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Mismatch Repair Protein Expression in Colorectal Carcinoma by a Four-Antibody Immunohistochemical Panel (MLH1, MSH2, MSH6 & PMS2) & its Correlation with Histomorphological Parameters

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Abstract:
Background: *Colorectal carcinomas (CRC) are one of the most common malignant neoplasms in industrialized countries and are responsible for a significant proportion of cancer related deaths. It is the fourth leading cause of cancer-related deaths in the world. The cause for CRC ranges from germline mutations such as the adenomatous polyposis coli genes to a completely environmental risk factor such as excess body mass index at the other end. Both MSI and the four antibody IHC have similar value in predicting germline mutation, the concordance rate between these two tests is less than perfect. The aim of our study is to assess the mismatch repair deficiency in colorectal cancer by a four antibody immunohistochemical panel and to compare the mismatch repair deficiency study by immunohistochemical panel with histopathological parameters in colorectal carcinoma.*
Materials and Methods: *A cross-sectional study was conducted in the Department of Pathology in collaboration with the Department of Surgery, Regional Institute of Medical Science, Imphal. All Colon & Rectum specimens received in the department of Pathology for histopathological examination, RIMS, Imphal were included in the study. A four-antibody panel of MMR proteins including MLH1, MSH2, MSH6 and PMS2 was performed by using DAKO EnVision method on the representative paraffin-fixed tissue blocks.*
Results: *Sex distribution of the CRC cases showed a M: F ratio of 1.7:1. Most of the patients were ≥50 years of age. In our study, MMR protein loss was observed in one third of the study population cases. MMR protein loss was more common in males, older patients & right sided colon cancer. Perineural Invasion & lymphovascular invasion was rarely seen in these cases. Mostly constituted with low grade tumours with highest number of not otherwise specific variant of Tumour.*
Conclusion: *This study demonstrates high frequency of MMR protein loss in colorectal cancer of Manipuri population as compared to other Indian Studies. MMR protein loss was more common in males, older patients & right sided colon cancer. Focal necrosis and tumour infiltrating lymphocyte were present in majority of the tumours. Although they add a big burden on the pathologists, all clinicopathological and histological parameters should be assessed in all CRC for the sake of predicting MSI.*
Key words: *Colorectal carcinoma, MMR proteins, MSI*

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I. Introduction

Colorectal carcinomas (CRC) are one of the most common malignant neoplasms in industrialized countries and are responsible for a significant proportion of cancer related deaths. It is the third most common cancer in men (10,06,019 cases, 10.9% of the total cancers) and the second in women (794958 cases, 9.5% of the total cases) worldwide. Almost 55% of the cases occur in more developed regions. It's the fourth leading cause of cancer-related deaths in the world. Incidence rates of CRC vary 10- fold in both sexes worldwide, the highest rates being estimated in Australia/New Zealand and Western Europe, the lowest in Africa (except Southern Africa) and South-Central Asia. [1]

Within Asia, the incidence rates of CRC vary widely and are uniformly low in all south Asian countries and high in all developed Asian countries like Japan, South Korea and Singapore. CRC is considered one of the clearest markers of the cancer transition, whereby countries undergoing rapid societal and economic changes show rapid increases in cancers.[2]

Fortunately, the age adjusted incidence rates of CRC in all the Indian cancer registries are among the lowest rates in the world.[3] In India, the Age adjusted rates (AARs) for colon cancer and rectal cancer in men are 4.4 and 4.1 per 100000, respectively. In the 2013 report, the highest AAR in men for CRCs was recorded in Thiruvananthapuram followed by Bangalore and Mumbai. Population based cancer registries show a rising trend in the incidence of CRC in India .[4] The incidence rates of CRC in Indian immigrants to the United Kingdom and USA are much higher, suggesting that life styles and dietary habits are important in the causation of the CRC. It indicates that the economic transition from a low income to middle incomeeconomy, there will be a big increase in the burden of CRC in India. Worldwide its burden is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030. [3]

The incidence of colorectal carcinomas (CRC) is still low in Manipur with a prevalence of 3.09 in 100000 population. Higher incidences of rectal involvement have been observed. CRC occurs at younger age group in Manipur which might be due to hidden risk factors.[5]

With advancement in medical sciences & screening for adenomas and early detection of CRC using fecal occult blood, flexible sigmoidoscopy, colonoscopy & other modalities have helped to prevent further progression of CRC which reduces mortality & morbidity.[6]

The cause for CRC ranges from germline mutations such as the adenomatous polyposis coli genes to a completely environmental risk factor such as excess body mass index at the other end. Life style and dietary factors are responsible for over two thirds of all CRC. There is a hot pursuit to identify the genes causing CRC, in the post-genomic era.[7]

Molecular Pathways behind Colorectal Cancer:

There are at least three main molecular pathways underlying the development of colorectal cancer (CRC):

1. Chromosomal instability (CIN)pathway:

CIN is the most prevalent molecular cause of genomic instability in CRC, so it is an original genetic basis of about 65%–70% of all sporadic CRC tumors.[8-11]CIN is characterized with an imbalance in number of chromosomes (aneuploidy), chromosomal amplification, and a high frequency of loss of heterozygosity resulting in some deleterious mutationsin tumor suppressor genes such as APC and TP53, and oncogenes including KRAS.[12-13]

2. Microsatellite instability (MSI) pathway:

It includes about 8%–20% of all CRC tumors which is more common in stage II (20%) than stage III (12%) and stage IV (4%).[14-15] This genetic change is a molecular fingerprinting for DNA- mutation in mismatch repair (MMR) system deficiency because of germline mutations or epigenetic changes in MMR genes.[9,16]

3. CpG island methylator phenotype (CIMP) pathway:

This is the epigenetic molecular changes leading to alteration in gene expression or gene function without any change in its DNA sequence.[17] For instance, CIMP within specific sites of promoter could lead to silencing of some vital tumor suppressor genes concluding tumor development which is found in about 35% of CRC tumors.[18-19]

DNA Repair systems and MMR:

There are a number of repair mechanisms that are used to identify and remove the various specific types of DNA lesions. Cellular repair mechanisms are divided into direct and indirect ones according to the molecule used for repair and the time period of repair.

