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Pathology Section

Immunohistochemical Expression of MutS Homolog 6 (MSH6) and Postmeiotic Segregation 2 (PMS2) and Their Association with Clinicopathological Features in Colorectal Carcinoma: A Cross-sectional Study

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ABSTRACT

Introduction: Colorectal Carcinoma (CRC) is a gastrointestinal malignancy arising from colon or rectum. Adenocarcinoma is the most common type of CRC. It is one of the most commonly diagnosed cancer worldwide, ranking third only to lung cancer and female breast cancer. About 15% of CRC cases are caused by Microsatellite Instability (MSI) due to loss of Mismatch Repair (MMR) proteins-MutL protein homolog 1 (MLH1), MutS protein homolog 2 (MSH2), MSH6 and Postmeiotic Segregation increased 2 (PMS2) which can be diagnosed using Immunohistochemistry (IHC). If any of this MMR protein expression is absent, it means MMR deficient (d-MMR). If all four MMR proteins are expressed, it means Mismatch Repair proficient (p-MMR). CRC with MSI plays a crucial role in tumourigenesis and prognosis.

Aim: To evaluate the immunohistochemical expression of MSH6 and PMS2 in CRC and to assess their association with clinicopathological parameters.

Materials and Methods: This cross-sectional study was conducted in the Department of Pathology at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab, India, from January 2023 to March 2024. A total of 41 histopathologically confirmed cases of CRC were included. IHC for MSH6 and PMS2 was performed. Clinicopathological parameters including age, gender, tumour size, histological grade, lymph node status, lymphovascular invasion and depth of invasion were analysed. Statistical analysis was done using Statistical Package for Social Sciences (SPSS) (version 26.0), Chi-square test or Fisher's-exact test was applied and p-value ≤0.05 was considered statistically significant.

Results: Loss of PMS2 expression was observed in 15 out of 41 (36.59%) cases, while loss of MSH6 expression was seen in 6 out of 41 (14.63%) cases. Concurrent loss of both markers was noted in 6 out of 41 (14.63%) cases, while isolated PMS2 loss was seen in 9 out of 41 (21.95%) cases. MMR deficiency (d-MMR) was significantly associated with tumour size (p=0.040), histological grade (p=0.002), lymphovascular invasion (p<0.001), lymph node involvement (p <0.001) and depth of invasion (p <0.001). No significant association was observed with age, gender, or tumour site.

Conclusion: The d-MMR is significantly associated with adverse clinicopathological parameters. Early-stage CRC with MSI have a better prognosis than proficient CRC. So, there is a substantial role of MMR proteins in CRC as a prognostic marker and for target therapy. IHC using a two-antibody panel (MSH6 and PMS2) is a reliable and cost-effective method for screening MSI in CRC.

Keywords: Adenocarcinoma colon, Immunostaining, Microsatellite instability Mutator S, Prognostic indicators, Tumour biomarkers

INTRODUCTION

Colorectal carcinoma (CRC) is a gastrointestinal malignancy which has emerged as a significant health challenge worldwide. According to Global Cancer statistics for world for the year (GLOBOCAN) 2022, CRC accounts for approximately 1.9 million new cases and 0.9 million deaths annually, ranking third only to lung cancer (12.4%) and female breast cancer (11.6%). Currently CRC is the seventh most common cancer in India with 65,358 new cases which were reported in 2021. In India, CRC is an emerging malignancy, with an estimated 65,000-70,000 new cases reported annually and a rising incidence particularly among younger individuals [1].

