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Original Article

A Study of Molecular Subtypes (Profile) of Colorectal Cancer and Their Correlation with Clinical and Pathological Profile in a Tertiary Care Center in India

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Abstract

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Background Colorectal cancer (CRC) is a heterogeneous disease morphologically, histologically, and molecularly. Most of the studies are on this molecular heterogeneity and their clinicopathological correlation from the western world. Very few studies have been done in India.

Objectives The aims of this study were to evaluate the clinical and pathological profile of CRCs, to determine the frequency of molecular subtypes of CRCs, to correlate between the molecular subtypes and their clinicopathological features, and to determine the association between different molecular subtypes of CRC.

Materials and Methods A prospective noninvasive interventional study was done on 50 patients (both outpatients and inpatients) with newly diagnosed CRCs presenting to the Rajiv Gandhi Cancer Institute and Research Centre, Rohini, Delhi, from February 2019 to March 2020. Clinical and histopathological data were collected from case sheets as per the study proforma: history and physical examination, noninvasive and invasive imaging, and histopathological reports. Patients in whom tissue was insufficient or not available for testing for at least three of five molecular markers (KRAS, NRAS, BRAF, MSI, and MLH1 methylation) were excluded. The results were analyzed with SSPS 23.0 software. For comparison of the frequencies among groups, the chi-squared test and the Fisher exact test were used. A *p*-value of less than 0.05 was considered statistically significant.

Keywords
- clinical
- colorectal cancer
- correlation
- India
- molecular subtypes
- pathological
- profile

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Results The median age was 53 years. The majority of the males (54%) had CRC and 44% were right-sided colon tumors. Of the 50 patients with CRC, 40, 0, 4, and 4% had KRAS mutation, NRAS and BRAF mutation, and deficient mismatch repair (dMMR), respectively. KRAS mutation was significantly associated with upfront liver metastases ($p = 0.02$) and well/moderate differentiation ($p = 0.02$). BRAF wild-type tumors were likely to be well differentiated ($p = 0.02$), and moreover, half of them (52%) had MLH1 promoter methylation. The proportion of dMMR was higher in male patients ($p = 0.04$). Deficient mismatch repair was associated with well/moderate differentiation ($p = 0.02$), early stage ($p = 0.02$), and and mild peritumoral lymphocytes ($p = 0.01$). None of the dMMR patients had stage IV CRC. In all, 27% of the patients (3/11) with dMMR tumors had germline mutation of the dMMR genes. The majority of dMMR tumors (43%, 3 out of 7) had MLH1 promoter methylation. Overall, 45% (5/11) of dMMR tumors harbored KRAS mutation.

Conclusion In conclusion, this is a prospective study evaluating the correlations between RAS/BRAF mutation and dMMR status with clinicopathological characteristics in Indian CRC patients, which is slightly similar to worldwide reports with some exceptions. To the best of our knowledge, this is the first study to evaluate the molecular marker combinations in CRC in India.

Introduction

The incidence of colorectal carcinoma (CRC) worldwide is 19.7 per 1,00,0000 population, with 23.6/1,00,000 in males and 16.3/1,00,000 in females.[1] According to GLOBOCAN 2018,[1] CRC ranks third in worldwide newly diagnosed cancer cases and accounts for 9.2% cancer related deaths. It is also the third most common cause of cancer-specific mortality in the Asian continent.[1]

CRC is a biologically, histologically, and epidemiologically heterogeneous disease. Recent studies showed that the molecular profile of CRC is also different according to the tumor site.[2,3] Many studies were done in the western world and few Asian countries on the different molecular types of CRCs and their correlation clinically and pathologically. Somatic MLH1 DNA methylation is associated with older age, females, proximal tumors, and more likely to be BRAF V600E mutated.[4] The pathological features with MSI-H CRC are mucinous histology (predominantly signet ring cell type), plenty of tumor-infiltrating lymphocytes, and poor differentiation.[5] BRAF-mutated tumors occur in advanced age, females, smokers, and those with right-sided tumors.[6] In CRC, most of the BRAF mutations are sporadic MSI tumors due to MLH1 promoter methylation.[6,7] Very few mismatch repair (dMMR) tumors are due to germline mutations such as in Lynch's syndrome (LS).[8,9] RAS-mutated CRCs were more common in males and had adenocarcinoma histology with well and moderately differentiated tumors with a microsatellite stable molecular type.[10]

To date, very few studies have been done on the clinical and pathological profiles of CRCs and their correlation with molecular profiles in India. The purpose of this study was to classify CRCs according to molecular subtypes and to correlate the molecular markers with the clinicopathological profile.