A. Direct Repair:

1. Proof reading
2. O6-methylguanine DNA methyltransferase (MGMT)
3. Pyrimidine dimers

B. Indirect Repairs:

1. Excision repair system
2. Recombination repair system
3. Mismatch repair system[20]

Mismatch repair (MMR) system:

This system recognizes and repairs small loops within the duplex DNA that arise from nucleotide misincorporation – either by base–base mismatches or by insertion/deletion loops. Defects in MMR lead to genome-wide instability, particularly in simple repetitive sequences (known as MSI).[15] A genetic loss of this system leads to a reduced repair mechanism and hence leads to an accelerated accumulation of potential mutations. Because of the loss of this repair mechanism, the error rate during replication is increased to 100–1000-fold, with the loss of a mechanism that plays an important role in maintaining the structure and function of

DNA. Increased predisposition to certain types of cancers has been linked to the MMR system, especially to CRC, where genetic failure of the MMR system is responsible for 15–20% of cases.[20,21, 22]

Functions of MMR:

The MMR system serves as an excision/resynthesis system, the functioning of which can be divided into four distinct steps:

a. Recognition of the mismatch within the repetitive duplex DNA by MutS proteins.
b. Recruitment of the enzymes that function to repair the lesion in the mismatched DNA.
c. Excision of the mismatch base or incorrect sequence.
d. Resynthesis of the DNA along the parental template strand by DNA polymerase.[23]

What is Microsatellite Instability (MSI)?

Microsatellite instability (MSI) is a hypermutable phenotype caused by the loss of DNA mismatch repair activity. Changes of any length due to either insertions or deletions of repeating units in a microsatellite within a tumour compared to normal tissue. Seen in different tumors such as colorectal, stomach, endometrium, ovarian, sebaceous carcinoma, glioblastomas, and lymphomas.[24-25]

Mechanism of MSI:

Underlying defect in the MMR system accelerates accumulation of single nucleotide mutations and alterations in the length of these simple repetitive sequences. Microsatellite regions have been found to be at high risk of mutations because of the slippage of the DNA polymerase during the replication process (Box 1). As a consequence of these 'replication errors', either frameshift mutations, leading to the nonfunctional proteins, or protein truncations occur in the affected cells. Replication errors commonly affect genes that include or are linked to microsatellite repeats regions. Such genes are TGFbRII, ILGF, E2F-4, and BAX.[24,25]

MSI in colorectal carcinoma

MSI is a hallmark of averagely 15% of CRCs and more than 90% of colorectal cancers that arise in patients with Lynch syndrome. Most of MSI CRC tumors are sporadic usually due to epigenetic silencing of MLH1 promoter because of somatic hypermethylation. These contain about 12%–15% of all CRCs in which lack of MLH1 function could lead to fast accumulation of mutations in other genes like TGF‐β and BAX resulting in tumor development.[19] Meanwhile, a somatic hereditable hypermethylation of MSH2 gene promoter has been also recently reported which is rarely occurred by some large deletion mutations in last exon of EPCAM, a gene located next to MSH2, or EPCAM‐MSH2 locus.[26] A few of MSI‐ CRC tumors including about 2%–3% of the all CRC tumors is related to Lynch syndrome (LS), a hereditary predisposing cancer syndrome, which is mainly due to a germline mutation in one of the four DNA‐MMR genes: MLH1, MSH2, PMS2, and MSH6.[27] The colorectal carcinoma microsatellite profile provides useful prognostic information, showing the patients with microsatellite unstable neoplasms have a better overall survival rate and a modified response to conventional chemotherapy.[28]

Box 1: Location of important MMR genes on human chromosomes
MLH1 (MutL homolog 1) is located on chromosome 3p21
MSH2 (MutS homolog 2) on chromosome 2p21–22
PMS2 (postmeiotic segregation 2) on chromosome 7p22
MSH6 (MutS homolog 6) on chromosome 2p16

What are Clinical Applications of Microsatellite Instability testing in colorectal Cancer?

Historically, at least three clinical applications could be considered for MSI testing in CRC:

1. Prognostic, 2. Diagnostic & 3. Predictive applications.

Detection MSI and MMR defects:

A. Clinical criteria:

The recognition that certain types of cancers cluster in families with HNPCC and that cancer develops at relatively early ages compared with the general population provided the rationale for development of criteria that could be used to aid in the diagnosis. Two sets of criteria (the Amsterdam criteria and Bethesda guidelines) developed by a consensus of experts, have been most widely accepted and best studied.

a) Amsterdem II criteria:
-Three or more relatives with histologically confirmed colorectal cancer or cancer of the endometrium, small bowel, ureter or renal pelvis one affected relative being a first degree relative of the other two; FAP should be excluded
-Two or more successive generations are affected
-At least one relative was diagnosed before the age of 50 years.[29,30]

a) Revised Bethesda guidelines:
One or more of the following criteria must be met:
-Colorectal cancer before the age of 50 years
-Synchronous or metachronous colorectal cancer or other HNPCC-related tumours, regardless of age
-Colorectal cancer with MSI-high morphology before the age of 60 years
-Colorectal cancer (regardless of age) and a first-degree relative with colorectal cancer or an HNPCC-related tumour before the age of 50 years
-Colorectal cancer (regardless of age) and two or more first or second-degree relative diagnosed with colorectal cancer or an HNPCC-related (regardless of age)[29,30]

A. Clinical testing for MSI and MMR

Because of the limitations of relying on clinical criteria to guide testing, it has been proposed that tumours from patients with colorectal cancer be evaluated for markers of HNPCC regardless of the family history. Although genetic testing remains the gold standard for detecting MSI, the College of American Pathologists (CAP) recommends an initial IHC workup using a four-antibody panel including MLH1, MSH2, MSH6, and PMS2 which detects the presence or absence of protein products.

