Correlation of Ki-67 Expression with the Stage of Disease in Patients of Colorectal Carcinoma

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Abstract: Colorectal carcinoma (CRC) is a multifactorial disease process with several factors influencing prognosis. CRC is associated with the expression of multiple cell proliferating markers such as Ki-67/MIB-1. This study was aimed to examine possible correlations between Ki-67 expression and the stage of colorectal carcinoma. This was a single centre prospective study including 93 patients who underwent surgery for colorectal carcinoma. Expression of Ki-67 was assessed by immunohistochemistry on formalin-fixed paraffin-embedded tumour tissue blocks. Categorical variables data were presented as number with corresponding percentage. Continuous data were analysed using parametric tests as applicable and categorical data using nonparametric tests. The level of significance $\alpha = 0.05$ and P-value < 0.05 was considered statistically significant. The average Ki-67 expression was 77.66% (SD [standard deviation] = 9.68%) with a range of 60 to 90%. Patients with nodal involvement and larger size had a higher Ki-67 expression. To assess statistical significance, the cut-off for Ki-67 expression was set at 70%. Of 66, 48 (72.7%) adenocarcinomas and 12/18 (66.66%) mucinous adenocarcinomas had Ki-67 expression above cut-off as compared to signet ring cell variety. Ki-67 expression in colorectal carcinoma signifies mitotic activity of the tumour. Thus, it could be used as an adjunct to the existing diagnostic arsenal to help overcome its limitation in gauging the functional status of tissues.

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Introduction

Colorectal carcinoma (CRC) is the most common malignancy of the gastrointestinal system (Kumar et al., 2015). As per Global Cancer Statistics 2020, it is third in terms of incidence, but second in terms of mortality (Kumar et al., 2015). CRC incidence rates have been steadily rising in the developing world (Sung et al., 2021). Previous studies have reported that its 5-year survival rate is about 64% (Li et al., 2020). However, the 5-year survival rate of metastatic CRC is only 12% (Li et al., 2020). Thus, it is very important to diagnose CRC at an early stage. Investigation into newer diagnostic markers and modalities are thus warranted.

CRC is a multifactorial disease process, and several factors influence the prognosis, including clinical, histopathological, and biological factors related to the TNM (tumour, node, metastasis) stage of the tumour. Therefore, investigations into the molecular mechanisms of CRC can lead to novel biomarkers, which can optimize diagnosis and/or treatment regimens.

Colorectal carcinoma is associated with the expression of multiple cell proliferating markers such as Ki-67/MIB-1, which have been linked to biological features and clinical behaviour of these tumours. The cell proliferation marker Ki-67 was identified as a nuclear nonhistone protein, found in perinucleolar region and nucleoplasm of dividing cells (Schlüter et al., 1993; Scholzen and Gerdes, 2000). Ki-67 labelling index (Ki-67LI): percentage of invasive cancer cell nuclei that are positive for Ki-67 immunostaining over total invasive cancer cell nuclei present in a histological sample – has become a routine practice in clinical pathology to estimate the growth fraction of a tumour (Melling et al., 2016). However, only a few studies exist on the prognostic role of Ki-67 in CRC and have produced contradictory results (Saleh et al., 2000).

Due to the paucity of definitive guidelines and inconsistent results in available literature further analysis into Ki-67 expression in CRC is needed. This study aimed to look for the correlation between Ki-67 expression and the grade of CRC.

Methods

Study design

This was a single centre prospective study conducted by the Department of Surgery from October 2019 to September 2021. We studied 93 cases of colorectal cancer which were included on accrual basis. The protocol was approved by the ethical review board of the institute (ethics number: LHMC/IEC/2019/76). According to the principles of the declaration of Helsinki 1975 and its later modifications, written, informed consent was obtained from all the participants.