Aims and Objectives

- To study the clinical and epidemiological profile of CRCs in a tertiary cancer care hospital in India.
- To study the molecular subtypes (profile) of CRCs and their correlation with the clinicopathological profile in a tertiary cancer care hospital in India.
  - To evaluate the clinical and pathological profile of CRCs.
  - To determine the frequency of molecular subtypes of CRCs.
  - To correlate between the molecular subtypes and their clinicopathological features.
  - To determine the association between different molecular subtypes of CRCs.

Materials and Methods

A prospective noninvasive interventional study was done in patients (both inpatients and outpatients) of CRC (-localized/locally advanced/metastatic) who came to our institute (Rajiv Gandhi Cancer Institute and Research Centre, Rohini, Delhi) from February 2019 to March 2020. Patients who younger than 18 years and in whom tissue was insufficient or not available for testing for at least three molecular markers out of 5 (KRAS, NRAS, BRAF, MSI, and MLH1 methylation) were excluded from this study. A sample size of 43 patients would be sufficient to detect 19.7% of incidence cases of CRC[1] with assumptions, 5% level of significance, and 12% minimum allowable error. Sample size calculation was done using nMaster 2.0 software (CMC Vellore). The sample size is small due to financial constraints. We had collected data of total 50 patients for this study.

Data were collected from case sheets as per the proforma attached, which included history taking, physical examination,

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investigations (blood tests—complete blood count, kidney function tests, and liver function tests), imaging (-colonoscopy/sigmoidoscopy, contrast-enhanced computed tomography of the abdomen, whole body positron emission tomography with computed tomography), and histopathology. Mismatch repair (dMMR) protein analysis expression was tested using immunohistochemistry (IHC; BenchMark XT, Ventana Medical Systems, Inc., Tucson, AZ, United States). Germline mutation analysis in MSH2, MLH1, PMS2, and MSH6 was performed based on the results of dMMR protein analysis. KRAS, NRAS, and BRAF V600E mutation analysis was done by reverse transcription polymerase chain reaction (PCR). Methylation of the CpG islands of MLH1 was done using pyrosequencing.

Statistical Analysis

Descriptive analysis was presented in mean ± SD or median (interquartile range) according to the distribution of data. Graphs such as bar charts, pie charts, and histograms are presented. The chi-squared and Fischer exact tests were applied for data analysis using SSPS 23.0. A *p*-value of less than 0.05 was considered to be statistically significant.

Ethical Implication

Informed consent was taken from patients undergoing blood tests for germline testing. Scientific committee and ethics committee approval was given for this study.

Financial Implication

The study has been funded by the Research Fund from the Rajiv Gandhi Cancer Institute and Research Centre, Delhi.

Results

Clinical and Epidemiological Characteristics

The baseline characteristics of the CRC patients are presented in ►Table 1. The median age at presentation was 53 years and the majority of the patients were males (54%). Tumor sites were the right colon (44%), left colon (38%), and rectum (18%). Of the 50 patients with CRC, 30 and 70% were current/former smokers and nonsmokers, respectively. Eight out of 50 patients consumed greater than 200 mL of alcohol daily. The majority of them (74%) had normal a body mass index (18–25), while only two patients had obesity (≥30). None of the patients had stage I CRC, while the majority (82%) had stage III and IV CRCs. Thirty percent and 14% of patients underwent upfront resection and neoadjuvant chemotherapy with or without radiotherapy followed by resection, respectively, while the rest never underwent resection due to either metastatic disease or progressive disease following neoadjuvant treatment.

Pathological Characteristics

The predominant histologic subtype was classical adenocarcinoma (68%). Most of the CRCs were moderately differentiated (72%). The tumor sites were the right colon (44%), left colon (38%), and rectum (18%). Further distribution according to tumor site is depicted in ►Fig. 1.

Table 1 Baseline characteristics of patients with colorectal cancer patients

	No. of patients ($n = 50$)
Age (y), median (range)	53 (25–74)
Gender	
Male	27 (54%)
Female	23 (46%)
Tumor site	
Right colon	44%
Left colon	38%
Rectum	18%
Stage (clinical and pathological)	
I	0 (0%)
II	9 (18%)
III	14 (28%)
IV	27 (54%)
Upfront obstruction/perforation	15/50 (30%)
Upfront liver metastases	13/27 (48%)

Half of the patients had a proliferative type of tumor morphology as seen by colonoscopy, sigmoidoscopy, or histopathological examination in the resected specimens.