Immunohistochemistry (IHC) testing

Pathogenic mutations in MMR proteins usually lead to the absence of a detectable gene product providing the rational for immunohistochemistry testing to determine loss of expression. Tumours from patients suspected to have MSI can be stained for MMR proteins and the surrounding normal tissues can be used as a positive control. IHC has an advantage over MSI analysis as it is much easier to perform and less expensive. Moreover, it provides gene specific information to direct further genetic analysis

B. Histomorphological Analysis.

a. Tumor features:

i. Mucinous histology:

Extracellular mucin accumulation bounded either by neoplastic epithelium or stroma. Tumors were subgrouped as mucinous histology being absent, <10%, 10–50%, and >50% of tumor area involved.

ii. Signet ring cells:
Presence of tumor cells with intracytoplasmic mucin and peripherally displaced crescent-shaped nucleus, whether present within extracellular mucin pools or infiltrating stroma.

iii. Cribriform growth pattern:
Neoplastic epithelial islands with sharp punched out glandular spaces. Semi-quantitative subgrouping into 10–50% and >50% was done.

iv. Poor differentiation:
Solid or sheet-like pattern of tumor cells in more than 70% of tumor.

v. Medullary pattern:
Sheets, trabeculae, or nests of small- to medium-sized tumor cells exhibiting syncytial pattern, frequent mitosis, and abundant stromal lymphocytic infiltration.

vi. Mixed growth pattern:
Distinct and different growth patterns adjacent to each other in the same histological section.

vii. Necrosis:
Presence of dirty necrosis. Subgrouped into focal and widespread.[31]

b. Host immune response features Crohn's-like peri-tumoral reaction:

Pronounced lymphoid reaction to tumor, composed of lymphoid follicles with germinal centres at tumor edges, not associated with either mucosa or pre- existing lymph node. Two or more large lymphoid aggregates in a section were required for the presence of this feature.
Intra-tumoral lymphocytic infiltrate:

The presence of small round lymphocytes within neoplastic epithelial cells. This category was subgrouped into mild to moderate (up to three intra-epithelial lymphocytes (IEL/HPF) and marked (>3 IEL/HPF) in accordance with the CAP guidelines.[32]

Need for the Immunohistochemistry study:

Both MSI and the four antibody IHC have similar value in predicting germline mutation, the concordance rate between these two tests is less than perfect. The sensitivity of IHC in predicting MSI is about 92%. This is because both tests may miss cases that are detectable by the other. Specifically, MSI may detect cases that have abnormalities in MMR genes that are not covered by the IHC antibody panel and therefore not detectable by IHC; on the other hand, IHC can detect MSH6 mutation cases that may not show high frequency MSI and therefore can be missed by MSI testing. The main reasons for choosing IHC are as follows:

a. Easily available:

- IHC is available as part of the routine service in the general pathology laboratories, it constitutes a convenient technique to general pathologists.

b. Inexpensive technique:

- The cost of IHC and MSI testing may vary among different countries and health care systems. Earlier analysis indicated that IHC was about 2-3less expensive than MSI testing.

c. Helps to identify the mutated Gene:

- IHC reveals which particular MMR gene may be defective, and as such it enables efficient mutation analysis on the target gene. Such ability is not possessed by MSI testing. The ability of IHC to identify the mutated gene also encourages the use of alternative procedures in cases in which standard methods fail.

d. IHC May Detect MMR-deficient cases that can be missed by MSI testing:

- Mutations in MSH6 tend to result in weaker or no MSI in the tumors.

Such MSH6 cases may be missed by MSI testing but can be detectable by MSH6 IHC.[32]

Literature review suggests that IHC with MLH1/MSH2 has a lower sensitivity than MSI testing in predicting gene mutation; however, inclusion of PMS2 and MSH6 significantly increases the sensitivity of IHC, resulting in a predictive value that is virtually equivalent to that of MSI testing.[33] This study is planned to assess the mismatch repair deficiency in colorectal cancer by a four antibody immunohistochemical panel and to compare the mismatch repair deficiency study by immunohistochemical panel with histopathological parameters in colorectal carcinoma

II. Materials And Methods

Study design: Cross-sectional study.

Study location: Study was carried out in the Department of Pathology in collaboration with the Department of Surgery, Regional Institute of Medical Science, Imphal.

Duration of study: Two years from August 2018 to July 2020.

Study population: All Colon & Rectum specimens received in the department of Pathology for histopathological examination, RIMS, Imphal were included in the study.

Inclusion criteria:

i) Histopathologically confirmed colorectal cases.

ii) Those who have given written consent for undergoing the study was also included in the study.

Exclusion criteria:

i. Patient with Colorectal Carcinoma who are being treated with chemotherapy and/or radiotherapy.

ii. Inadequate sample

Sample size: 46

(All the colorectal samples received in the department of pathology, RIMS in the study period of 2 years were included in the study)

Procedure Methodology:

I. IMMUNOHISTOCHEMICAL EVALUATION:

A four-antibody panel of MMR proteins including MLH1, MSH2, MSH6, and PMS2 was performed by using DAKO EnVision method on the representative paraffin-fixed tissue blocks. According to the CAP protocol for immunohistochemistry interpretation, any nuclear staining even patchy is taken as"no loss of expression" and only absolute absence of nuclear staining shall be considered "loss of expression" provided internal controls are positive. Hence, carcinoma was considered dMMR when there was absence of nuclear staining for at least one protein. Adjacent normal colonic epithelium, lymphocytes, and stromal cells was used

as positive internal controls. Expression of proteins was grouped into six categories: no loss of expression, loss of expression of all four proteins, combined loss of MLH1/PMS2, combined loss of MSH2/MSH6, isolated loss of PMS2 and isolated loss of MSH6.

Procedure methodology:
Samples was collected as per the guidelines of inclusion and exclusion criteria. The preparation of the tissues preserved in buffered formal saline for light microscopy involves the following steps:

- **Fixation:** The specimens received after gross examinations were fixed in 10% formal saline overnight. In case of any abnormal appearing area on gross examination, some additional sections were taken.
- **Processing:** The tissue pieces were wrapped in filter paper and put inside the tissue basket. Then it was dehydrated with ascending grades of ethyl alcohol and cleared in xylene in automated tissue processor.
- **Block making:** After clearing, the tissue was impregnated with molten paraffin and Leuckhart's L-Blocks were used to make paraffin blocks.
- **Section cutting:** With the help of rotary microtome, the tissue blocks were cut into sections of 3-5µm thickness. The sections were put into floatation bath and subsequently transferred to albumenised glass slides.
- **Deparaffinisation:** Sections were treated with xylene to deparaffinise.
  - Xylene was washed off with graded alcohol (100% - 90% - 70%and water)
- Staining with Haematoxylin and Eosin was done. Special staining was adopted when needed.