Participants

The patients were selected from those attending the out-patient department at the hospital. The biopsy proven cases of colorectal carcinoma above the age of 18 years were included in the study. Exclusion criteria were: the patients who received chemotherapy/targeted immunotherapy, and also the cases with a history of other conditions with increased Ki-67 expression such as carcinoma breast and cervix were excluded from the study. Initially 96 patients were enrolled for the study, 2 patients had received neoadjuvant chemoradiotherapy while 1 patient had a history of carcinoma breast, and therefore, 3 total cases were excluded from the study.

Patients were clinically evaluated by detailed history during routine examination on initial contact. All the preoperative routine investigations were performed. Staging of disease was done using radiological and histopathological investigations.

Routine investigations include complete blood count, liver and renal function tests, serum electrolyte levels, blood total protein and albumin levels, electrocardiography, and chest X-ray.

Special investigations for diagnosis and planning of management include fecal occult blood test, carcinoembryonic antigen level, colonoscopy, biopsy and histopathological examination and contrast-enhanced computed tomography (CECT) of the abdomen. CECT chest was done in patients with findings of metastasis on chest X-ray. Disease was staged based on TNM staging system.

Patients meeting the inclusion criteria were given the standard treatment which includes surgery and chemotherapy. All surgeries were performed by the same team of two senior surgeons (including authors) and help from the third senior surgeon was taken in case of difficulty in the intraoperative period. Radical surgery, removing the tumour *en masse* with vessels first (medial to lateral) approach, was done in all the cases and R0 resection (microscopically negative resection margins) was achieved in all the operated cases. Chemotherapy was given based on the stage of the disease, and patient parameters.

Histopathological examination (HPE) (gross and microscopic) was performed on specimens collected in definitive surgery. Expression of Ki-67 was assessed by immunohistochemistry on formalin-fixed paraffin-embedded tumour tissue blocks. Representative tissue sections were examined for Ki-67 expression. Ki-67 labelling index was counted as fraction of the immunohistochemistry (IHC) positive nuclei observed

in viable tissue areas. All viable tissue areas (excluding the necrotic foci) in the representative sections were considered in this calculation.

The details and specifications of materials used for immunohistochemistry in the study were as follows

1) The VENTANA BenchMark XT autostainer
is composed of four main components that work together as a system comprised of the computer and its software, stainer assembly, automated fluidics assembly and waste bottle assembly. The Stainer assembly comprising of reagent carousel and various other parts performs processing of all slides which are identified by the system using unique bar codes.

2) Materials required
Poly-L-lysine coated slides, slide cradle, milk protein powder, any mild detergent, 100% alcohol, xylene, mounting medium

3) Labels and reporters
Antibodies used: ANTI: KI67, company: VENTANA, immunogen: Rabbit Monoclonal antibody, clone: SP6, type: ready to use, detection kit: Ultraview DAB IHC Detection Kit, control tissue: tonsil

Statistical analysis
Data was entered in excel sheets, compiled, validated and analysed using 27th (June 2019) version of SPSS software. Continuous variables were expressed as mean and standard deviations (SD). The presentation of the categorical variables was done in number and percentage (%). Continuous data were analysed using parametric tests as applicable and categorical data using nonparametric tests. The level of significance $\alpha = 0.05$ and P-value < 0.05 was considered statistically significant.

Results

A total of 93 diagnosed cases of CRC were included in this study. The average age of patients was 52.39 years (SD = 15.10) with a range of 53 years (27–80 years). There was a slight male preponderance in the study, with 48 (51.61%) male patients (Table 1).

The most common site of involvement was the recto-sigmoid region seen to be involved in 45 patients (48.39%) followed by right side involvement in 39 (41.94%).

Stage 3 disease was seen in 64.52% of the patients at the time of presentation. The most common stage was stage 3b seen in 36 patients (38.71%).

The average size (largest dimension) of the lesion was 5.54 cm (SD = 2.35 cm) with the maximum dimension of 11 cm seen in the study.