LVI and PNI were not assessed in seven patients due to biopsy from the metastatic site. Nearly half of the patients (22 of 43) had LVI, while around 40% had PNI. Both LVI and PNI were seen in 23% patients. In 17 patients, peritumoral lymphocytic infiltration could not be assessed as they were small biopsies. Intraepithelial lymphocytes were absent (0/HPF), low (<3/HPF), and high (≥3/HPF) in 60, 20, and 20% of the patients, respectively. Mild to moderate intratumoral lymphocytes (1–25%) were seen in 37 of 50 patients.

Almost half of the patients (49%, 16/33) did not have any peritumoral lymphocytes (PTLs). Of the 26 resected CRC patients, 52, 15, and 23% had low, intermediate, and high tumor budding scores (TBSs).

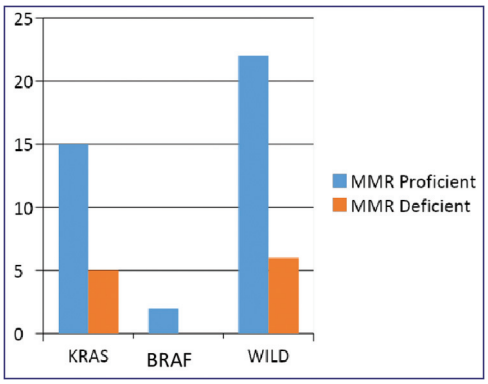

Fig. 1 Mismatch repair (dMMR) expression in RAS/BRAD mutant and wild-type colorectal cancers.

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Table 2 Frequency of KRAS/NRAS/BRAF mutation, dMMR expression, and MLH1 methylation

Molecular marker	No. of patients ($n = 50$)
KRAS/NRAS	
Wild	30 (60%)
Mutant	20 (40%)
BRAF	
Wild	48 (96%)
Mutant	2 (4%)
dMMR	
Proficient	39 (78%)
Deficient	11 (22%)
MLH1 promoter region ($n = 27$)	
Methylation	14 (52%)
Unmethylation	13 (48%)

Molecular Characteristics

There were 40% of KRAS mutation (n =20), 0% of NRAS mutation ($n = 0$), 4% of BRAF mutation ($n = 2$), 56% of wild-type CRCs ($n = 28$), and 22% of deficient mismatch repair (dMMR) CRCs ($n = 11$), as shown in ►Table 2. Five patients with dMMR were also KRAS mutated.

None of the dMMR patients had BRAF mutation. Three patients (3 out of 9) were found to be having germline deficiency in the MLH1 gene. None of the patients had any personal/past/family history of CRCs and LS-related cancers prior to their diagnosis.

Association between RAS Mutation and Clinicopathological Characteristics

In total, 30 CRCs were RAS wild-type tumors (60%). KRAS wild-type carcinomas were seen in 56% of patients older than 50 years and 63% of males, which were not statistically different from those for mutated KRAS carcinoma at 44 and 37%, respectively. RAS-mutated patients are less likely to present with upfront obstruction symptoms and advanced stages (stages III and IV) even though the numbers did not reach statistical significance. RAS-mutated tumors were associated with upfront liver metastases (69 vs. 31%; $p = 0.019$) and were poorly differentiated (92 vs. 50%, $p = 0.020$) as compared with RAS wild-type tumors, as shown in ►Table 3.

Association between BRAF Mutation Status and Clinicopathological Features

BRAF wild-type CRCs were significantly poorly differentiated than BRAF mutant-type CRCs (92 and 8%, respectively; $p = 0.020$).

Association between dMMR Protein Expression and Clinicopathological Features

dMMR status was done in all 50 patients. Eleven (22%) patients had dMMR. MLH1, PMS2, MSH2, and MSH6 deficiency rates were 16% (8/50), 2% (1/50), 4% (2/50), and 0% (0/50), respectively. MLH1/PMS2 and MSH2/MSH6 deficiency was seen in 18% (9/50) and 4% (2/50) of CRCs, respectively. The correlation of clinicopathological characteristics with dMMR status is presented in ►Table 3.

Females had a higher proportion of pMMR (91 vs. 67%, $p < 0.046$). pMMR status was also associated with well differentiation ($p < 0.022$), right-sided colonic tumors ($p = 0.024$), and absence of PTLs. There was a definite male preponderance, with 81% of dMMR tumors detected in males. The majority of dMMR tumors were stage II cancers (67%, $p < 0.001$). dMMR patients had a lower propensity to invade the bowel wall ($p = 0.010$), nodal metastases ($p = 0.006$), and distant metastases. Most dMMR tumors (72%) were located on the right side of the colon. Almost half (5/11) of the dMMR tumors were mucin producing and the rest were found to have classical adenocarcinomas.