Histopathological Features

One H&E slide per case was reviewed. Histopathological evaluation of tumor features and host response was done using the following criteria.

Tumor features

a. Mucinous histology
Extracellular mucin accumulation bounded either by neoplastic epithelium or stroma. Tumors was subgrouped as mucinous histology being absent,
<10%, 10–50% and >50% of tumor area involved.[13]

b. Signet ring cells
Presence of tumor cells with intracytoplasmic mucin and peripherally displaced crescent-shaped nucleus, whether present within extracellular mucin pools or infiltrating stroma.

c. Cribriform growth pattern
Neoplastic epithelial islands with sharp punched out glandular spaces. Semi-quantitatively subgrouping was done as 10–50% and >50%.

d. Poor differentiation
Solid or sheet-like pattern of tumor cells in more than 70% of tumor.

e. Medullary pattern
Sheets, trabeculae, or nests of small- to medium-sized tumor cells exhibiting syncytial pattern, frequent mitosis, and abundant stromal lymphocytic infiltration.

f. Mixed growth pattern
Distinct and different growth patterns adjacent to each other in the same histological section.

g. Necrosis
Presence of dirty necrosis. Sub-grouped into focal and widespread.

h. Host immune response features:

- **Crohn's-like peri-tumoral reaction:**

Pronounced lymphoid reaction to tumor, composed of lymphoid follicles with germinal centres at tumor edges, not associated with either mucosa or pre-existing lymph node. Two or more large lymphoid aggregates in a section was required for the presence of this feature.[14]

- **Intra-tumoral lymphocytic infiltrate**

The presence of small round lymphocytes within neoplastic epithelial cells. This category was sub-grouped into mild to moderate (up to three intraepithelial lymphocytes (IEL)/HPF) and marked (>3 IEL/HPF) in accordance with the CAP guidelines.

Statistical Analysis:
Data entry and analysis was done using SPSS Version 21.0 (Armonk NY: IBM Inc.). Descriptive statistics like mean, standard deviation, percentage and proportion were used.

III. Results

A total 46 resected specimens of colorectal carcinoma sent for histopathological examinations were studied, the following results were recorded and analysed.

Table-1: Socio Demographic Profile (N=46)

Variables		No. of Cases	Percentage (%)
Sex	Male	29	63
	Female	17	37
Age	<50 years	17	37
	>50 years	29	63
Religion	Hinduism	33	71.7
	Christianity	10	21.7
	Islam	03	06.6
Family History	Yes	01	02.1
	No	45	97.9
Marital Status	Yes	46	100
	No	00	00
Occupation	Business	10	21.7
	Farmer	06	13
	House Wife	13	28.3
	Labourer	02	04.3
	Service Holder	15	32.7

Total 29 males and 17 females were included in our study. The present sample comprises of 63% of males & 37% of females and all are married. 37% cases were below 50 years and 63% were ≥50 years. The mean age of the patient was 54.2 years, with age ranges from 22 years to 80 years. Manipur is a Hindu dominated state and therefore Hinduism has highest percentage (71.7%). Service holders and housewives are the commonest occupations with respective percentage of 32.7%, 28.3% and next to them were the business (21.7%), Farmers (13%) and labourer (4.3%) respectively. All the above findings are illustrated in the Table-1.

Table-2: Clinicopathological characteristics of CRC included in the study (N=46)

Features		N (%)
Laterity	Right Sided	22 (47.8)
	Left Sided	24 (52.2)
LVI*	Present	10 (21.7)
	Absent	36 (78.3)
PNI**	Present	02 (04.3)
	Absent	44 (95.7)
Grade	I	03 (06.6)
	II	35 (76.0)
	III	08 (17.4)
Variant Tumour	Adenocarcinoma NOS ***	38 (82.6)
	Mucinous	07 (15.3)

	Signet Ring	01 (02.1)
T stage	T1	01 (02.1)
	T2	04 (08.7)
	T3	38(82.6)
	T4	03(06.6)
N stage	N0	23(50)
	N1	10(21.7)
	N2a	07(15.3)
	N2b	06(13)

*(*LVI: Lymphovascular Invasion, **PNI: perineural Invasion, ***NOS: Not otherwise specified;)*
Our study comprises with 22(47.8%) CRC of right sided and 24(52.2%) were left sided (Fig-2A & Fig-2B). Lymphovascular Invasion was present in only 10(21.7%) cases and perineural invasion was present in only 2(4.3%) cases. The study population mainly comprises of Grade II CRCs with 35(76%) cases followed by grade III with 8(17.4%) cases. The most common type of CRC was adenocarcinoma NOS with 38(82.6%) cases followed by 7(15.3%) cases of mucinous carcinoma and 1(2.1%) case of signet ring carcinoma. The most common T stage was T3 with 38(82.6%) cases followed by T2 stage with 4(8.7%) cases. N0 stage with 23(50%) cases were the most common according to N staging. All the above findings are illustrated in Table-2.

Fig-1A: Photograph of a gross specimen of Carcinoma Rectum(APR) which includes part of sigmoid colon, rectum and anus without perforation (Complete TME Grade-III). The tumour was taken as left sided because it was situated between the splenic flexure to the rectum.

Fig-1B: Photograph of cut Section of APR Specimen- An Infiltrative type of tumor originating from rectum and involving circumferentially.

