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Original Research Article

Ki67 Protein expression and correlation with the histological grade and pTNM stage of colorectal carcinoma

Neepamanjari Barman[1], Indroneel Bhattacharya[1,*], Ranu Sarkar[1]

[1] *Dept. of Pathology, Nil Ratan Sarkar Medical College and Hospital, Kolkata, West Bengal, India*

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ABSTRACT

Background: Colorectal cancer, is third most frequently encountered malignancy worldwide. Histological grade, stage, and proliferative index act as vital prognostic markers, playing a decisive role in patient care and prognosis. While histopathological examination can determine grade and stage, Ki67 protein expression by immunohistochemistry represents the proliferative capacity of the malignant cell population.
Aim: This Study was conducted to immunohistochemically analyze expression of Ki67 protein and its change with respect to different grades and stage of colorectal adenocarcinoma.
Materials and Methods: A total of 66 histologically diagnosed cases of colorectal carcinoma underwent histopathological examination followed by immunohistochemistry for Ki67 protein.
Result: A statistically significant (p=0.04) correlation was obtained between Ki67 labelling index and histopathological grade, with higher values of Ki67 labelling index in poorly differentiated carcinomas (43.2±1.7). The Ki67 labelling index value was lowest in stage IV disease (11.4±2.4) with metastatic burden, with higher values in lower stage diseases, however this correlation was not found to be significant statistically (p=0.07).
Conclusion: The rate of cell division and proliferation measured by Ki-67 antibody is related to histological grade of the malignancy, demonstrating higher mitotic activity with loss of differentiation and anaplasia. Stage IV disease have lower mitotic activity, thus may be less amenable to cytostatic chemotherapy drugs, and require multimodality treatment with addition of radiotherapy and other drug regimes.

1. Introduction

In view of the various malignancies of the Gastro intestinal tract, adenocarcinoma of colon ranks first both in incidence and as a major cause of mortality and morbidity worldwide. Globally colorectal adenocarcinoma accounts for approximately 1.2 million new cases, and 600,000 deaths each year.[1] In India, the incidence of colorectal cancer is 4.3 and 3.4 per 100,000 male and female respectively.[2]

Colorectal cancer can develop both sporadically (85%) or as a component of hereditary cancer syndromes (15%). Accumulation of molecular alteration, including mutation in Kirsten-ras (K-ras), p53 and adenomatous polyposis coli (APC), contribute to colorectal carcinogenesis.[3]

One of the most efficient prognostic tools for colorectal malignancies is the TNM staging (AJCC; 8^{th} edition) which not only groups patients into ordered risk strata's, but also plays a decisive role in determining stage specific, efficient treatment strategies.[4] The histological appearance and grade of colorectal carcinoma may vary considerably and is essentially an estimate of the pace of growth and the aggressiveness of tumour cells.[5] Both the estimation of histopathological grade and the TNM stage of colorectal cancers enable grouping of cases into favourable and

* *Corresponding author*.
E-mail address: indroneelb87@Gmail.com (I. Bhattacharya).

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unfavourable outcomes.

Proliferation is a key feature of malignant neoplasms. Ki-67 is a proliferation associated nuclear antigen expressed in all cycling cells except resting cells in the G0 phase, and in particular it reflects cells in the S/G2+M phase. The Ki-67 gene is present on the long arm of the human chromosome 10 (10q25).[6] Ki67 protein has wide-ranging effects on chromatin organisation, it spatially organises heterochromatin, thereby controlling gene expression.[7] The serine threonine kinase enzymes activate Ki67 protein. Inactivation of Ki67 by dephosphorylation leads to cessation of cell division.[3]

Ki67 expression is measured on the basis of percentage of tumour cells stained by the antibody, the nuclear staining is considered the most important criteria in this regard. MIB-1, which is a monoclonal antibody recognises nuclear antigen in the cells of formalin fixed embedded tissue sections.[8]

It has been demonstrated in previous studies that Ki67 expression and proliferation score was higher in Duke's stage B than in Dukes' stage C.[9] Thus, further corroborating as well as providing evidence to the fact that Ki67 expression and proliferative index is an important prognostic marker in colorectal malignancies. Colorectal adenocarcinomas are classified into three grades based on the arrangement of cells and tubular (acinar) formation. Tumours with higher grade have worse prognosis, as well as high Ki67 proliferative index.[10]

2. Aims and Objectives

The aim of this study is to (1) Assess the proliferation index (PI) in formalin-fixed, paraffin- embedded tissue sections of histopathologically diagnosed cases of colorectal adenocarcinomas and, (2) Investigate the relationship between the proliferative activity of colorectal carcinoma, with the help of IHC examination using Ki67, with the histological grade and the pathological stage (pTNM) of the tumour.