Moreover, a high number of intraepithelial lymphocytes and mild peritumoral lymphocytic infiltration was statistically associated with dMMR (40 vs. 17%, $p = 0.072$ and 62 vs. 38%, $p = 0.010$, respectively) as compared to pMMR CRCs. As seen in ►Table 3, at the time of resection and biopsy, fewer dMMR tumors showed lymphovascular or perineural invasion and many had low lymph node harvest ratio (LNR) and TBS although they were not statistically significant.

Association between dMMR and RAS/BRAF Mutation Status

Five (25%) of the 20 CRCs with KRAS mutations were dMMR, whereas most of the CRCs (78%) in the other two subgroups (BRAF mutant and RAS/BRAF wild type) were dMMR proficient (►Table 4). These associations were statistically insignificant ($p = 0.714$).

Association between MLH1 Methylation and dMMR, KRAS, and BRAF

MLH1 methylation data of 27 patients were analyzed. MLH1 promoter methylation at the 5′ site was seen in 14 of 27 patients (52%). Of the seven dMMR tumors, three had MLH1 methylation and the rest were nonmethylated. The majority of dMMR proficient tumors were MLH1 methylated (55%). KRAS/BRAF mutation status and MLH1 methylation had no significant association (►Table 4).

Discussion

This study demonstrates that abnormalities of the KRAS gene are an important finding in colorectal neoplasia in the Indian population. The data correlate with the KRAS mutation prevalence from different countries such as United States (44%),[10] Japan (33.5%),[11] China (40.4%),[12] and other studies from India.[13] In contrast to our results, Bisht et al[14] found a lower prevalence of KRAS mutation (23.5%) in Indian CRCs. Although statistically insignificant, we found the frequency of RAS mutations to be low in males and younger patients with CRC (<50 years). This is dissimilar to the findings in the Bisht et al[14] study, where KRAS mutations were significantly higher in older patients and females. This variable prevalence

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Table 3 Association between molecular subtypes and clinicopathological characteristics in colorectal cancer patients

Clinicopathological characteristics	KRAS/NRAS				BRAF				dMMR			
	No. of patient (%)	Mutant, 40%	Wild, 60%	*p*-value	No. of patients (%)	Mutant, 4%	Wild, 96%	*p*-value	No. of patients (%)	Deficient, 22%	Proficient, 78%	*p*-value
Age	50 (100)			0.538	50 (100)			0.322	50 (100)			0.728
<50 y	16 (32)	5 (31)	11 (69)		16 (32)	0 (0)	16 (100)		16 (32)	4 (25)	12 (75)	
≥50 y	34 (68)	15 (44)	19 (56)		34 (68)	2 (6)	32 (94)		34 (68)	7 (20)	27 (80)	
Gender	50(100)			0.774	50 (100)			0..493	50(100)			0.046
Male	27 (54)	10 (37)	17 (63)		27 (54)	2 (74)	25 (26)		27 (54)	9 (33)	18 (67)	
Female	23 (46)	10 (43)	13 (57)		23 (46)	0 (0)	23 (100)		23 (46)	2 (9)	21 (91)	
Smoking status	50 (100)			1.000	50 (100)			0.528	50 (100)			0.205
Former/current smokers	15 (30)	6 (40)	9 (60)		15 (30)	1 (67)	14 (33)		15 (30)	5 (33)	10 (67)	
Never smokers	35 (70)	14 (40)	21 (60)		35 (70)	1 (3)	34 (97)		35 (70)	6 (17)	29 (83)	
Alcohol	50 (100)			0.875	50 (100)			0.297	50 (100)			0.823
Yes	8 (16)	3 (38)	5 (62)		8 (16)	1 (12)	7 (88)		8 (16)	2 (25)	6 (75)	
No	42 (84)	17 (40)	25 (60)		42 (84)	1 (2)	41 (98)		42 (84)	9 (21)	33 (79)	
BMI	50 (100)			0.198	50 (100)			0.899	50 (100)			0.722
<18	2 (4)	2(100)	0 (0)		2 (4)	0 (0)	2		2 (4)	0 (0)	2 (100)	
18–25	37 (74)	13 (35)	24 (65)		37 (74)	2 (5)	35 (95)		37 (74)	9 (24)	28 (76)	
25–30	8 (16)	3 (37)	5 (63)		8 (16)	0 (0)	8 (100)		8 (16)	1 (12)	7 (88)	
>30	3 (6)	2 (67)	1 (33)		3 (6)	0 (0)	3 (100)		3 (6)	1 (33)	2 (67)	
Upfront obstruction	50 (100)			1.000	50(100)			0.345	50 (100)			0.823
Yes	15 (30)	6 (40)	9 (60)		15 (30)	0 (0)	15 (100)		15 (30)	3 (20)	12 (80)	
No	35 (70)	14 (40)	21 (60)		35 (70)	2 (6)	33 (94)		35 (70)	8 (23)	27 (77)	
Upfront liver metastases	26 (52)			0.019	26 (52)				26 (52)			0.000
Yes	13 (50)	9 (69)	4 (31)		10	1 (10)	9 (90)	0.605	13 (50)	0 (0)	13 (100)	
No	13 (50)	2 (15)	11 (85)		16	0 (0)	16 (100)		13 (50)	0 (0)	13 (100)	
Stage	50 (100)			0.454	50(100)			0.499	50 (100)			0.002
I + II	9 (18)	5 (56)	4 (44)		9 (18)	0 (0)	9 (100)		9 (18)	6 (67)	3 (33)	
III + IV	41 (82)	15 (36)	26 (64)		41 (82)	2 (5)	39 (95)		41 (82)	5 (12)	36 (88)	