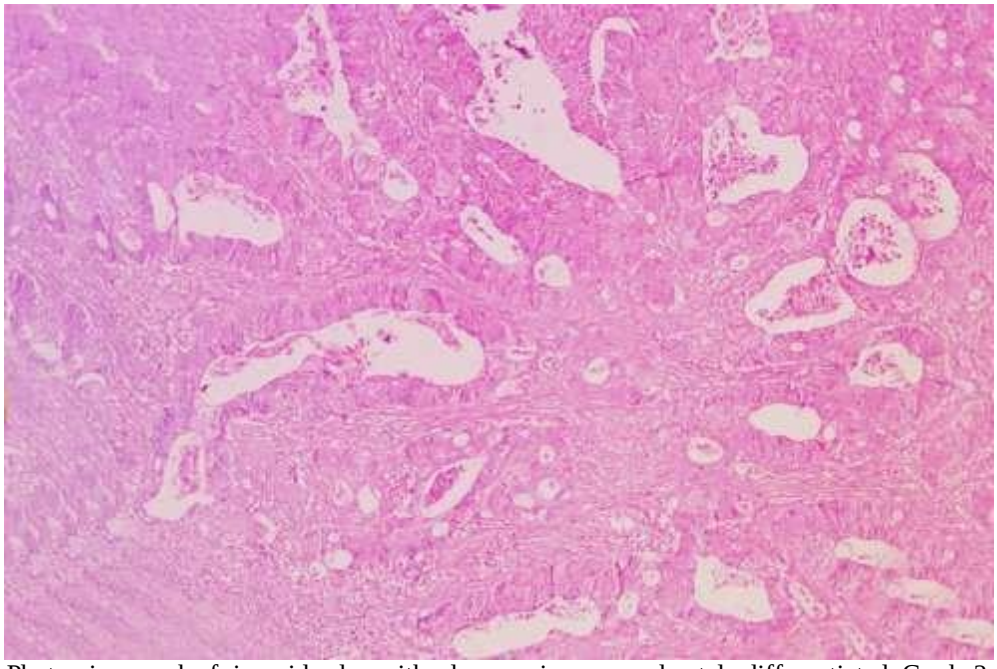

Fig-2A: Photomicrograph of sigmoid colon with adenocarcinoma, moderately differentiated, Grade 2 showing malignant cells arranged in glandularpattern. (10x, H & E Staining)

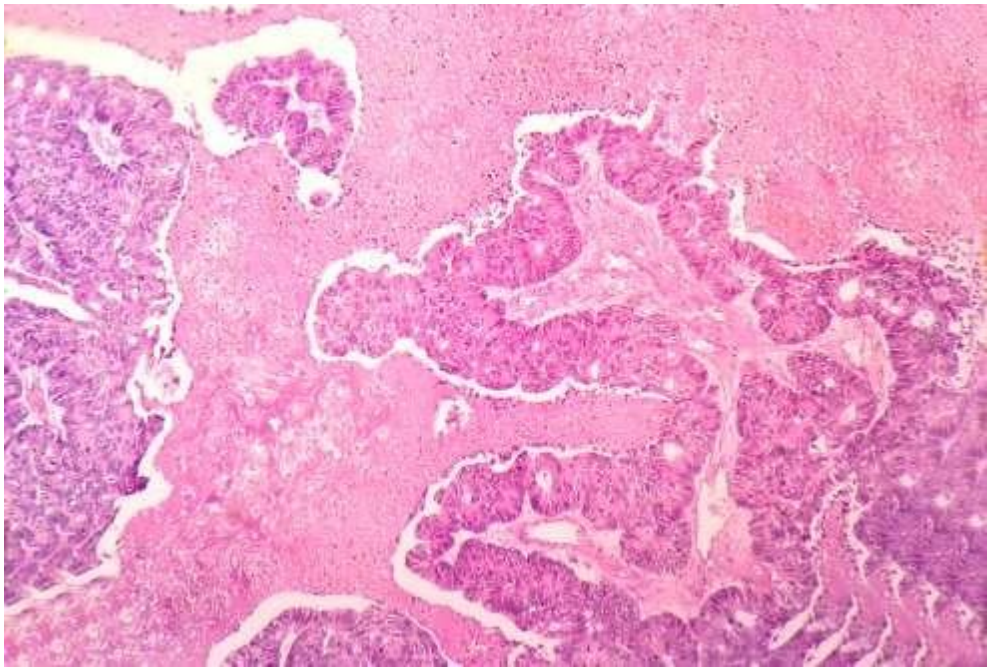

Fig-2B: Photomicrograph of descending colon with adenocarcinoma, moderately differentiated, grade 2, showing malignant cells arranged in glandular pattern with widespread areas of necrosis. (10x, H & E Staining)

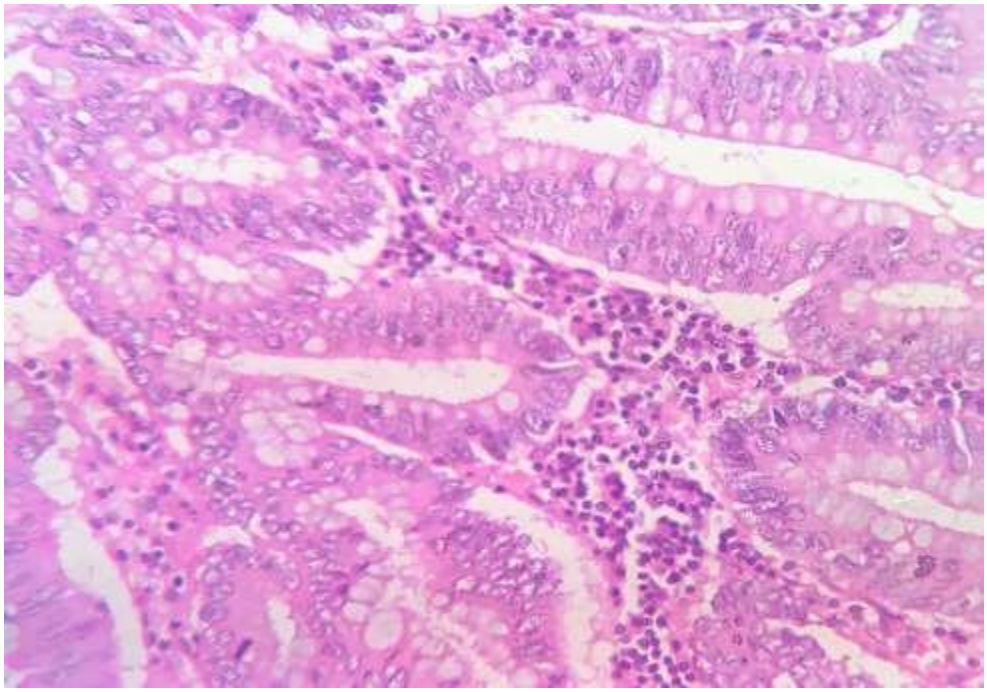

Fig-2C: Photomicrograph of colonic tissue with adenocarcinoma showing malignant cells arranged in glandular pattern. Some of the tumor cells show intracellular mucin, many intra tumoural lymphocytes and peritumoural lymphocytes in between the malignant glands. (40x, H & E Staining)

