Immunohistochemical analysis of Mismatch repair-deficient gene expression in unselected Colorectal Cancer: An update of tertiary cancer center from North India

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Abstract

Colorectal cancer (CRC) is the third most deadly and fourth most commonly diagnosed cancer in the world. The present study has been carried out to see the frequency of lost MMR protein expression and its histopathological characteristics in unselected colorectal cancer patients from north India. 103 consecutive patients of colorectal cancer who underwent surgery between 2014-2018 in a tertiary care teaching hospital in North India were included in the study. The study was approved by the Institute's Ethical Committee. MMR protein status was determined by Immunohistochemistry (IHC) to examine the expression of MLH1, PMS2, MSH2 and MSH6 on paraffin-embedded tissues. Patients with MLH1deficient protein were further followed by BRAF V600E testing. The histopathological features were correlated with MMR protein expression.

IHC results revealed a loss of different MMR protein expression in 33 (32%) patients. the most frequent loss was lost MSH2 12(36.4%) followed by MLH1 10(30.3%), PMS2 8(24.2%) and MSH6 3(9.1%); Out of 10 MLH1 deficient cases, 6 (60%) were BRAF V600E mutant and 4 (40%) were BRAF-wild-type. We have found significance with medullary histological phenotype, poor histological grade and TILS. In our study, the frequency of MMR protein loss was found in 32% of patients of CRC. The loss was significantly associated with medullary phenotype, degree of differentiation and presence of tumor infiltrating lymphocytes (TILS).

Keywords: Colorectal cancer (CRC), Mismatch repair (MMR) proteins, Immuno-histochemistry, Histopathological features.

Introduction

Colorectal cancer (CRC) is the fourth most commonly diagnosed and third most deadly cancer worldwide. According to GLOBOCAN 2020, CRC ranked third in terms of new cases and fourth in terms of mortality. In India, the number of new cases of CRC was 40,408 in males (6.3%) and in females it was 24,950 (3.7%) of total cancers.[25] CRC incidence has been steadily rising especially in developing countries due to the adoption of western lifestyles including dietary habits. Initially, this was thought to be uncommon cancer in India with an incidence of 4 per 100,000 but it is increasing over time (currently 9.2 per 100,000).[14]

Initially reported to be a disease of the elderly, now it is being reported in the young population too.[21,24] Risk factors include advanced age, familial predisposition, obesity, physical inactivity, smoking, alcohol consumption and red meat. It can be sporadic or hereditary. Sporadic form is the most common type but genetic and familial associations are well known. Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC) comes under the most common genetic type. This syndrome is caused by mismatch repair (MMR) genes mutations which are characterized by autosomal dominant inheritance, predominance for right side cancer and early age of onset.[2]

Clinical criteria (Amsterdam I and II) [27] were initially suggested to make the clinical diagnosis of Lynch syndrome but later revised Bethesda guidelines [22,28] were used to select patients for subjecting to screening tests. The diagnosis is clinched by genetic testing which is expensive and not readily available everywhere. Hence, before performing genetic testing, screening tests are performed to select the patients likely to benefit from genetic testing. This can be done by either testing for MMR protein expression using immuno-histochemistry (IHC) or by microsatellite instability (MSI) testing based on the revised Bethesda criteria. However, a large number of patients who have a loss of MMR protein expression did not satisfy the revised Bethesda criteria for the diagnosis of Lynch syndrome. These patients pose a diagnostic dilemma and require special attention.

Due to the limitations of clinical criteria to guide genetic testing in patients with family history, some authorities have proposed that tumors from patients with colorectal cancer can be evaluated for markers of HNPCC despite the fact of the family history.[31] One of the studies with a higher sample size of 1,066 patients with colorectal cancer tumors was tested for MSI.[7] Patients with suggestive microsatellite unstable phenotype were then tested for germline mutations in the mismatch repair genes MLH1, MSH2, MSH6 and

PMS2 by IHC, genomic sequencing and deletion studies. Approximately 15% of CRC cases are accountable for MMR deficiency.

The most common cause of MMR deficiency is MLH1 hypermethylation. MLH1-deficient CRC is more often due to sporadic than genetic origin. BRAF gene is a member of the Raf kinase family of serine/threonine protein kinases. BRAF V600E mutation is generally found in 5–10% of patients with metastatic CRC and also acts as an adverse prognostic factor with a median survival of 9–14 months. The deficiency of MLH1 prompted BRAF V600E testing.