Adenocarcinoma was the most common histological subtype (70.97%) observed in the study, followed by mucinous adenocarcinoma (19.3%) and signet ring carcinoma (9.6%). Grade 1 (61.29%) disease was the most common occurrence at presentation followed by grade 2 (constituting 22.58%) and grade 3 lesions (16.13%). Nodal involvement was seen in 54 (58.06%) patients. Out of these 54 patients, only 3 patients had nodal status as N2, and N1 status was the more frequent occurrence.

Ki-67 expression analysis
The average Ki-67 expression was 77.66 (SD = 9.68%) with a range 60 to 90%. The Ki-67 percentage expression for the patients was assessed and

Table 1: General characteristics and tumour parameters

Parameter	Observation		
Age	<45 years	>45 years	
	33	60	
Site	right	left	rectosigmoid
	39	9	45
Stage	stage 1	stage 2	stage 3
	0	33	60
Node	positive	negative	
	54	39	
HPE type	adenocarcinoma	mucinous adenocarcinoma	signet cell
	66	18	9
Grade	grade 1	grade 2	grade 3
	57	21	15

HPE – histopathological examination

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correlated with different clinical and demographic parameters. For correlation with different parameters and to assess statistical significance, the cut-off for Ki-67 expression was set at 70%.

Patient age

Patient age was found to be positively correlated to Ki-67 expression with a correlation coefficient of R=0.23. However, the results were not significant statistically (P=0.2176). On dividing the data into subgroups according to level of Ki-67 expression, 39/60 (65%) patients of age > 45 years had 80–90% Ki-67 expression, as compared to just 3/33 patients of age < 45 years. This result was found to be statistically significant (chi-square, df 11.39, 2) (P=0.0034).

Patient gender

There was no significant difference between the two genders in terms of the Ki-67 expression (77.83 vs. 77.50%, P=0.9212).

Tumour site

There was no significant difference between the lesions based on site in terms of Ki-67 expression. The difference was not significant statistically (P=0.8587).

Disease stage at presentation

It was seen that the Ki-67 expression was slightly higher for the stage 2 patients (78.41%) compared to stage 3 (77.25%). The difference was not significant statistically (P=0.7555).

Lymph node status

Patients with nodal involvement had a higher Ki-67 expression (78.33%) compared to those with no nodal involvement (76.73%). But the P-value was not suggestive of any statistical significance (P=0.6658).

Tumour size

It was seen that as the size of the lesion increased, the Ki-67 expression percentage also increased. The correlation coefficient R was 0.21 suggestive of a positive correlation. The P-value for this analysis was 0.3361.

Table 2: Histopathological subtypes vs. KiLI

	Histopathological subtypes	
KiLI	**adenocarcinoma**	**mucinous**
60–70	18	6
70–80	18	0
80–90	30	12
>90	0	0
	chi-square, df 8.369, 4, P-value 0.079	

KiLI – Ki labelling index

Histopathological type

It was seen that the signet ring carcinoma was associated with a lower Ki-67 average expression (66.67%) compared to the adenocarcinoma (79.05%) and mucinous carcinoma subtypes (78.75%). The results were not significant statistically (P=0.1329).

But on using 70% expression value as cut-off, significant difference was noted. 48 out of 66 (72.7%) adenocarcinomas and 12 of 18 (66.66%) mucinous adenocarcinomas had Ki-67 expression above cut-off as compared to signet ring cell variety, all (9 out of 9) of which had expressions below the cut-off. These differences were found to be statistically significant (P-value – 0.0470, chi-square – 6.115) (Table 2).

Tumour grade

It was observed that the average Ki-67 expression was highest for the grade 1 patients (79.47%) and decreased as the grade changed to grade 3 (70.50%). The difference was not significant statistically (P=0.2256).