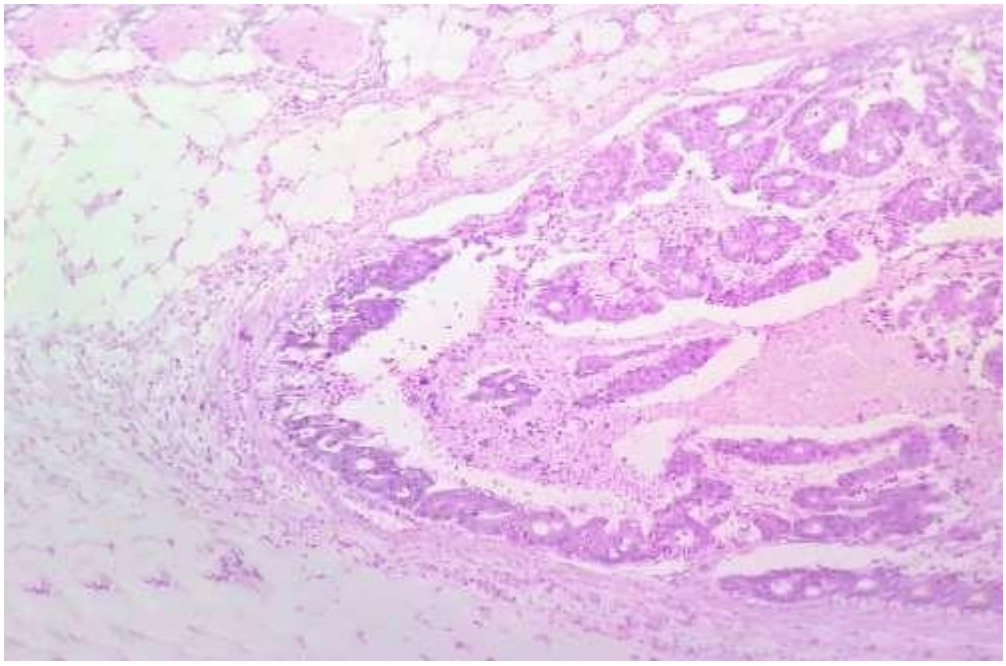

Fig-2D: Photomicrograph showing a lymphnode where adenocarcinoma colon metastasized. (H & E staining, 10x)

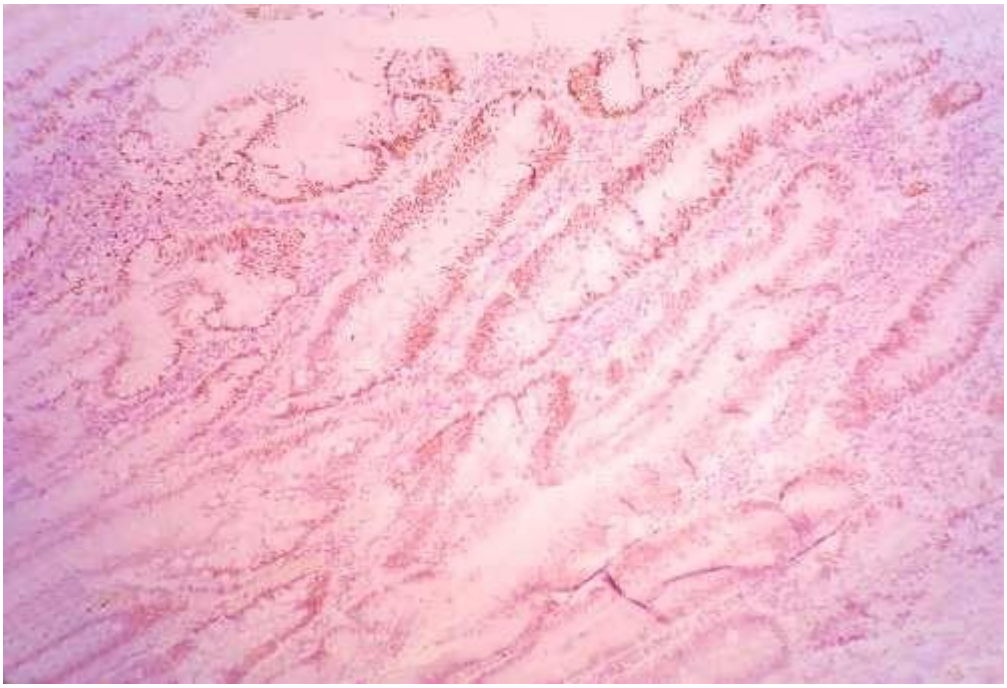

Fig-3A: Photomicrograph showing immunohistochemical stain positivity for MSH2 marker, more than 60% malignant cells nucleus are stained positive with negative internal controls. (IHC staining for MSH2 marker, 10x)