Further testing was stopped once mutation detected since it was unlikely that the cancer was due to HNPCC. If wild-type BRAF mutation was found in MLH1-deficient patients, sequencing of MLH1 was performed. If combined MLH1-PMS2 deficiency was detected in any patient, then sequencing of PMS2 was also performed as well if MLH1 sequencing was normal. HNPCC was detected in 23 patients (2.2%) of whom 10 were older than 50 years of age and the rest 5 did not meet the Bethesda or Amsterdam guidelines.

So as per given data, the Bethesda or Amsterdam criteria's alone may miss as many as 22% of patients with HNPCC. However, only 5 additional individuals from the cohort of 1,066 patients would have been identified by routine molecular analysis of all colon cancers that were fulfilling the Bethesda criteria, these were practically expensive for routine clinical use. Therefore; most proved and expert guidelines on screening of HNPCC suggest a combination of sequential laboratory testing in patients who fulfil the Amsterdam criteria or Bethesda guidelines to minimize costs and maximize test accuracy.[3,5]

Approaches based on above strategy have been considered to be cost-effective.[20] Currently proposed strategies include initial testing of tumors for IHC with or without MSI or/and testing of MSI with or without IHC for loss of expression of mismatch repair proteins with germ-line gene sequencing for patients with suggestive results.

The present study has been carried out to find the frequency of MMR protein loss in colorectal cancer patients from north India and also to see if there is any correlation with clinical and histopathological features which might be useful to select the patients with Lynch syndrome.

Material and Methods

A prospective study was carried on colorectal cancer patients who were surgically treated at Sanjay Gandhi Post Graduate Institute of Medical Sciences, India. This study was approved by the Institute's ethics committee (IEC). Informed consent was taken from all patients. Patients who are known to have Familial Adenomatous Polyposis (FAP) were excluded from the study. During the period of study (May 2014 to June 2018), samples were collected from all 117 patients who were admitted for surgery with the diagnosis of colorectal cancer. 14 patients were excluded after the final histology report which revealed benign conditions like TB and Crohn's disease. Finally, a total of 103 colorectal cancer patients who were above 18 years and willing to participate in the study were included. Patients were categorized into two groups based on revised NIH, Bethesda guidelines[28]: those who fulfilled the criteria and others who did not. MMR status was determined by Immuno-histochemistry (IHC) staining to examine the expression of MLH1, PMS2, MSH2 and MSH6 on paraffin-embedded tissues as per the standard method.[8]

MMR expression was considered lost only when there was a complete absence of nuclear staining in the presence of positive control. Patients with MLH1deficient protein were further followed by BRAF V600E testing. The various clinicopathological factors (age, sex, location of the tumor, degree of differentiation, presence and absence of mucin, LVI, PNI, TILS) were analyzed to see its effect on MMR protein expression. Normal tissue adjacent to the tumor (minimum 5cm away from the lesion) was taken as a positive control.

Statistical analysis: Continuous data were shown as mean or median and discrete data were reported in percentage. Univariate analysis was performed using the 2-tailed student t-test for continuous non-normally distributed variables and categorical variables were compared using the chi-square test or Fisher's exact test. Multivariate correlation analysis was performed using the logistic regression test by using SPSS version 16.0 (IBM Corporation, Armonk, NY, USA). A P-value less than 0.05 (<0.05) was considered as statistically significant.

Histopathology: The histopathological features of each slide were reviewed by a pathologist as regards the prognostic factors (mucinous, signet ring, medullary and poorly differentiated etc. Mucinous adenocarcinoma was defined according to the WHO classification which is >50% of the tumor lesion composed of pools of extracellular mucin; tumor with <50% of the lesion composed of mucin is categorized as having mucinous component. The presence or absence of tumor-infiltrating lymphocytes (TILS), lymphovascular invasion (LVI), perineural invasion (PNI) was also recorded. The tumor was staged after the final histopathology as per TNM AJCC 8th staging.