Three main pathways leading to the carcinogenesis of CRC are Chromosomal Instability (CIN), MSI and CpG methylator phenotype (CIMP) [2]. Microsatellites, also known as Short Tandem Repeats (STRs) are repeated sequences of 1-6 nucleotides. Each microsatellite has two parts, the central core and the peripheral flanks and is usually located near the coding region or intron region. MSI is due to mutation in MMR proteins of Deoxyribonucleic Acid (DNA) i.e., MLH1 (MutL homologue 1), MSH2 {Mutator S (MutS) homologue 2} , MSH6 (MutS homologue 6) and PMS2 (postmeiotic segregation 2). Mutations leading to MSI can be sporadic or germline as seen in Lynch syndrome. MSI can be of two types- High-MSI (MSI-H) and Low-MSI (MSI-L). MSI-H is defined as instability at ≥30% of loci studied and MSI-L is defined as instability at 1% to 29% of loci. MSI-H CRC accounts for 15% of all CRCs of which 12% are sporadic and 3% are Lynch syndrome [3,4].

Patients with deficient MMR CRC have distinct clinicopathological characteristics such as predominance in the proximal colon, poor differentiation, mucinous histology and increased lymphocytic infiltration. MSI-H CRCs also demonstrate distinct prognostic and therapeutic implications compared to microsatellite stable tumours [5,6].

Detection of MMR protein expression can be done by DNA- based MSI testing or by IHC. Both MSI testing and IHC are complementary to each other i.e., loss of MMR protein expression by IHC has been shown to correspond with DNA-based MSI testing with a good sensitivity (>90%) and an excellent specificity (100%). IHC has an advantage over Dna-based testing in that it is more widely available,

does not require molecular laboratory for testing and can also direct germline testing to that specific gene which helps in the identification of patients with Lynch syndrome [3,4,7].

If any of this MMR protein expression is absent, it is labelled as d-MMR which corresponds to MSI. If all the four MMR proteins are expressed, it is labelled as p-MMR and are expected to be Microsatellite Stable (MSS) [3].

The MMR proteins form heterodimer complexes, MLH1 with PMS2 and MSH2 with MSH6. Out of these MLH1 and MSH2 are the essential partners which stabilises the minor partners i.e., PMS2 and MSH6. Hence, loss of MLH1 and MSH2 leads to degradation of MSH6 and PMS2. But the reverse is not true as essential partners can bind with other minor MMRs. Therefore, loss of PMS2 and MSH6 will not always lead to loss of MLH1 and MSH2. As a result of this binding pattern, it has been proposed to use a two panel IHC test comprising of MSH6 and PMS2 instead of four panel IHC study [5].

Given the important role of MMR proteins and their deficiency leading to tumourigenesis and disease progression, their detection has become a relevant and promising target for anticancer therapies. So, there is substantial role of detection of MMR protein deficiency in CRC as a prognostic marker [8].

Despite the established role of d-MMR in CRC, limited data are available from the Indian population evaluating the diagnostic utility of a two-antibody panel. The selection of MSH6 and PMS2 is based on their role as secondary binding partners in MMR heterodimers, allowing effective detection of both MLH1- and MSH2-associated defects using a limited antibody panel. Therefore, the present study was undertaken to assess the immunohistochemical expression of MSH6 and PMS2 and to evaluate their association with clinicopathological parameters.

MATERIALS AND METHODS

The present study was a cross-sectional study and was conducted between 1st January 2023 to 31st March 2024 in Department of Pathology at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab, India on 41 histopathologically diagnosed cases of CRC in colectomy specimens. The study was approved by Institutional Ethics Committee (IEC No: SGRD/IEC/22-123).

Sample size: The sample size was calculated by analysing the number of colectomy specimen received in Pathology Department in last three years.

Inclusion criteria: Histopathologically proven cases of CRCs in colectomy specimens were included in the study.

Exclusion criteria: Inadequate tissue, small biopsies and previously treated cases (chemotherapy/radiotherapy) were excluded from the study.

Study Procedure

Tissue specimens were fixed in 10% neutral buffered formalin, processed and embedded in paraffin. Sections of 3-5 µm thickness were stained with Haematoxylin and Eosin (H&E) for histopathological evaluation. IHC was done to find out the expression of MSH6 and PMS2 using primary antibody Mouse Monoclonal Antibody (BIOCARE MEDICAL).