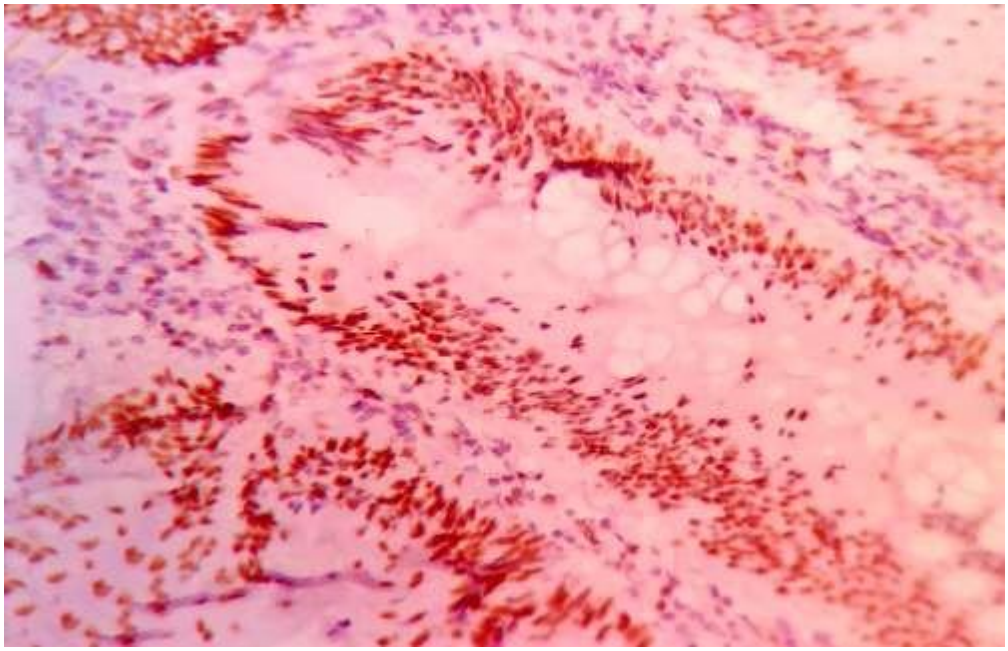

Fig-3b: Photomicrograph showing immunohistochemical stain positive for MSH2 marker, more than 80% nucleus of malignant cells are stained positive with negative internal controls. (IHC staining for MSH2 marker, 40x)

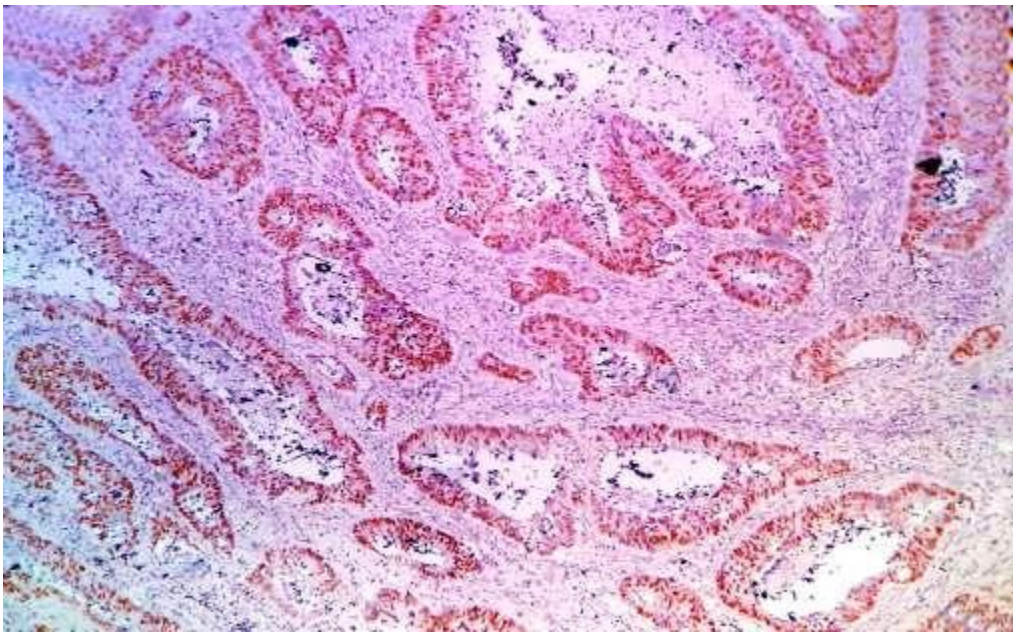

Fig- 4A: Photomicrograph showing immunohistochemical stain positivity for MLH1 marker, more than 90% nucleus of malignant cells are stained positive with negative internal controls. (IHC staining for MLH1 marker, 10x)

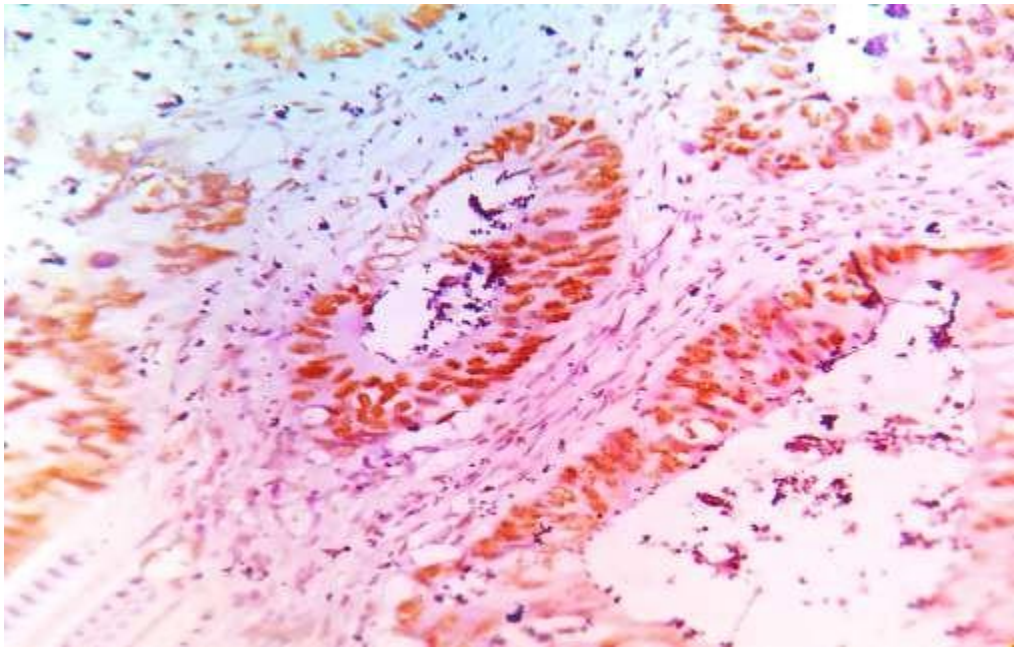

Fig- 4B: Photomicrograph showing immunohistochemical stain positive for MLH1 marker, more than 90% nucleus of malignant cells are stained positive with negative internal controls. (IHC staining for MLH1 marker, 40x)