2.1. Inclusion criteria

Specimens obtained by colorectal endoscopic biopsies and surgical procedures including hemicolectomies, anterior resection and abdominoperineal resection for colorectal growth were included in the study.

2.2. Exclusion criteria

Specimens of patients with a history of chemotherapy, recurrence, familial syndromes and specimens with a histopathological diagnosis of non-epithelial colorectal neoplasms were excluded from the study.

3. Materials and Methods

An institutional based cross-sectional observational study was conducted in a tertiary care center, from February 2019 to July March 2020 in the Department of Pathology in collaboration with Department of General Surgery. With the exclusion of all cases meeting the inclusion criteria within the study period were included. A total of 66 cases could be enrolled in the study. Data collected were cautiously evaluated for variables like Ki67 expression and proliferation index by MIB-1 labelling using IHC technique along with corresponding clinicopathological profile including dietary history, macroscopic findings, histological grade, histological type, stage and other relevant parameters. Reporting was done by Pathology experts of the same institute.

3.1. Histopathology

After histological confirmation of the diagnosis following parameters like histological type, degree of differentiation, tumor grade, lymphovascular and perineurial invasion, nodal involvement, status of margins, and staging [American Joint Committee on Cancer 2018] were analyzed.

3.2. Immunohistochemistry

Immunohistochemical examination for Ki67 protein was performed. Sections (4 μ) from formalin fixed paraffin embedded tissue blocks were stained by standard immunohistochemical methods using horseradish peroxidase-linked antibody. Primary anti-Ki67 rabbit monoclonal antibody supplied by CELL MARQUE with a dilution factor is 1 in 100 was used for this purpose.

Ki67 labelling index: Result of Ki-67, immunostaining index was interpreted as labelling index (Ki-67 LI) = Number of nuclei showing positive staining (brown colour)/total number of nuclei × 100%. A positive nuclear staining was observed in the epithelial cells of normal colonic mucosa and in the lymphoid cells which served as internal positive control.

3.3. Statistical analysis

Data was entered in MS excel. For descriptive purposes mean ± SD, range and percentage were used. Statistical analysis was performed on the Windows based SPSS software, version 19.0 (Statistical Package for the Social Sciences Inc., Chicago, IL, USA). Student t test (two tailed, independent) and Chi-square test have been used to find the significance of study parameters on categorical scale between two or more groups. Significance level was considered at p value < 0.05.

Ethical permit for this scientific endeavor duly obtained from the institutional ethical committee before

commencement of the study on 18.12.2018, vide letter memo No. 184/12.

4. Results

The average age of the participants was estimated to be 61.62±11.11 (mean ±SD) years with a range of 45 (77-32) years. About 29% of the participants were female. Majority (57.14%) had no addiction for tobacco. About one third (30.16%) were obese and 17.63% reportedly had inclination towards alcohol consumption. Only, 14.29% had at least one family member affected by cancer. In 40% (27) of the colectomy specimens, the tumor was located in the rectum, followed by 35% (24) of the cases in cecum and the ascending colon.

Left sided hemicolectomy was the most common specimen received for histopathological examination (65.55%) followed by abdominoperineal and anterior resection (15.90%). Nearly two third (66%) of the cases had a polypoid exophytic appearance. The size of the malignant neoplasms ranged from 2 to 10 cm with a mean of 3.50 ± 1.50 cm. Macroscopic tumor perforation was seen only in 6% of the specimens.

During histopathological grading, 14(21.21%) cases were found to be well differentiated adenocarcinomas (Figure 1a), 46(70%) were graded as moderately differentiated adenocarcinoma (Figure 1b), and the remaining 6(9%) were graded as poorly differentiated adenocarcinoma of large intestine (Figure 1c). On pathological staging (pTNM staging), one third were either in stage I or stage II and the rest in stage III or stage IV. Microscopic evidence of lymphovascular and perineurial invasion was found in 44 and 22 cases respectively.

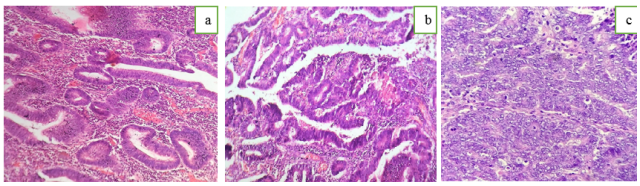

Fig. 1: a: Photomicrograph of histopathological section of well differentiated adenocarcinoma of rectum (H&E, X10); **b:** Photomicrograph of histopathological section of moderately differentiated adenocarcinoma cecum (H&E method, X10); **c:** Photomicrograph of histopathological section of poorly differentiated adenocarcinoma cecum (H&E method, X10).