(*Continued*)

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Table 3 (*Continued*)

Clinicopathological characteristics	KRAS/NRAS				BRAF				dMMR			
	No. of patient (%)	Mutant, 40%	Wild, 60%	*p*-value	No. of patients (%)	Mutant, 4%	Wild, 96%	*p*-value	No. of patients (%)	Deficient, 22%	Proficient, 78%	*p*-value
Tumor site	50 (100)			0.687	50(100)			0.790	50 (100)			0.056
Right colon	22 (44)	10 (45)	12 (55)		22 (44)	1 (5)	21 (95)		22 (44)	8 (36)	14 (64)	
Left colon	19 (38)	6 (31)	12 (69)		19 (38)	1 (5)	18 (95)		19 (38)	1 (5)	18 (95)	
Rectum	9 (18)	3 (33)	6 (67)		9 (18)	0 (0)	9 (100)		9 (18)	2 (22)	7 (78)	
Clinicopathological characteristics	**KRAS/NRAS**				**BRAF**				**dMMR**			
	No. of patients (%)	Mutant	Wild	*p*-value	No. of patients (%)	Mutant	Wild	*p*-value	No. of patients (%)	Deficient	Proficient	*p*-value
T stage	50 (100)			0.904	50 (100)			0.530	50 (100)			0.010
T3	18 (36)	7 (39)	11 (61)		18 (36)	0 (0)	18 (100)		18 (36)	8 (44)	10 (56)	
T4	32 (74)	13 (40)	19 (60)		32 (74)	2 (6)	30 (94)		32 (74)	3 (9)	29 (91)	
N stage	50 (100)			0.454	50 (100)			0.499	50 (100)			0.002
N0	9 (18)	5 (56)	4 (44)		9 (18)	0 (0)	9 (100)		9 (18)	6 (67)	3 (33)	
N1or N2	41 (82)	15 (36)	26 (64)		41 (82)	2 (5)	39 (95)		41 (82)	5 (12)	36 (88)	
M stage	50 (100)			0.728	50 (100)			0.954	50 (100)			0.000
M0	24 (48)	9 (37)	15 (63)		24 (48)	1 (4)	23 (96)		24 (48)	11 (46)	13 (54)	
M1	26 (52)	11 (42)	15 (58)		26 (52)	1 (4)	25 (96)		26 (52)	0 (0)	26 (100)	
Tumor morphology	50 (100)			1.000	50 (100)			1.000	50 (100)			0.496
Ulcerative	25 (50)	10 (40)	15 (60)		25 (50)	1 (4)	24 (96)		25 (50)	4 (16)	21 (84)	
Proliferative	25 (50)	10 (40)	15 (60)		25 (50)	1 (4)	24 (96)		25 (50)	7 (28)	18 (72)	
Tumor histology	50 (100)			0.462	50 (100)			0.612	50 (100)			0.410
Classic adenocarcinoma	34 (68)	13 (38)	21 (62)		34 (68)	2 (6)	32 (94)		34 (68)	6 (18)	28 (82)	
Mucinous/signet cell	15 (30)	6 (40)	9 (60)		15 (30)	0 (0)	15 (100)		15 (30)	5 (33)	10 (67)	
Serrated adenocarcinoma	1 (2)	1 (100)	0 (0)		1 (2)	0 (0)	1 (100)		1 (2)	0 (0)	1 (100)	
Tumor grade	50 (100)			0.020	50 (100)			0.020	50 (100)			0.019
Well/moderately differentiated	36 (72)	18 (50)	18 (50)		36 (72)	0 (0)	36 (100)		36 (72)	11 (30)	25 (70)	
Poorly differentiated	24 (28)	2 (8)	12 (92)		24 (28)	2 (8)	12 (92)		24 (28)	0 (0)	14 (100)	

