0−, 1+) in patients with pT4 stage with or without lymphovascular invasion and/or distant metastasis. Among different subtype (histological variant)/grade of carcinoma, CDX2 expression showed different scores [Table 3]. Maximum score observed was 3+++ in 18 out of IHC proven 38 carcinomas; among which Signet ring-cell carcinoma was 4 of 6, adenocarcinoma was 12 of 20, and mucinous carcinoma was 2 out of 6 cases. All the cases in the current study were

found to be positive for CDX2 expression (60 cases, 100%). CDX2 is identified with elevated mean expression levels in adenomatous polyp and carcinoma compare to normal tissue though the expression is reduced in PT4 tumor. Only carcinoma cases were found to be highest in number having high score and moderate to strong intensity of expression of this marker, which was statistically significant ($P = 0.008$, using Chi-square test) indicating a strong association.

Though Ki67 staining had no correlation with age, gender or tumor location; high Ki67 index (2++ and 3+++) was found in higher grade and stage of tumor. Ki67 was found usually in the lower third of normal colonic crypts. Patients with colorectal polyps (non-neoplastic) revealed increased positivity index (2++) and distribution was with medium intensity while neoplastic polyps showed uniformly high intensity and high score (2++, 3+++) like that of carcinomas. Considering different histological subtypes of carcinoma; Ki67 showed variable expression with highest percentage in carcinoma cases showing high intensity and score without any case of negative expression [Figure 3]. In our study majority carcinoma cases are showed score 2++ and 3+++ i.e., $n = 15$ (39.47%) each. Signet ring-cell carcinoma: highest cases had score of 3+++ (3 cases out of 6 cases), Adenocarcinoma: maximum cases had score of 3+++ (10 out of 20 cases) whereas Mucinous carcinoma: showed highest score of 2++ immunoexpression of Ki67 antigen (3 out of 6 cases). Like that of CDX2 staining, all 60 cases were found to have positive Ki-67 expression. None of our case had negative expression.

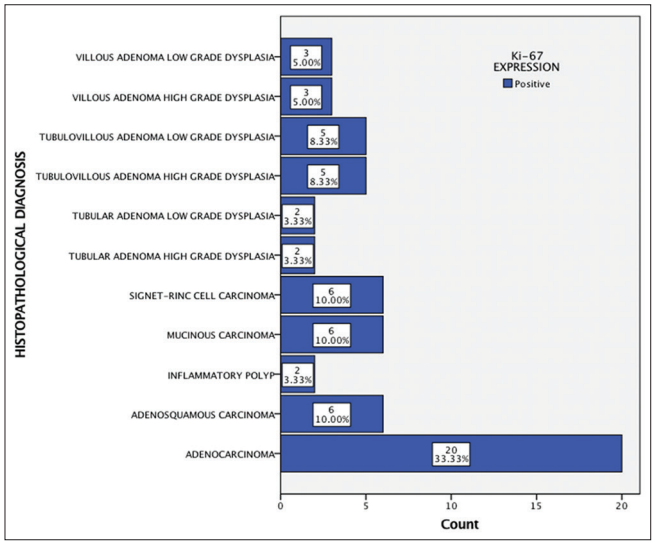

Figure 3: Ki67 expression among cases subjected for immunohistochemistry ($n = 60$)

Discussion

In India, the annual incidence rates (AARs) for colon cancer and rectal cancer in men are 4.4 and 4.1/1 lakh respectively accounting for eighth and ninth rank. In women, the AAR for colon cancer is 3.9/1 lakh with the rank ninth whereas, rectal cancer does not figure in the top 10 cancers.[10] Risk factors for CRC are broadly divided into genetic and environmental/life style factors including sedentary habit, consumption of red meat, low fat diet, alcohol, tobacco. Carcinogenesis of CRC is heterogeneous resulting from different pathways. Majority of the CRCs are sporadic, and tumorigenesis occurs in a