Immunohistochemistry: Formalin-fixed paraffin-embedded CRC tissue blocks were sectioned at 3µm thickness and collected on poly-lysine coated glass slides (Poly-L-lysine coatings were done for 6 hours to provide adhesion to slides for better tissue fixation). Before immunostaining, sections were deparaffinized in xylene and rehydrated in an alcohol series (100%, 70% and 30% and then with water). Antigen retrieval was done in 1X EDTA (Ethylene diamine tetraacetic acid) buffer (pH 9.0) at 90°C for 30 minutes in the microwave. Slides were cooled at room temperature. Endogenous peroxidase activity was blocked

by putting the slides in 3% H_2O_2 in methanol for 25 minutes. Protein blocking will be done by protein blocking reagent e.g. 0.5% bovine serum albumin or dry milk prevents non-specific binding of antibodies to tissue. Slides were incubated with the Anti-MMR antibodies (MLH1-1:50 dilution, MSH2- 1:50 dilution, PMS2- 1:50 dilution, MSH6-1:50 dilution; Ready-to-use) at room temperature for 2 hours.

Slides were washed with tris-buffer saline (TBS). The slides were then incubated with the secondary antibody (Leica Biosystems, Ready-to-use) for one hour at room temperature followed by washing with TBS. Slides were then incubated with 3,3'-Diaminobenzidine (DAB) for 10 minutes (DAB is a chromogenic substrate that stains antigen-antibody sites 'brown'). Counterstaining was done by haematoxylin. We have used marginal non-malignant tissue (normal colonic mucosa) as a positive control in every batch for comparison; the complete absence of nuclear staining of tumor cells was regarded as lost MMR protein expression.

Results

A total of 103 patients (72 males and 31 females) with colorectal cancer underwent resection and formed the study group. The median age of the patient was 53 years (range 15-81 years). Forty-three (48%) patients were younger than 50 years. Colon cancer was found in 69 (67%) patients and rectal cancer in 34(33%). The right-sided colonic lesion was found in 53(76%) patients. Histopathological examination revealed well-differentiated carcinoma in 33 (32%), moderately differentiated in 14 (13.6%) and poorly differentiated lesion in 56 (54.4%) patients. A family history of malignancy was present in 9 (8.7%) patients. Seven of these patients were first-degree relatives and 2 were second-degree relatives. Positive family history of cancer was more in younger patients than older (55.6% vs 44.4%). Patient demographics, tumor location and histopathological characteristics are shown in table 1.

MMR protein loss was found in 33 (32%) patients (Table 2a). Of these, 17 were younger than 50 years of age. Out of 33 patients with loss of MMR protein expression, 21 (64%) patients had right-sided colon cancer, 6 (18%) left-sided colon cancer and 6 (18 %) had rectal cancer. The frequency of MMR loss was equally distributed in patients <50 years of age vs patients > 50 years of age (51.5% and 48.5% respectively). However, younger patients (< 50years of age) showed more advanced tumors (58.1% vs. 40%; p=0.076) and signet ring cell histology (20.9% vs. 13.3%; p=0.42) than older patients (> 50 year of age). All six patients who had additional malignancy were MMR deficient (p=0.001).

Table 1
Demographic details of the patients (n=103)

S. N.	Features	No. of patients (%)
	Gender	
	Males	72 (69.9)
	Females	31 (30.1)
2	**Age**	
	Median Age (Years)	53
	Age Range (Years)	15-81
	No. of Cases (≤50)	43 (41.7)
	No. of cases (>50)	60 (58.3)
3	**Location of tumor**	
	Caecum	10 (9.7)
	Ascending colon	37 (35.9)
	Transverse Colon	6 (5.8)
	Descending Colon	8 (7.8)
	Sigmoid Colon	8 (7.8)
	Rectum	34 (33.0)
4	**Synchronous lesion**	11 (10.7)
5	**Metachronous lesion**	5 (4.9)
6	**Revised Bethesda**	
	Fulfilled	46 (44.7)
	Not Fulfilled	57 (55.3)
7	**History of CRC in the family**	9 (8.7%)
	First Degree Relatives (FDR)	7 (77.8)
	Second Degree Relatives (SDR)	2(22.2)

In our study (n=33), the most frequent loss was lost MSH2 12(36.4%) followed by MLH1 10(30.3%), PMS2 8(24.2%) and MSH6 3(9.1%) (Figure1). Isolated loss of PMS2 was found in 8 (24.2%) and MSH6 was found in 3(9.1%) patients. The combined loss of MLH1 and PMS2 was found in 30.3% and of MSH2 and MSH6 in 36.4% of patients. Out of 10 MLH1 deficient cases, 6 (60%) were BRAF V600E mutant and 4 (40%) were BRAF-wild-type (Figure 2). The late-stage (III/IV) of the tumor was significantly associated with MMR protein loss as compared to the early stage (Stage I/II) (p=0.034). 21(63.6%) patients with MMR protein loss had a poorer degree of differentiation.