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Table 3 (*Continued*)

Clinicopathological characteristics	KRAS/NRAS				BRAF				dMMR			
	No. of patient (%)	Mutant, 40%	Wild, 60%	*p*-value	No. of patients (%)	Mutant, 4%	Wild, 96%	*p*-value	No. of patients (%)	Deficient, 22%	Proficient, 78%	*p*-value
LVI	43 (86)			0.374	43 (86)			0.323	43 (86)			0.795
Present	18 (42)	6 (33)	12 (67)		22 (42)	1 (4)	21 (96)		22 (52)	6 (33)	16 (67)	
Absent	25 (58)	10 (40)	15 (60)		21 (58)	0 (0)	21 (100)		21 (48)	5 (20)	16 (80)	
PNI	43 (86)			0.052	43 (86)			0.413	43 (86)			0.728
Present	17 (40)	3 (18)	14 (82)		17 (40)	0 (0)	17 (100)		17 (40)	5 (29)	12 (71)	
Absent	26 (60)	13 (50)	13 (50)		26 (60))	1 (4)	25 (96)		26 (60)	6 (23)	20 (77)	
LN harvest ratio	26 (52)			0.224	26 (52)			NA	26 (52)			0.114
0	12 (46)	6 (50)	6 (50)		12 (46)	0 (0)	12(100)		12 (46)	7 (58)	5 (42)	
0.01–0.17	8 (30)	1 (12)	7 (88)		8 (30)	0 (0)	6 (100)		8 (30)	1 (12)	7 (88)	
>0.17	6 (24)	2 (33)	4 (67)		6 (24)	0 (0)	8 (100)		6 (24)	2 (33)	4 (67)	
Tumor budding score	26 (52)			0.898	26 (52)			NA	26 (52)			0.483
Low	16 (62)	6 (37)	10 (63)		16 (62)	0 (0)	16 (100)		16 (62)	7 (44)	9 (56)	
Intermediate/high	10 (38)	4 (40)	6 (60)		10 (38)	0 (0)	10 (100)		10 (38)	3 (30)	7 (70)	
IEL	50 (100)			0.470	50 (100)			0.470	50 (100)			0.072
Absent/low	40 (80)	17 (42)	23 (58)		40 (80)	2 (5)	38 (95)		40 (80)	7 (17)	34 (83)	
High	10 (20)	3 (30)	7 (70)		10 (20)	0 (0)	10 (100)		10 (20)	4 (40)	5 (50)	
ITL	50 (100)			0.652	50 (100)			0.382	50 (100)			0.419
Absent	12 (24)	4 (33)	8 (67)		12 (24)	0 (0)	12 (100)		12 (24)	1 (8)	11 (92)	
Mild/minimal	26 (52)	12 (46)	14 (54)		26 (52)	2 (8)	24 (92)		26 (52)	7 (30)	19 (70)	
Moderate/high	12 (24)	4 (33)	8 (67)		12 (24)	0 (0)	12 (100)		12 (24)	3 (25)	9 (75)	
PTL	33 (66)			0.560	33 (66)			NA	33 (66)			0.010

Abbreviations: BMI, Body Mass Index; BRAF, V-Raf Murine Sarcoma Viral Oncogene Homolog B, IEL, Intra-Epithelial Lymphocytes; ITL, Intra -Tumoral Lymphocytes; KRAS, Kirsten RAt sarcoma; LVI, Lympho-Vascular Invasion; M, Metastasis; NRAS, Neuroblastoma RAt Sarcoma; N, Node; PNI, Peri-Neural Invasion; PTL, Peri-Tumoral Lymphocytes; T, Tumour.