4.1. Ki67 proliferating index

The highest Ki67 labelling index was noted in the sections with a histopathological diagnosis of poorly differentiated adenocarcinoma, (43.2±1.7), (Figure 2a), followed by those histologically diagnosed as moderately differentiated carcinomas, (32.3±3.69), (Figure 2b), and the lowest labelling index value was obtained in sections diagnosed as well differentiated carcinoma, (25.4±2.23), (Figure 2c), of colon and rectum. The gradual increase in the Ki67 labelling index values with higher grades of adenocarcinoma was found to be statistically significant, at p=0.04.

Table 1: Ki67 proliferating index in association with the grade and TNM stage of colorectal cancer.

Histopathological grade of colorectal cancer (n)	**Mean Ki67 proliferating index (Ki67PI ± SD)**
Well differentiated (16)	25.4±2.23
Moderately differentiated (46)	32.3±3.69
Poorly differentiated (4)	43.2±1.7
Student t test, p = 0.04	
AJCC/TNM stage of colorectal cancer (n)	**Mean Ki67 proliferating index (Ki67PI ± SD)**
Stage 1	25.1±5.8
Stage II	26.4±5.4
Stage III	16.1±5.1
Stage IV	11.4±2.4
Student t test, p = 0.07	

On pathological staging, the lowest value of Ki67 labelling index was noted in stage IV disease, (11.4±2.4), followed by stage III, (16.1±5.1) disease, the Ki67 labelling index was higher in stage II, (26.4±5.4), than in stage I (25.1±5.8), disease. Stage IV disease with metastatic burden was least mitotically active, with the lowest value of Ki67 labelling index, however this observation was not found to be statistically significant with a p=0.07 (p>0.05).

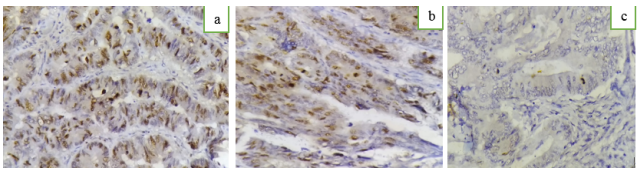

Fig. 2: a: Photomicrograph of poorly differentiated adenocarcinoma of colon stained with Ki-67 immunostain, with highest Ki67 Labelling index; **b:** Photomicrograph of moderately differentiated adenocarcinoma of colon stained with Ki-67 immunostain; **c:** Photomicrograph of well differentiated adenocarcinoma of colon stained with Ki-67 immunostain with low Ki67 labelling index.

5. Discussion

Ki67 labelling index has frequently been used as an indicator in proliferating cells. Several diagnostic applications for Ki67 labelling index been explained, the value of which is significantly higher in malignant neoplasms. Ki67 labelling index gradually increases with a decrease in tissue differentiation and higher rate of anaplasia in malignant neoplasms. MIB-1 is a monoclonal antibody that recognises a fixation resistant epitope of Ki67 antigen and it is used to estimate the proliferative fraction of neoplasia. Immunostaining by Ki67/MIB-1 has been

introduced to determine the rate of proliferation during histopathological examination.

Keeping this in mind the aim of this study was to evaluate the proliferation index (PI) in formalin fixed, paraffin embedded tissue sections of colorectal carcinomas using monoclonal MIB-1 (Ki67) antibody and to assess the relationship between proliferative index (PI) and vital prognostic markers of colorectal carcinoma including histological grade, and pTNM stage.

In the present study, the mean age of the study population was found to be 61.62±11.11 (mean ± SD) years with a range of 45 (77-32) years. This is consistent with the figures mentioned by Weiser et al[4] and Pretlow et al.[11] Distal colon followed by cecum were the commonly involved sites in the present study which is in agreement with Ishida et al.[12] In our study, moderately differentiated adenocarcinomas were the commonest type accounting for 70% of cases. Well differentiated and poorly differentiated cases formed 21% and 9% respectively, which is in congruence to Peedikayil et al.[13]