Immunohistochemistry (IHC): Paraffin-embedded tissue sections of 3-5 µm thickness were mounted on poly-L-lysine coated slides and dried overnight at 37°C. Sections were deparaffinised in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed using Ethylenediaminetetraacetic Acid (EDTA) buffer (pH 8.0) in a decloaker at 95°C for 60 minutes, followed by cooling to room temperature.

Endogenous peroxidase activity was blocked using hydrogen peroxide for five minutes. Sections were washed with Phosphate-Buffered Saline (PBS)/Tris buffer and incubated with protein block for five minutes to prevent non specific binding. Mouse monoclonal antibodies against MSH6 and PMS2 (Biocare Medical, USA) were used as per manufacturer's instructions and incubated for 1 hour in a moist chamber at room temperature.

After washing, sections were incubated with post-primary block for 30 minutes, followed by polymer for 30 minutes, with intermediate PBS washes. Immunoreactivity was visualised using Diaminobenzidine (DAB) as chromogen for 2-3 minutes. Sections were then washed, counterstained with haematoxylin, dehydrated through graded alcohols, cleared in xylene and mounted with Dibutylphthalate Polystyrene Xylene (DPX).

The stained slides were examined under light microscopy. The primary outcome was assessment of MSH6 and PMS2 expression. Secondary outcomes included correlation of their expression with histological grade and clinicopathological parameters such as age, tumour size, lymph node involvement and depth of invasion.

Interpretation of the IHC scoring: Positive controls were run with every batch of IHC. Nuclear immunostaining of normal epithelial cells, lymphocytes and stromal cells served as internal positive controls in each case. Interpretation was based on whether the cells were/were not showing MSH6 and PMS2 expression. Tumours showing complete loss of nuclear MSH6 and PMS2 were classified as MSH6 negative and PMS2 negative respectively. Carcinomas with normal expression of MSH6 or PMS2 were classified as MSH6 positive and PMS2 positive, respectively [9].

The results were interpreted as MSH6 positive: Nuclear expression is retained: MMR Proficient (MSS); MSH6 negative: Absent nuclear expression: MMR Deficient (MSI); PMS2 positive: Nuclear expression is retained: MMR Proficient (MSS); PMS2 negative: Absent nuclear expression: MMR Deficient (MSI).

Tumour was p-MMR; hence interpreted as negative for MSI. The negative nuclear expression of either MSH6 and PMS2 i.e., d-MMR was interpreted as positive for MSI [9].

The study evaluated age, gender, tumour size, histological grade, lymphovascular invasion, lymph node involvement and depth of tumour invasion.

STATISTICAL ANALYSIS

Data from the present study was systematically collected, compiled in Microsoft Excel and statistically analysed using Statistical Package of Social Sciences (SPSS version 26.0) to draw relevant conclusions. The observations were tabulated in the form of numbers and percentages. Categorical data was analysed using Chi-square test or Fisher's-exact test as appropriate. Level of significance was determined as p-value ≤0.05 as significant and p-value <0.001 as highly significant.

RESULTS

A total of 41 histopathologically confirmed cases of CRC were evaluated. The majority of patients were aged >50 years (24/41; 58.54%), with a female predominance (24/41; 58.54%). The most common tumour site was the ascending colon (21/41; 51.22%). Tumour size ≤5 cm was observed in 25/41 (60.98%) cases.