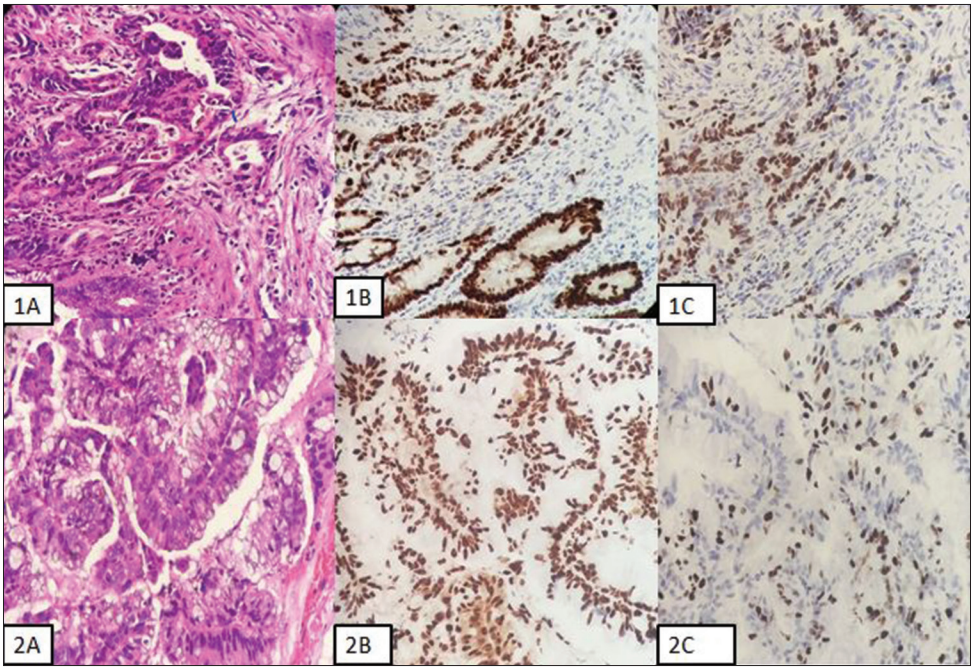

Figure 4: (1A) Photomicrograph showing a case of Intermediate grade Adenocrcinoma with moderate degree of nuclear pleomorphism (H and E, ×100), (1B) CDX2 and (1C) Ki67. (2A) Photomicrograph showing a case of Low grade Adenocrcinoma with papillary pattern and mild nuclear pleomorphism (H and E, ×100), (2B) CDX2 and (2C) Ki67

Table 2: Type of lesion as per histopathological diagnosis of the cases (*n*=367)

	Type of cancer	Frequency (%)
Nonneoplastic	Hamartomatous polyp	4 (1.1)
	Hyperplastic polyp	6 (1.6)
	Inflammatory polyp	13 (3.5)
	Juvenile polyp	15 (4.1)
	Sub-total	38 (10.35)
Adenoma (neoplastic polyp)	Tubular adenoma with high-grade dysplasia	13 (3.5)
	Tubular adenoma with low-grade dysplasia	21 (5.7)
	Tubulovillous adenoma with high-grade dysplasia	7 (1.9)
	Tubulovillous adenoma with low-grade dysplasia	29 (7.9)
	Tubulovillous high with grade dysplasia	28 (7.6)
	Tubulovillous with low grade dysplasia	58 (15.8)
	Villous adenoma with high-grade dysplasia	35 (9.5)
	Villous adenoma with low-grade dysplasia	27 (7.4)
	Sub-total	218 (59.40)
Carcinoma	Adenocarcinoma	63 (17.2)
	Adenosquamous carcinoma	6 (1.6)
	Mucinous carcinoma	33 (9.0)
	Signet-ring cell carcinoma	9 (2.5)
	Sub-total	111 (30.24)
	Total	367 (100.0)

Table 3: CDX2 and Ki67 expression among cases subjected for immunohistochemistry (*n*=60)

Histopathology	*n*	Marker	Positivity index			
			0-	1+	2+	3+
Mucinous adenocaricnoma	6	CDX2	0	1	3	2
		Ki67	0	1	3	2
Adenocarcinoma	20	CDX2	0	2	6	12
		Ki67	0	4	6	10
Adenosquamous carcinoma	6	CDX2	1	4	1	0
		Ki67	3	2	1	0
Signet ring cell carcinoma	6	CDX2	0	0	2	4
		Ki67	0	0	3	3
Tubulovillous adenoma with dysplasia						
High grade	10	CDX2	0	1	5	4
		Ki67	0	0	4	6
Low grade	10	CDX2	2	4	4	0
		Ki67	0	4	4	2
Inflammatory polyp	2	CDX2	0	0	2	0
		Ki67	0	0	2	0

conventional pathway in the stepwise manner called adenoma carcinoma sequence where adenoma is the early lesion. This process is associated with mutations in genes such as APC, p53, KRAS, SMAD2, SMAD4 or MMR; particularly KRAS was reported in up to 50% of villous adenoma and up to 18% of tubular adenoma.[11] Adenoma development and recurrence are complex genetic processes driven by multiple gene copy number changes and CDX2 identified as a potential marker of recurrence.[12] In human, CDX2 (caudal type homeobox 2), a the transcription factor that is expressed in the nucleus of intestinal epithelial cells and the gene encoding it functions as a tumor suppressor. Normally CdX2 expressed throughout the small and large intestines, beginning from the duodenum, peaks in the proximal colon and decreases caudally with even expression along the crypt-cuff axis of the colonic crypt. It regulates the development, differentiation, and maintenance of colonic epithelium. Hence, CDX2 was used as a diagnostic marker of colonic origin for many years and is found to be positive in majority of colon and appendix carcinomas. In recent era, several studies have demonstrated that the lack of CDX2 expression in CRC and associated with an aggressive behavior, poor prognosis, high tumor grade, high tumor stage, BRAF mutation, MSI high phenotype, owing to the tumor-suppressor action.[13,14]

Hence, we carried out this study to observe the heterogeneity in the expression pattern of CDX2 and Ki67 in different types of colorectal lesions, also among the different histological subtypes, grades and stages of CRC which were then correlated with similar studies. The current study included both neoplastic as well as nonneoplastic polyps and CRC of total 367 Cases whose demographic data like age, sex, locations, and presenting symptoms were also compared.