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Table 4 Association between MLH1 promoter methylation status and RAS/BRAF mutation status

Molecular marker	MLH1 promoter		
	Methylation ($n = 14$)	Unmethylation ($n = 13$)	*p*-value
dMMR			
Deficient ($n = 7$)	3 (43)	4 (57)	0.2500
Proficient ($n = 20$)	11 (55)	9 (45)	
KRAS/NRAS			
Wild ($n = 8$)	8(53)	7(47)	1.0000
Mutant ($n = 6$)	6(50)	6(50)	
BRAF			
Wild ($n = 27$)	14 (52)	13 (48)	1.0000
Mutant ($n = 0$)	0 (0)	0 (0)	

of KRAS mutation can be attributed to genetic factors, dietary factors, environmental factors, testing method, and quality of the sample.

The KRAS-mutated tumors were mostly seen in patients with classical adenocarcinoma in western studies.[10] On the contrary, we found it to be not statistically significant with adenocarcinoma histology. In our study, KRAS wild-type tumors were associated with well-differentiated tumors unlike the observation by Veldore et al.[13] This dissimilarity could be due to the small sample size. Being a rare mutation,[15] we could also not find any NRAS mutation in our study.

BRAF mutation frequency was found to be 4% in this study, which is higher than the reported frequencies in China (2.3%) and lower than that reported in India (9.8%). The small sample size and different sensitivities for the molecular techniques used can explain the disparity. In our study, BRAF wild-type tumors were more commonly associated with well differentiation. These findings are inconsistent with the study by Bisht et al.[14] As in the Li et al[16] study, there was no significant difference with BRAFV600E-mutated tumors according to age and sex. Furthermore, in line with prior studies and literature, none of the KRAS mutation cases had concomitant BRAF mutations, which indicates the mutually exclusive nature of these mutations. KRAS- and BRAFV600E-mutated tumors were more advanced tumors with $\geq$4 positive lymph nodes and higher TNM stages. However, this observation was not consistent with our results. The smaller size of the BRAFV600E mutation and KRAS mutation subgroups can explain this variation.

The 22% of CRCs with dMMR was in accordance with data from an Indian study (29%).[17] Reports from other Asian countries showed only 10% MSI-H CRCs.[5,18] This discrepancy can be due to the different molecular tests used and their sensitivities to some extent. Compared with PCR-based MSI testing, IHC is easy to perform and the turnaround time is very less. Most importantly, IHC helps in picking up and may detect few dMMR cases that may have been missed by PCR-based MSI testing.[19] Correlations between dMMR status and clinicopathological features were contrary with prior studies. It might be due to the different inclusion criteria, environmental factors, and the variable specificity and sensitivity of the different tests. Several studies showed a significant association of dMMR colorectal tumors with a lower TNM stage, poor differentiation, high intraepithelial lymphocytes, the presence of several intratumoral lymphocytic, and PTLs, and N0 nodal stage. We could not find any association between dMMR expression and LNR. This is in contrary to the study by Berg et al[20] where the authors found a significant association between MSI-H status and adequate lymph node harvest (>12).

MLH1 loss accounted for the majority dMMR CRCs (72%) and 40% of this deficiency (2 out of 5 tumors with MLH1 loss) was caused by MLH1 promoter methylation, which separates sporadic dMMR CRCs from germline mutation LS cases. This is dissimilar to Hampel et al's[21] study in which around 70% were sporadic tumors. Another curious finding in our study is that the family history is deceptive and misleading.[21] Therefore, all newly diagnosed CRC patients should be screened for LS using an IHC-based algorithm[22] (►**Fig. 2**) rather than on family history. In our study, dMMR/KRAS mutation, dMMR/KRAS wild-type, pMMR/KRAS mutation, and pMMR/KRAS wild-type tumors were 10, 12, 30, and 48%, respectively, which is similar to the that reported by Ye et al.[23] This tumor subgrouping according to molecular subtypes is prognostic as MSS/KRAS mutant tumors had the worst survival.[24] Therefore, dMMR and KRAS markers will be key for the development of a molecular prognostic scoring system for CRC in the future.