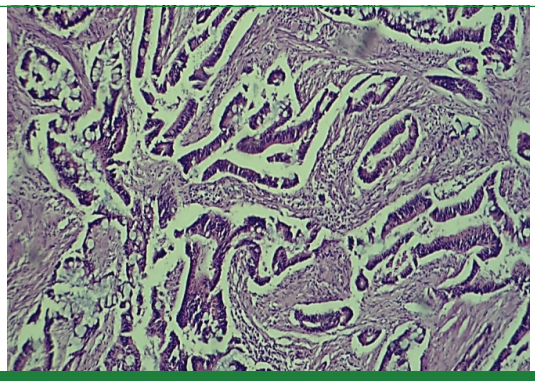
On histopathological examination, most tumours were moderately-differentiated i.e., Grade II (30/41; 73.17%), followed by well-differentiated i.e., Grade I (6/41; 14.63%) and poorly-differentiated i.e., Grade III (5/41; 12.20%) carcinomas. Mucinous/signet ring cell differentiation was identified in nine out of 41 (21.95%) cases. Lymphovascular invasion was present in 21/41 (51.22%) cases, all of which showed lymph node metastasis. Among these, 6/41 (14.63%) and 15/41 (36.59%) cases were categorised as N1 and N2 stage, respectively. Regarding depth of invasion, majority of tumours were staged as T3 (23/41; 56.10%).

Immunohistochemical analysis revealed loss of PMS2 expression in 15/41 (36.59%) cases and loss of MSH6 expression in 6/41 (14.63%) cases. Concurrent loss of both PMS2 and MSH6 was observed in 6/41 (14.63%) cases, while isolated loss of PMS2 was noted in 9/41 (21.95%) cases. No case demonstrated isolated loss of MSH6. Overall, d-MMR was identified in 15/41 (36.59%) cases, whereas 26/41 (63.41%) cases showed p-MMR status.

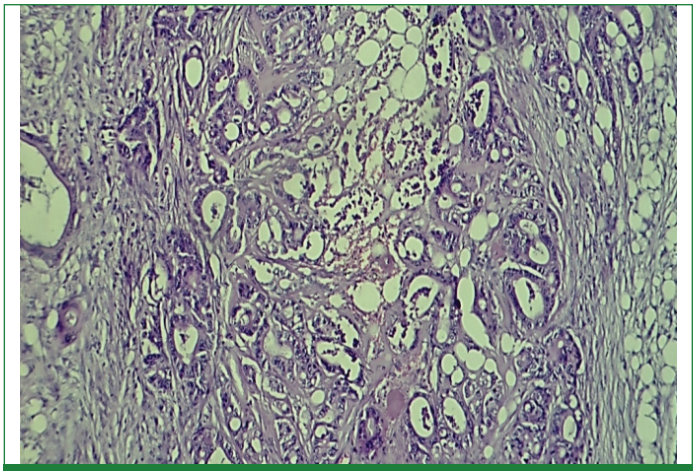
The d-MMR showed a statistically significant association with tumour size >5 cm (9/15; 60.00%; p=0.040), higher histological grade, with 10/15 (66.67%) cases being moderately-differentiated and 5/15 (33.33%) poorly-differentiated (p=0.002), lymphovascular invasion (14/15; 93.33%; p <0.001), lymph node involvement (14/15; 93.33%; p <0.001) and advanced tumour stage (p <0.001). Among d-MMR cases, the majority were staged as T3 (12/15; 80.00%), followed by T4 (3/15; 20.00%). No statistically significant association was observed between MMR status and age (p=0.697), gender (p=0.607), or tumour site [Table/Fig-1]. Representative histopathological and immunohistochemical findings are illustrated in [Table/Fig-2-9].