Subdividing the grade 1 tumour according to stage at presentation, it was noted that 27/39 (69.2%) stage 3 tumours had Ki-67 expression more than 70% as compared to 9/18 (50%) of stage 2 tumours. Within grade 2 tumours 9/9 of stage 3 tumours had Ki-67 expression above 70% as compared to 9/12 (75%) of stage 2 tumours. This difference was not statistically significant. Similar analysis on grade 3 tumours however yielded a statistically significant difference.

Table 3: Tumour stage vs. KiLI

	Grade 1 tumours		Grade 2 tumours		Grade 3 tumours
KiLI	stage 2	stage 3	stage 2	stage 3	stage 2
<70%	9	12	3	0	0
≥70%	9	27	9	9	3
	P-value 0.4192 chi-square 0.6525		P-value 0.3496 chi-square 0.8750		P-value 0.0253 chi-square 5

KiLI – Ki labelling index

All 12 of stage 3 tumours in our study had less than 70% Ki-67 expression as compared to higher expression in the single stage 2 tumour (Table 3).

Discussion

Ki-67 immunostaining patterns have been found to correlate well with tumour growth fraction and S phase fraction in various human malignancies; however, correlation with clinicopathologic parameters has been inconsistent (Scholzen and Gerdes, 2000). Some studies showed a significant correlation of Ki-67LI with clinically important prognostic pathologic parameters in colorectal carcinomas such as tumour differentiation, metastatic disease, and local invasiveness, whereas other studies showed no such correlation (Saleh et al., 2000; Melling et al., 2016). This lack of correlation is owing to the considerable heterogeneity of colon carcinoma (Saleh et al., 2000).

In the present study, Ki-67LI with a cut-off of around 70% yielded meaningful results. This cut-off is towards the higher side according to existing literature where cut-offs range from 5 to 62% (Scopa et al., 2003; Yoshimura et al., 2003).

We found a statistically significant higher expression of Ki-67 in tumours of patients > 45 years age as compared to those < 45 years (P=0.0034). Our finding of a positive correlation of Ki-67 expression with the increasing age of the patient (R=0.23) is similar to what has been reported previously by various authors (Lin et al., 2008; Li et al., 2016). This finding however is contradictory to our hypothesis in the sense that classically CRC in younger patients is known to be aggressive and thus further study is required to better analyse the correlation.

Our results found no statistically significant correlation between the site of lesion and Ki-67 expression. This again is in concordance with results in available literature (Melling et al., 2016). Carcinomas involving the rectosigmoid region were found to have a higher Ki-67 expression as compared to the other sites. This finding was not statistically significant. This finding has not been observed earlier by other authors and requires further evaluation.

We did not get any statistically significant difference in Ki-67 expression between TNM stage 2 and stage 3 tumours. Stage 2 tumours had slightly higher Ki-67 expression as compared to stage 3 tumours (average 78.41% vs. 77.25%). A large study on 1,800 patients reported the same finding with statistically significant results, whereas another study reported that high Ki-67 expression is associated with a higher tumour stage (Melling et al., 2016; Gayyed et al., 2021). Although overall results of the present study did not reveal any major trend, looking into various subgroups revealed the correlation between high Ki-67 and higher stage at presentation.

A positive correlation was observed between the tumour size and Ki-67 expression in our study (R=0.21), signifying that tumours with a higher mitotic rate tend to present with a bulkier disease. This result is in line with the previous reports (Saleh et al., 2000; Nussrat et al., 2011). In an existing study on colorectal adenomas, it has been reported that higher Ki-67 correlates well with size even in the case of adenomas (Nussrat et al., 2011).

In our study, we found that adenocarcinomas and mucinous adenocarcinomas had higher Ki-67 expression as compared to signet ring cell type. This difference was also found to be statistically significant (P=0.047) with 72% and 66% of adenocarcinomas and mucinous adenocarcinomas, respectively having Ki-67 expression > 70%, as compared to none of signet ring subtype. Similar findings could not be found in the existing literature.