This study has its limitations. There are missing histopathological and molecular data for few parameters in view of small biopsies or biopsies from metastatic sites. A relatively small sample size could have under- or overestimated the significance of the association between the molecular markers and the clinicopathological characteristics. Nevertheless, aside from the TBS, lymph node ratio, PTLs, and MLH1 methylation, the rest of the collected data were accurate to around 95% with less than 5% of missed information. Another drawback is the consideration of molecular testing in all CRC patients. However, the low incidence of CRCs in India and the short time period available for completion of this study could explain this. On the other hand,

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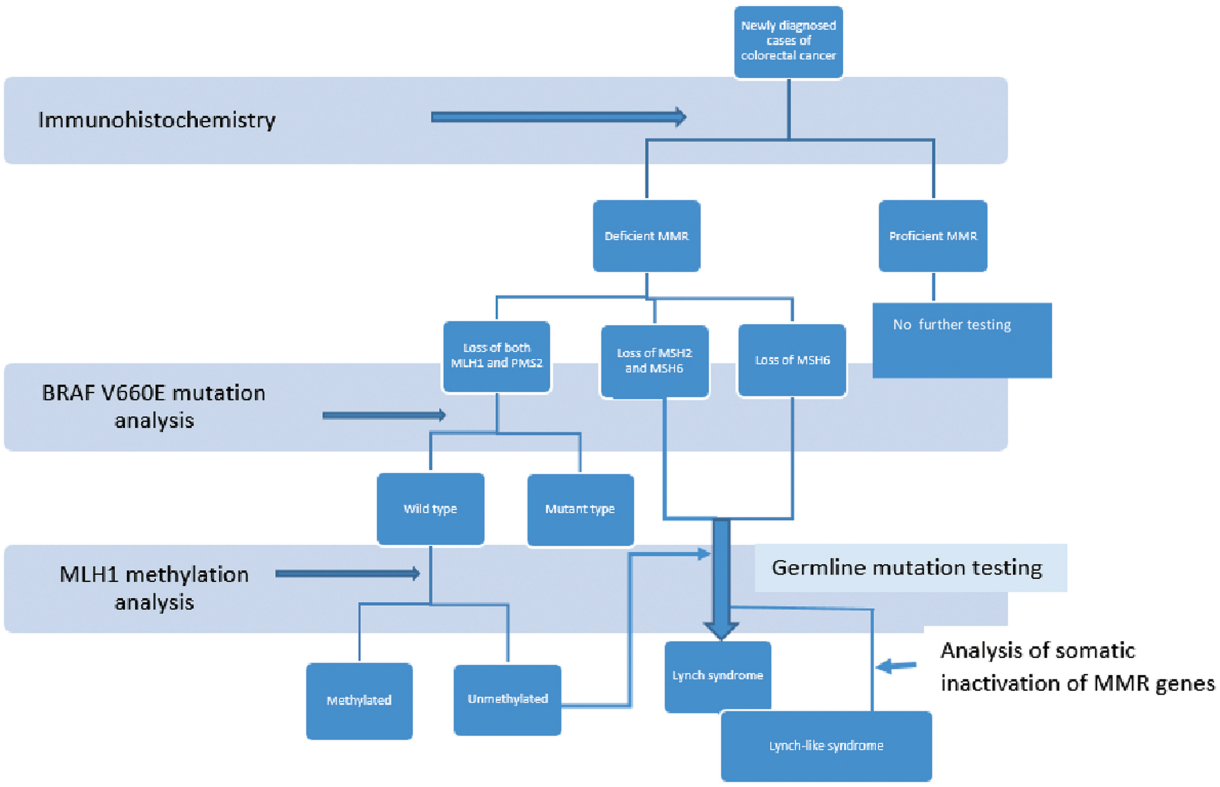

Fig. 2 Flowchart of universal screening for Lynch's syndrome (LS) by immunohistochemistry in mismatch repair (dMMR) proteins in newly diagnosed cases of colorectal cancer.

the strength of our study was prospective data collection and statistical correlation of data from one of the cohorts of CRCs ($n = 50$). Above all, this is the first Indian study to correlate complete RAS and BRAF analysis, dMMR status, and MLH1 methylation with CRCs' clinicopathological features and also the association between different molecular subtypes. It helped pick up additional cases for germline testing for LS.

Conclusion

In conclusion, 40, 4, and approximately one-quarter (22%) of the colorectal tumors were KRAS mutant, BRAF mutant, and dMMR, respectively. In particular, KRAS, BRAF mutation status, dMMR expression, and MLH1 methylation have unique clinical, pathological, and molecular characteristics, which must be kept in mind when assessing in clinical trials the prognosis values of different molecular markers in CRCs. Further studies including larger cohorts of CRC patients should be done to confirm these associations.

Previous Presentation

The abstract of the study was presented as poster at WGI–ESMO 2022 in Barcelona, Spain.

Ethical Approval

The study was approved by the Ethics committee of the Rajiv Gandhi Cancer Institute and Research Centre.

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Conflict of Interest

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