Characteristics	IHC normal n=26 (MMR-proficient)	IHC abnormal n=15 (MMR deficient)	Total n=41	p-value
Age				
≤50 years	11 (42.31%)	6 (40.00%)	17	0.697
>50 years	15 (57.69%)	9 (60.00%)	24	
Gender				
Male	10 (38.46%)	07 (46.66%)	17	0.607
Female	16 (61.53%)	08 (53.33%)	24	
Size*				
≤5 cm	19 (73.07%)	06 (40.00%)	25	0.040
>5 cm	07 (26.92%)	09 (60.00%)	16	
Tumour grade				
I	06 (23.08%)	0	06	0.002
II	20 (76.92%)	10 (66.67%)	30	
III	0 (0)	5 (33.33%)	5	
Lymphovascular invasion				
Present	07 (26.92%)	14 (93.33%)	21	<0.001
Absent	19 (73.07%)	01 (6.67%)	20	
Lymph node involvement				
Involved	07 (26.92%)	14 (93.33%)	21	<0.001
Not involved	19 (73.07%)	01 (6.67%)	20	
N stage				
N0	19 (73.08%)	1 (6.67%)	20	<0.001
N1	03 (11.53%)	3 (20.00%)	06	
N2	04 (15.38%)	11 (73.33%)	15	
Depth of invasion				
T1	01 (3.84%)	0	01	<0.001
T2	14 (53.84%)	0	14	
T3	11 (42.30%)	12 (80.00%)	23	
T4	0 (0)	3 (20.00%)	03	
Mucinous/signet ring cell differentiation				
Present	02 (7.69%)	07 (46.67%)	09	0.003
Absent	24 (92.30%)	08 (53.33%)	32	

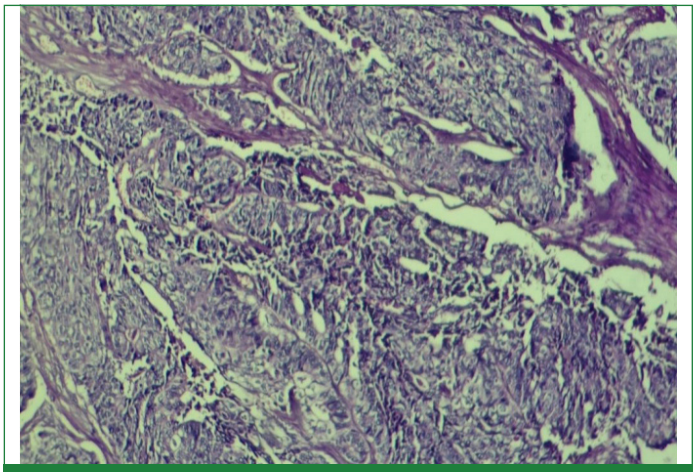
[Table/Fig-1]: The comparison of clinicopathological parameters between deficient and proficient MMR groups.
Tumour size was categorised using a 5 cm threshold in accordance with previous studies evaluating the association between tumour size and mismatch repair status and clinicopathological characteristics in colorectal carcinoma [14].



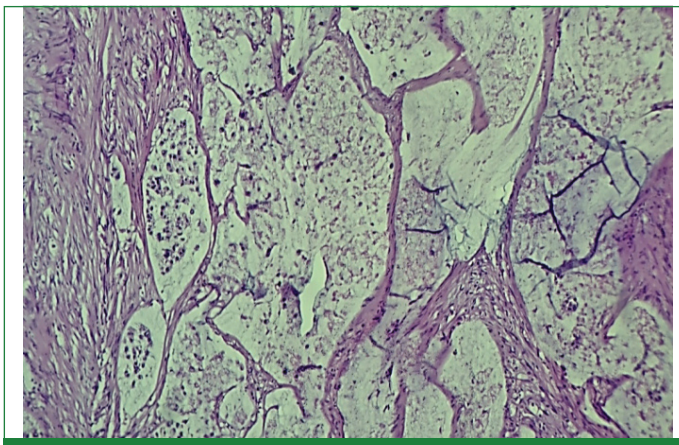
[Table/Fig-2]: Well-differentiated adenocarcinoma (H&E, 100x).