Various factors significantly associated with MMR protein expression on univariate analysis were colonic lesion, late stage of the disease and histopathological features like mucin secretion, medullary pattern, poorly differentiated tumors and tumor-infiltrating lymphocytes (TILS). However, on multivariate analysis, three factors were found significant. These were medullary histology, poor degree of differentiation and tumor-infiltrating lymphocytes (Table 2b). There was no significant difference found between MMR protein loss with patient age, gender, family history of malignancy and histopathological factors like LVI, PNI, TILS, signet ring cells and mucinous histology.

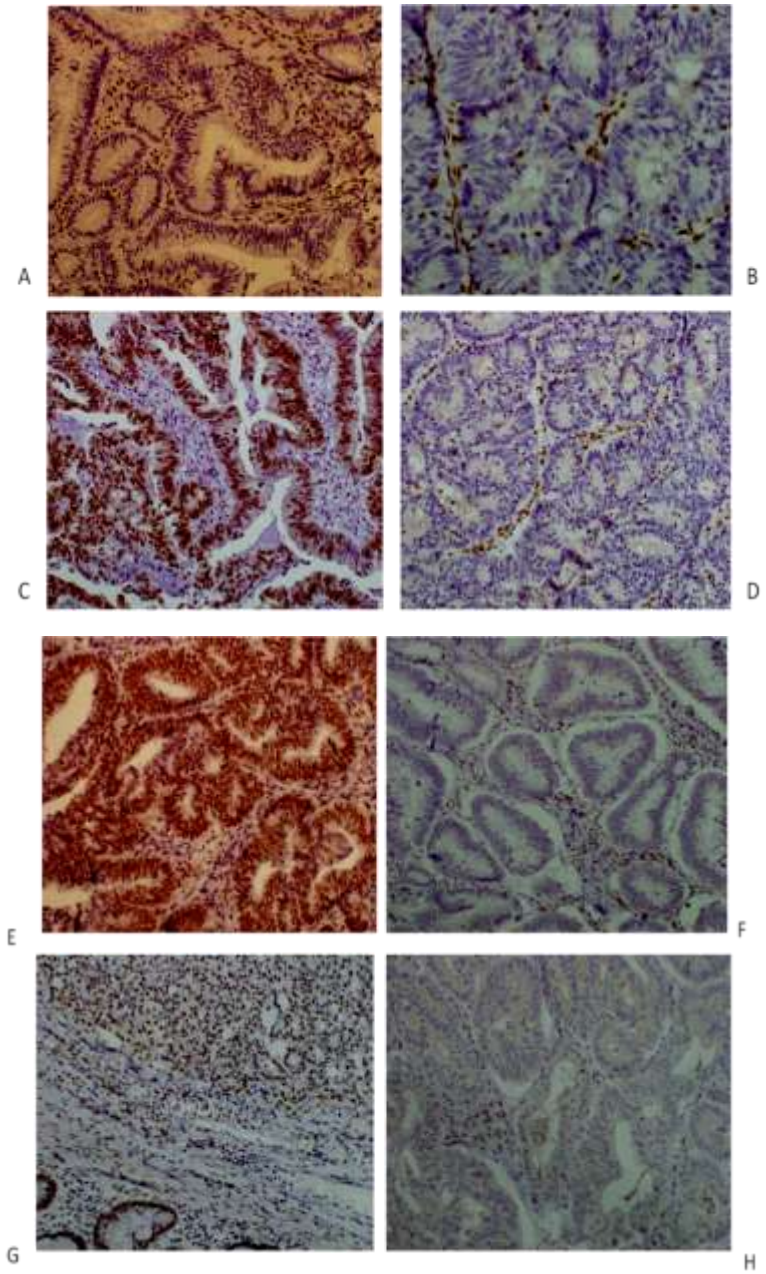

Figure 1: **Immunohistochemical tumor testing for MMR proteins (A) MLH1: The nuclei stained brown in MLH1 intact tumors (40X magnification) (B) Blue colour of haematoxylin showing MLH1 lost tumors (40X magnification); C: MSH2: The nuclei stained brown in MSH2 intact tumors (20X magnification) (D) showing MSH2 protein loss (20X magnification) E: Intact MSH6 (20X magnification) F: Lost expression of MSH6(20X magnification) G: Intact PMS2 (40X magnification) and H: Lost PMS2 (40X magnification)**