After histopathological diagnosis of the cases as colorectal adenocarcinoma, immunostaining with Ki67 monoclonal antibody was performed, and Ki67 labelling index was calculated. Poorly differentiated carcinomas had the highest Ki67 labelling index, (43.2±1.7) followed by moderately (32.3±3.69) and well (25.4±2.23) differentiated adenocarcinomas, with a p value of 0.04. A statistically significant relationship between Ki67 labelling index and histopathological grade of colon carcinoma was obtained, higher mitotic activity with loss of differentiation and anaplasia was noted. This is in concordance with Saleh et al,[14] which performed a study on 52 cases of colorectal carcinomas. In this study well and moderately differentiated carcinomas were grouped together (n=42) while poorly differentiated cases (n=10) were grouped separately. Ki-67 immunostaining was performed and concluded that Ki-67 Proliferative Index (PI) appeared to increase with decreasing degree of differentiation of carcinoma: 35.7±9.5 in well /moderately differentiated vs. 48.3±11.7 in poorly differentiated carcinomas (p=0.0007).

However, a study conducted by Ishida et al[15] in Japan reported 207 invasive colorectal carcinoma without metastases, and 82 invasive colorectal carcinomas with metastases and concluded that among 207 invasive carcinomas, the positive rate of Ki-67 antibody in poorly differentiated adenocarcinoma (46.6±24.5) was significantly lower than in well differentiated (57.7±23.24) and moderately differentiated adenocarcinomas (60.9±23.8), suggesting that proliferative activity is low in cancers with poor differentiation, which is not in congruence to our finding in this study.

In this study stage IV disease with metastatic burden demonstrated the lowest Ki67 labelling index, (11.4±2.4), with higher values for stage III (16.1±5.1), stage II (26.4±5.4), and stage I (25.1±5.8) disease, this conclusion establishes the possibility of lower proliferative index and mitotic activity in high stage disease, with metastasis or very high potential thereof. The statistical significance of lower mitotic rates represented by lower Ki67 labelling index value in high stage disease could not be established in this study (p=0.07). This is in concordance with the findings of Nabi et al[16] who reported that Ki67 labelling index was high in Dukes' stage B (Mean Ki-67 LI 360.13±90.03) than in Dukes' stage C (Mean Ki-67-LI 241.66±101.31), showing that tumours in advanced stage (Dukes' C) have a low proliferating index than tumours in an early invasive stage (Dukes' B). Similar findings were elicited by Ishida et al[15] in which they concluded that Ki-67 proliferating index was significantly lower in carcinomas with subserosa or deeper invasion (55.2±21.4) than in carcinomas with submucosa (63.8±26.0) or muscularis propria (65.8±23.6) invasion.

In this study no correlation between Ki67 labelling index and the major epidemiological parameters like sex, age, race, location, risk factors (smoking and alcohol consumption), presenting complaints and growth pattern of neoplasm could be established. This is in concordance to the studies performed by Shepherd et al,[17] Kyzer et al[18] and Sahin et al.[19] Therefore, it was concluded that the proliferative index as determined by the Ki67 labelling index has no relation with the clinicopathological and the epidemiological parameters of colorectal carcinoma.

6. Conclusion

From this study it can be concluded that in colorectal carcinoma, that immunohistochemical technique employing Ki-67 antigen is simple and applicable to surgical specimens. The reproducibility with MIB-1 antibody staining is excellent even when paraffin embedded tissue sections are used. It was further observed that the Ki-67 proliferative index is high in poorly differentiated adenocarcinomas with loss of tumor differentiation and anaplasia and in early stage disease without extraintestinal metastatic tumor deposit. On the other hand, Ki-67 proliferating activity is low in well differentiated tumours and in an advanced stage IV disease with metastasis. There was a statistically significant association between Ki67 labelling index values and the histological grade of colon cancer, one of the most important and vital prognostic markers of colorectal adenocarcinomas. A lower Ki67 labelling index value in stage IV disease although not statistically significant in this study, does emphasise the poor prognosis of high stage metastatic disease and the possible reason behind their poor therapeutic response with cytostatic chemotherapy drugs. These high stage tumors require multimodality treatment, including radiotherapy and the use of more novel targeted therapies which can provide better therapeutic results than the conventional cytostatic drugs. As these cytostatic drugs primarily

target proliferating malignant cells, they demonstrate less therapeutic efficacy in high stage disease with low Ki67 labelling index.

7. Conflict of Interest

The authors declare that there are no conflicts of interest in this paper.

8. Source of Funding

None.

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Author biography

Neepamanjari Barman, Assistant Professor

Indroneel Bhattacharya, Senior Resident

Ranu Sarkar, Professor and HOD

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