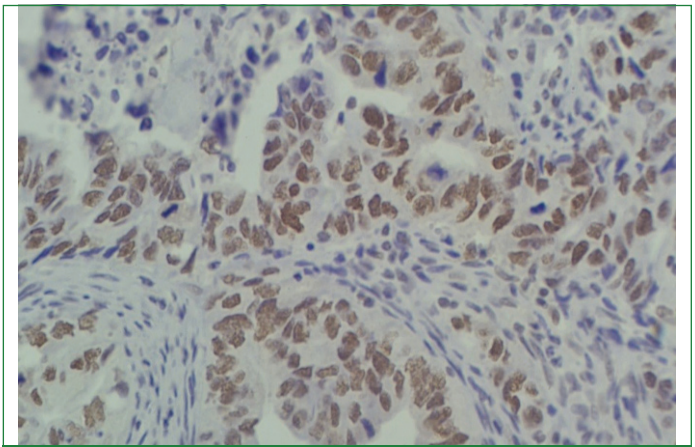
[Table/Fig-3]: Moderately-differentiated adenocarcinoma (H&E, 100x).



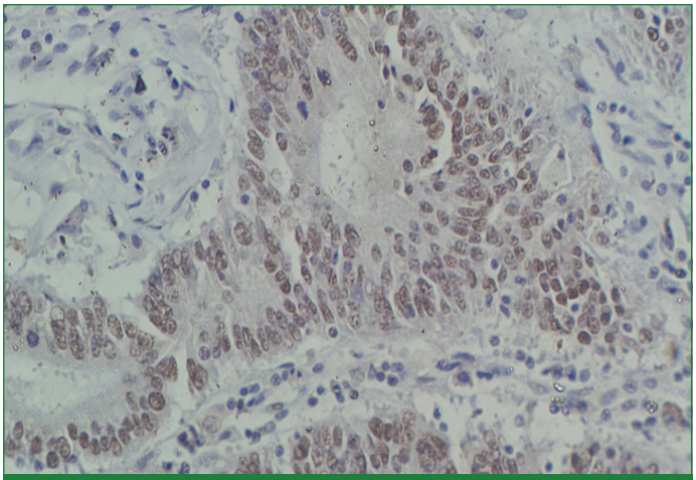
[Table/Fig-4]: Poorly-differentiated adenocarcinoma (H&E, 100x).



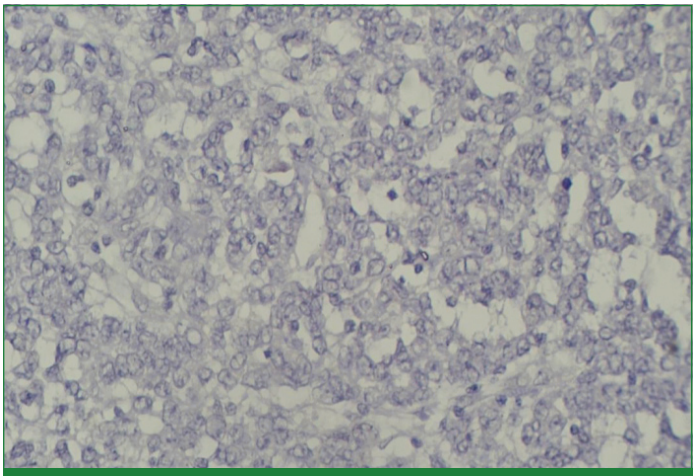
[Table/Fig-5]: Mucinous/signet ring cell differentiation in Colorectal Carcinoma (CRC) (H&E, 100x).



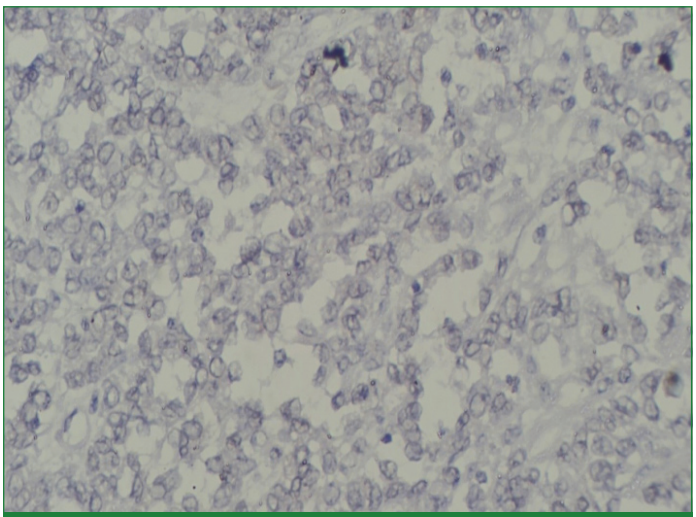
[Table/Fig-6]: Showing nuclear staining of PMS2 in tumour cells in Colorectal Carcinoma (CRC) (IHC, 400x).