Table 2a
Clinical and histopathological features and its association with MMR protein Expression (Univariate analysis):

Features	Total No. (n=103)	MMR Loss (n=33)	*p* – value
Age			
≤50 Years	43 (41.7%)	17 (51.5%)	0.201
>50 Years	60 (58.3%)	16 (48.5%)	
Gender			
Male	72 (69.9%)	19 (57.6%)	0.070
Female	31 (30.1%)	14 (42.4%)	
Location of tumor			
Colon	69 (67%)	27 (81.8%)	**0.028****
Rectum	34 (33%)	6 (18.2%)	
Family H/O Malignancy			
Present	9 (8.7%)	1 (2.8%)	0.265
Absent	94 (91.3%)	32 (97.2%)	
Revised Bethesda			
Fulfilled	46 (44.7%)	18 (54.5%)	0.204
Not fulfilled	57 (55.3%)	15 (45.5%)	
Lymphovascular Invasion (LVI)			
Present	31 (32%)	15(45.5%)	**0.020****
Absent	72 (68%)	18(54.5%)	
Perineural Invasion (PNI)			
Present	17(15.5%)	7(21.2%)	0.403
Absent	86 (84.5%)	26(78.8%)	
TILS			
Present	35 (34.0%)	19(57.6%)	**0.001****
Absent	68 (66.0%)	14(42.4%)	
Extracellular Mucin pool (Mucin <50%)			
Present	41(39.8%)	7(21.2%)	**0.017****
Absent	62(60.2%)	26(78.8%)	
Mucinous (mucin >50%)	20 (19.4%)	5(13.9%)	0.596
Signet Ring cell	17 (16.5%)	2(11.8%)	0.085
Medullary	11 (10.7%)	7(21.2%)	**0.035****
Stage			
Early (I/II)	54(53.4%)	12(36.4%)	**0.025****
Late (III/IV)	49(46.6%)	21(63.6%)	
Tumor Grade			
Well Differentiated	33 (32.0%)	4(16.7%)	**0.002****
Moderately Differentiated	14 (13.6%)	8(22.2%)	
Poorly Differentiated	56 (54.4%)	21(61.1%)	

Table 2b
Multivariate analysis

Effect	Odds ratio	95% wald confidence limits		P-value
Medullary pattern	12.401	2.044	75.250	**0.006*****
Degree of differentiation	4.791	1.227	18.401	**0.024*****
Tumor infilterating lymphocytes	4.592	1.053	10.760	**0.032*****

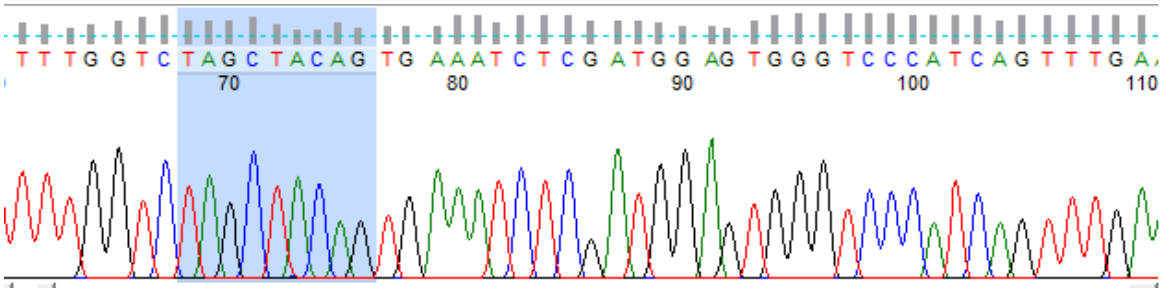

Figure 2: Electropherogram of MLH1 deficient patient showing wild type (WT) BRAF

Discussion

In India; the age-standardized incidence rates (ASR) for CRC are low and was 6.0 per 100,000 population in males and 3.7 per 100,000 populations in women (As per fact sheets by The Global Cancer Observatory, India 2020; https://gco.iarc.fr/today/data/factsheets/populations/356-india-fact-sheets.pdf). The 5-year survival of CRC in India is one of the lowest in the world at less than 40%. As per CONCORDE-2 study, the 5-year survival of rectal cancer in India is falling in some registries.[1] Colorectal cancer (CRC) is a disease of the older population, with more than 90% occurring after the age of 55 years. Off late, colorectal cancer incidence is increasing in young age patients and is associated with a poor outcome due to presentation at an advanced stage and poor histopathological features.