Tumours having less aggressive morphology but the higher stage at presentation were also found to have higher Ki-67 expression. This again might signify the value of Ki-67 expression as a marker of aggressive tumour behaviour in CRC and its ability to identify low-grade tumours with advanced stage and aggressive behaviour (Ma et al., 2010).

We also observed that 9/12 tumours with poor morphology (histopathological grade 3) had lower Ki-67 expression. Despite this, these tumours had a higher stage at presentation. (All 9 signet ring carcinomas in our study were grade 3, stage 3 tumours. All nine also had very low Ki-67 expression of below 70%.) This again signifies that poor histological grade does not necessarily mean higher mitotic activity within the tumour. These tumours are aggressive due to some other reason (like poor cellular cohesiveness) than higher mitotic index which needs further evaluation by a larger and detailed study.

Analysis of tumour grade revealed a negative correlation between the grade of tumour and Ki-67 expression. One study reported a statistically significant positive correlation (P=0.0007) (Saleh et al., 2000). Another previous study reported the opposite finding, but their result also was not statistically significant (P=0.21) (Gurzu et al., 2007). Another study reported a statistically significant positive correlation between Ki-67 and higher dysplasia grades (Stromar and Jakic-Razumovic, 2014).

Though our results were not statistically significant, we observed that a large proportion of these low-grade tumours also had a higher stage at presentation (48/72 of grade 1 and 2 tumours were stage 3 at presentation). This might be due to the difference

between mitotic activity and morphological grading of tumours and requires further evaluation with a larger study. One study in the literature search found a positive correlation between these two parameters, yet another reported results similar to our finding (Saleh et al., 2000; Ma et al., 2010). Another study failed to find any correlation (Melling et al., 2016). Higher Ki-67 expression in tumours with low HPE grade but the higher stage at presentation was found to be correlating. Advanced tumour stage at presentation despite low histopathological grade might signify that histopathological grading alone does not present the complete picture. High Ki-67 expression if considered separately indicates aggressive tumour behaviour (Scholzen and Gerdes, 2000). Using it as an adjunct in histopathology analysis might help risk-stratify patients in a better way. Histopathological grading only tells about the morphology of tumours, and regular staining studies can't detect the mitotic rate of tumour tissues (Scholzen and Gerdes, 2000). Measuring Ki-67 expression thus is important and can detect highly mitotic tumours with dormant morphology.

It was seen that the Ki-67 expression was slightly higher for the stage 2 patients (78.41%) compared to the stage 3 (77.25%). The difference was not significant statistically (P=0.7555). These observations might have occurred due to the small study population and also the fact that stage 3 patients constituted 64.5% (60/93) of the group. But as mentioned previously within the subgroups classical adenocarcinoma and grade 1 tumours higher stages were observed to be correlated to high Ki-67LI.

Lymph node involvement was also noted to be positively correlated with Ki-67 expression. This observation though statistically non-significant is in line with multiple previous studies (Ishida et al., 2004; Martins et al., 2015).

Our study though providing valuable insights is limited by a small sample size. Larger studies are thus needed to verify and standardise the hypothesis.

Conclusion

Finally, we conclude that rather than using it as a blanket all or non-prognostic marker, Ki-67 expression in CRC should be used as an adjunct to standard histopathology, as it measures the growth fraction of the neoplasm and reflects progression of the tumour. Existing standard diagnostic protocols are limited by morphological features of the tumour specimen. Morphological features classically used to describe aggressive tumours are not necessarily present in all mitotic tissue. Thus, using Ki-67 as a marker signifying cell division provides additional information, over and above the morphological features characteristic of malignant behaviour. This can help by identifying tumours which have seemingly less aggressive morphology, but are physiologically rapidly dividing. This subset of tumours would be labelled as less aggressive variants if only morphology is relied upon. This information might prove helpful to devise aggressive management protocols and prognostic criteria for the tumours having higher Ki-67 expression.

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