[Table/Fig-7]: Showing nuclear staining of MSH6 in tumour cells in Colorectal Carcinoma (CRC) (IHC, 400x).



[Table/Fig-8]: Showing absent nuclear staining of PMS2 in tumour cells in colorectal Carcinoma (CRC) (MMR Deficient) IHC, 400x.



[Table/Fig-9]: Showing absent nuclear staining of MSH6 in tumour cells in Colorectal Carcinoma (CRC) (MMR Deficient) IHC, 400x.

DISCUSSION

The present study evaluated the frequency of d-MMR using IHC for PMS2 and MSH6 in CRC and analysed its association with various clinicopathological parameters, including tumour grade, tumour size, lymph node status and depth of invasion.

In the present study, which was conducted on 41 histopathologically proven cases of CRC, d-MMR was observed in 15/41 (36.59%) cases. Among these, 6/15 (40.00%) cases demonstrated concurrent loss of PMS2 and MSH6, while 9/15 (60.00%) cases showed isolated loss of PMS2. No case exhibited isolated loss of MSH6. These findings are comparable to those reported by Grillo F et al., and Hashmi AA et al., where d-MMR was observed in 39.00% and 34.00% cases, respectively [10,11]. However, variations have been documented in other studies. Lee CT et al., reported isolated loss of PMS2 and MSH6 in 15.25% and 9.32% cases, respectively, with concurrent loss observed in only 1.69% cases [12]. Similarly, Zeng Z et al., reported 8.3% cases with PMS2 defects and 3.1% of patients had isolated PMS2 defects [13].

The MMR-deficient CRC exhibited distinct clinicopathological features. A majority of cases i.e., 9/15 (60.00%) had tumour size greater than 5 cm, which is in concordance with the findings of Zhao L [14]. Furthermore, 12/15 (80.00%) cases showed invasion up to the serosa (T3 stage), consistent with studies by Lee CT et al., and Hashmi AA et al., where 72% and 76.5% MMR-deficient tumours, respectively were classified as T3/T4 stage [11,12].

Regarding histological grade, 10/15 (66.67%) MMR-deficient cases were moderately-differentiated, while 5/15 (33.33%) were poorly-differentiated. Additionally, mucinous/signet ring cell differentiation was observed in 7/15 (46.66%) MMR-deficient cases, compared to 2/26 (7.69%) MMR-proficient cases. These findings are consistent with those reported by Chew MH et al., and Hashmi AA et al., which also demonstrated a higher frequency of mucinous differentiation i.e., 25.4% and 26.4%, respectively in MMR-deficient tumours [11,15].

A significant association was observed between d-MMR and lymphovascular invasion, with 14/15 (93.33%) cases showing lymphovascular invasion. Among these, the majority of cases belonged to higher nodal stages, with 11/15 (73.33%) cases classified as N2. These findings differ from those reported by Hashmi AA et al., and Ismael NE et al., where lower rates of lymphovascular invasion (27% and 40.9% respectively) were observed, suggesting possible regional and population-based differences [11,16].

Despite the relatively small sample size, a higher frequency of d-MMR CRC was observed, emphasising the importance of routine MMR screening, particularly in younger patients and in the context of hereditary syndromes such as Lynch syndrome. Given the limited data available from the Indian population, further large-scale studies incorporating molecular techniques such as MSI testing and mutation analysis are warranted.