The majority of CRC patients are sporadic and a small percentage of patients is hereditary. Hereditary conditions such as familial polyposis (FAP) and Lynch syndrome (LS) confer an extremely high lifetime risk of CRC but account for a minority of all CRCs. Early detection of CRC through screening with established modalities beginning at age 50 reduces CRC morbidity and mortality.[10] There are several criteria described in the literature to identify the patients with a high risk of developing CRC like revised Bethesda.

A patient diagnosed as having Lynch syndrome based on revised Bethesda criteria can have germline mutations in any one of several genes involved in DNA MMR proteins. A major amount of data has shown that tumors in patients with Lynch syndrome have defective MMR (dMMR) protein. Immuno-histochemistry is often used as the first-line screening tool which is followed up by microsatellite instability (MSI) testing to validate the results.[4,23]

Forty-three patients (41.7%) were under 50 years of age of which 67.4% were males. A large regional variation has been reported in India with regards to young age CRC patients (<50 years) which varies from 20-50%.[16] MMR protein loss was more commonly seen in right-sided tumors (63.6%) compared to left-sided (13.9%) and rectal tumors (13.9%). Colonic tumors were significantly associated with MMR protein expression as compared to rectal tumors (p=0.028*). Similar findings have been reported from other parts of the world.[12] This MMR deficiency was slightly more (51.8%) in young patients (<50 years of age). Studies from Japan[26] and the USA[17] have reported lower dMMRs of 8.4% and 10.7% respectively in young aged patients as compared to our findings. The possible reason for higher MMR loss in our young CRC patients might be due to genetic, environmental and dietary factors. These patients also presented an advanced stage of the disease.

MMR protein loss of 32% of our series emphasizes the importance of routine MMR protein testing. The various published series have also reported the MMR protein losses ranging from 13% to 30%. There is significant geographical and regional variation in the reporting. MMR protein loss of 32% in the present series seems to be higher than the reported series from the west as in a study from the UK, MMR protein loss of 21% has been reported while from Memorial Sloan-Kettering Cancer Centre, USA, it was 19%. Even the lower MMR loss of 7% has been reported from China by Li et al.[13]

Few Indian studies by Pandey et al[19] and Malhotra et al[14] have also reported lower MMR protein loss 17.8 and 19.9%. respectively whereas but the loss was approximately similar when compared from earlier Indian reports by Nayak et al[16] with (23%) and Rai et al[20] with 29%. Loss of PMS2 and MSH6 has been found as 44% and 42% respectively which seems higher than the published series. Loss of MMR protein was found more significantly associated with the presence of other cancers (p=0.001), colon cancer (p=0.028), advanced stage of the disease, poor degree of differentiation, presence of medullary histology and extracellular mucin.

Karahan et al[11] in the Turkish population reported a positive correlation between MMR protein loss with the colonic location and poorer histology. Association of MMR protein loss with medullary histology has also been reported by other authors.[9,30] A study from Mexico suggested medullary colonic carcinoma with MMR deficiency having lower survival compared with conventional colonic adenocarcinoma.[6] The finding of our study may serve as a prognostic marker for patients with colorectal cancer. We did not find any significant correlation when compared with MMR deficient tumors with signet ring cell and mucinous histology similar to the earlier report.[32]

Out of 33 patients having MMR protein loss, 18 (54.5%) patients met the revised Bethesda criteria whereas 15 (45.5%) patients did not satisfy the revised Bethesda criteria.

This is not a very big gap, hence, if testing had been done solely based on revised Bethesda criteria, half of the patients carrying the MMR protein loss would have been missed as per our study. Hence, genotypic screening is necessary over the clinical screening based on Bethesda criteria which may be a fallacious insignificant percentage of patients.

Conclusion

MMR protein loss was found in 32% of the patients in the present series. The loss was significantly associated with medullary phenotype, poor degree of differentiation and presence of tumor-infiltrating lymphocytes (TILS). MMR testing is relatively inexpensive, hence should be performed routinely in every colorectal cancer patient and their suspected family members.

Acknowledgement

Authors sincerely acknowledge Dr. Narendra Krishnani, Department of Pathology, SGPGIMS for his kind support. Integral University, Lucknow is also acknowledged.

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(Received 13th May 2021, accepted 28th July 2021)