The present study showed highest affection of colorectal lesions between 40 and 60 years of age with mean age = 49.62 years and male preponderance (male:female = 1.5:1) that was well correlated with the findings of Sen *et al*., Nayak *et al*., and Peedikayil *et al*.[9,15,16] In Nabi study, 88% of colorectal carcinoma were between 41 and 69 years.[17] Mesina *et al*. studied 82 cases of colorectal adenocarcinoma between 31 and 93 years with predominance of male. Location of both malignant polyps and carcinoma in their study was more in left side like our study.[8] Whereas Peedikayil *et al*. found 74% of the tumor located distal to the splenic flexure.[16] Nayak *et al*. reported sigmoid colon to be the most common site followed by the cecum and rectum.[15]

A study by Mesina *et al*. showed CDX2 expression in majority (84.70%) cases with variable intensity among which (45.12%) of tumors expressed high positivity index.[8] CDX2 positivity was also reported in >70% colorectal mucinous carcinomas by Kaimaktchiev *et al*. tissue microarrays study. Strong nuclear staining for CDX2 was observed in 86% of colonic adenocarcinomas of their study without significant difference in the staining of well (Grade I) and moderately differentiated (Grade II) tumor.[18] In another study by Bayrak *et al*., 97% CDX2 positive colorectal carcinomas was reported, of which 60% had high positivity index. Their study suggested no significant association between CDX2 expression and tumor grade/clinical stage of tumor in CRC because they found 98%

of low-grade tumors and 91% of high-grade tumors were positive for CDX2.[19] Other studies showed down regulation in CDX2 immunoexpression in higher stage, similar to our study.[8,20] Witek *et al*. found CDX2 over expression in most colorectal tumors compared to normal mucosa, similar feature was also observed by the current study and Sen *et al*.[9,21] Saad *et al*. suggested that CDX2 should not be used as the sole diagnostic marker for the primary GI tract adenocarcinoma and be used with other immunohistochemical markers.[22] Whereas Werling *et al*. reported that the high levels of CDX2 expression were found almost exclusively in adenocarcinomas of the colorectum, but the intermediate levels were found in adenocarcinomas arising elsewhere in the GI tract.[23]

While depicting Ki-67 LI, Georgescu *et al*. found that the Ki-67 LI increased with the histological grade of adenocarcinomas that had an agreement with our study where highest index of Ki67 was seen in Signet ring-cell carcinoma (high grade) and pT4 stage of tumor.[24] Nabi *et al*. had concluded that the proliferative activity measured by Ki-67 is related to histological type, grade and stage; Gurzu *et al*. found a significant increase of Ki67 median expression with poorer grade, age of patients and lymph node involvement.[17,25] Mesina *et al*. had disagreed to the fact that Ki67 immunoexpression in patients with CRC had no correlation with tumor proliferative capacity and tumor invasion.[8] Petrisor *et al*. found a wide range of Ki-67 LI in colonic carcinomas ranging from 5% to 95% and observed no relationship between Ki-67 LI of colonic adenocarcinomas and histopathology grade. However, existence of significant differences was advocated in their study, by comparing mean Ki-67 values with respect to different pathologic subtypes of rectal adenocarcinoma.[26]

From the above studies, it had been observed that CDX2 usually overexpressed in any colorectal lesion than normal colonic epithelium, both in form of score and intensity. However, comparing CRCs versus nonneoplastic polyps and adenomatous polyps; CRCs show high level expression (high score). While considering different histological variants like mucinous/nonmucinous/signet ring-cell type carcinomas or different grades like G1 (well differentiated)/G2 (moderately differentiated)/G3 `erentiated)/G4 (Undifferentiated) or even different stages (PT1-PT4), there were varied opinion. Considering Ki67 which is already in use as an established prognostic marker in many common cancers such as breast/ovarian/bladder cancers was also seen to have similar expression pattern in colorectal lesions found in the current study and several other studies. Steady rise of Ki67 was observed as the tumor progresses from low-to-high grade and stage. The limitation of our study was very less number of cases (n = 60) upon which we performed IHC, metastatic site IHC not done, also the survival analysis could not be analyzed which could have given a better impact on the study. As there is a significant rising trend of sporadic CRC in younger generation, emphasis is given for an early diagnosis by screening and to halt the process of spread at a lower stage for achievement of better survival.

Hence, CDX2 can act as a diagnostic marker though not a specific one and Ki67 can be utilized as a prognostic marker for CRC.

Conclusion

We concluded that CDX2 is a good indicator for any colorectal lesion, especially in all variants of adenocarcinoma but grade and stage are not directly correlated as that of Ki67. Hopefully these potential protein markers can be utilized in future to aid in assessing risk group and development of new consensus guidelines for individual prognosis and precession treatment in the clinical setup of India.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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