In the present study, PMS2 deficiency was more frequently observed than MSH6 deficiency. PMS2 loss is commonly associated with MLH1 deficiency, which may occur due to promoter hypermethylation or germline mutations. Therefore, additional molecular testing, such as BRAF mutation analysis, may be useful in distinguishing sporadic cases from a germline mutation in the MLH1 gene.

Previous studies by Yuan L et al., Shia J et al., and Hall G et al., have demonstrated that a two-antibody panel (PMS2 and MSH6) is highly effective in detecting d-MMR [17-19]. In these studies, all MLH1-deficient tumours also showed loss of PMS2 expression, while all MSH2-deficient tumours demonstrated loss of MSH6 expression. Notably, no cases showed isolated loss of MLH1 or MSH2. The sensitivity (100%) and specificity (98.2%) of the two-antibody panel with only PMS2 and MSH6 were found to be comparable to those of the conventional four-antibody panel, supporting its use as a cost-effective and efficient screening method.

Thus, comparing the present results with the previous similar studies, this two panel (MSH6 and PMS2) has the potential to possibly replace the existing four panel (MLH1, MSH2, MSH6 and PMS2) study. The findings of these studies, along with those of the present study, support the use of a two-antibody panel for routine screening of d-MMR, as summarised in [Table/Fig-10] [17-19].

Overall, the findings of the present study highlight the significant association between d-MMR and adverse clinicopathological features in CRC, underscoring the importance of routine MMR screening in clinical practice. The findings of the present study are in agreement with previous studies and further support the utility of a two-antibody panel and this two panel (MSH6 and PMS2) has the potential to possibly replace the existing four panel (MLH1, MSH2, MSH6 and PMS2) study.

Study	Number of patients	Total number of MMR deficient cases	PMS2 alone deficient n (% of MMR-deficient cases)	MSH6 alone deficient n (% of MMR-deficient cases)	Concurrent loss of PMS2 and MSH6 n (% of MMR-deficient cases)
Yuan L et al., (2015) [17]	296	72	01 (1.39%) (n=72)	6 (8.33%)	01 (1.38%)
Hall G et al., (2010) [19]	344	104	01 (0.96%) (n=104)	01 (0.96%)	02 (1.92%)
Shia J et al., (2009) [18]	232	70	09 (12.86%) (n=70)	06 (8.57%)	0
Present study, (2026)	41	15	09 (60.00%) (n=15)	0	06 (40.00%)

[Table/Fig-10]: Comparison of multiple studies depicting the efficacy of 2 panel IHC (PMS2 and MSH6) in detection of all patterns of MMR deficiency (d-MMR) [17-19].

Limitation(s)

The present study had certain limitations. The sample size was relatively small, which may limit the statistical power and generalisability of the findings. Being a single-centre study, the results may not fully represent the broader population. Molecular confirmation of MSI using Polymerase Chain Reaction (PCR) or next-generation sequencing was not performed due to resource constraints, which could have strengthened the diagnostic accuracy. Additionally, only a two-antibody panel (MSH6 and PMS2) was used and evaluation of the complete four-antibody panel (MLH1, MSH2, MSH6, PMS2) was not performed. Long-term follow-up data and survival analysis were also not included, limiting assessment of prognostic outcomes.

CONCLUSION(S)

The CRCs are slowly becoming one of the leading cancers in the world. MMR proteins (PMS2 and MSH6) show normal nuclear staining in the stromal cells and normal crypts. Lack of protein expression in tumour cells of at least one MMR protein is defined as MMR-deficient in which MSI is seen. The present study concluded that MMR status has got direct relationship with the size of the tumour, histological grade of the tumour, lymphovascular invasion, depth of tumour invasion and lymph node status. Early stage CRC with MSI have better prognosis than proficient CRC as they have improved disease-free survival and overall survival with treatment. So, there is a substantial role of MMR proteins in CRC as a prognostic marker and for target therapy. Thus, with MMR proteins, we can help to provide the patient with prognostic information and better treatment modalities.

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