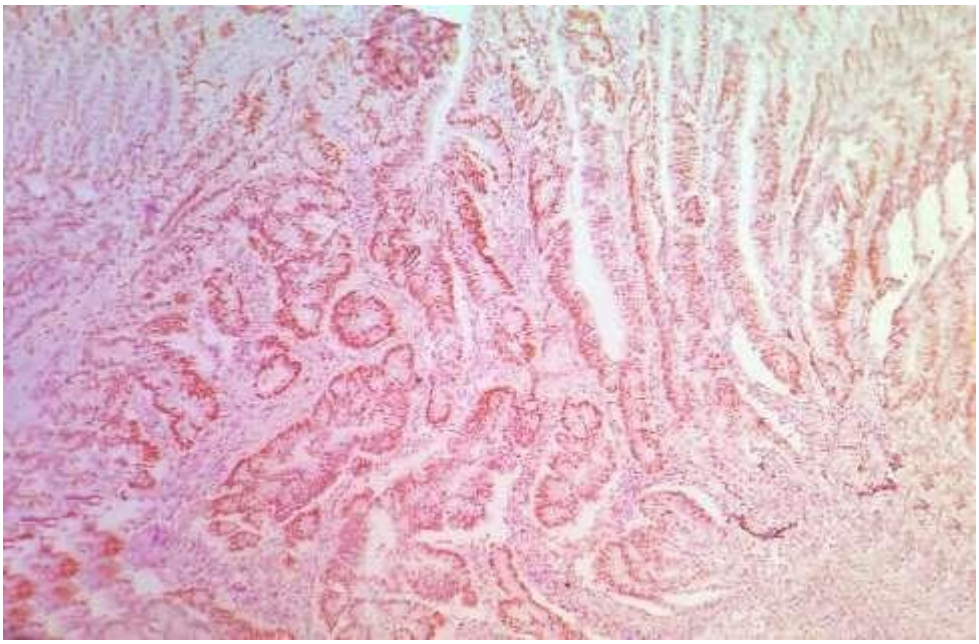

Fig-5A: Photomicrograph showing immunohistochemical stain positivity for MSH6 marker, more than 90% nucleus of malignant cells are stained positive with negative internal controls can be compared. (IHC staining for MSH6 marker, 10x)

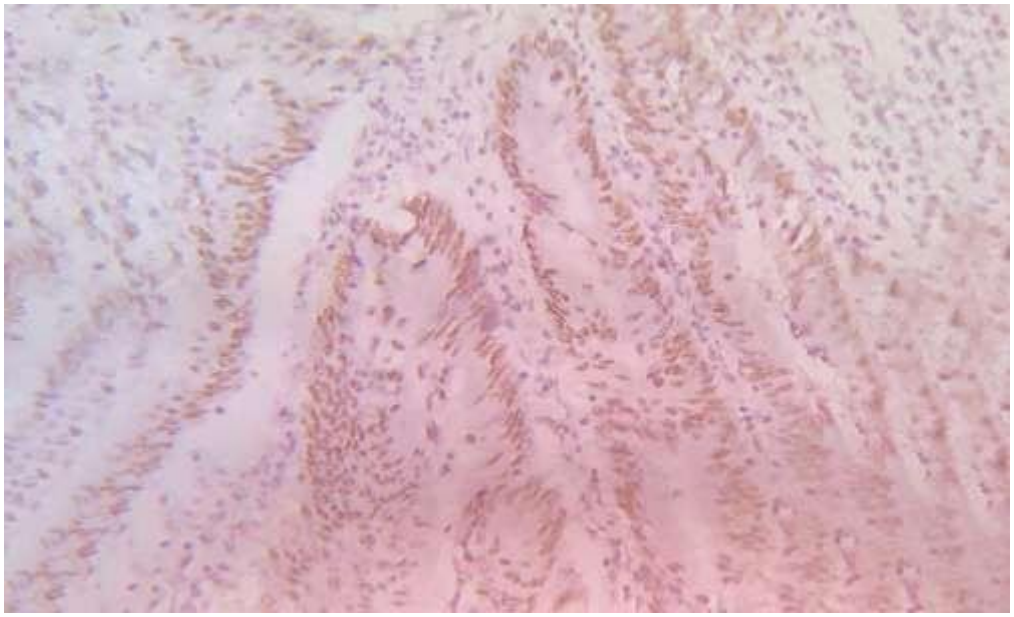

Fig-5B: Photomicrograph showing immunohistochemical stain positivity for MSH6 marker. Here more than 90% nucleus malignant cells are stained positive with negative internal controls. (IHC staining for MSH6 marker, 40x)

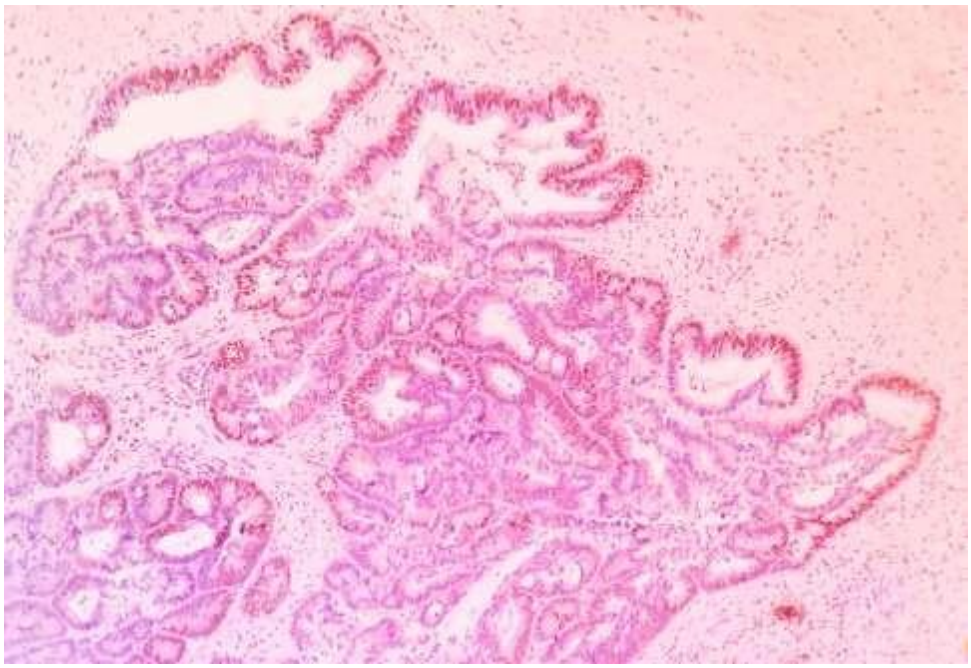

Fig-6A: Photomicrograph showing immunohistochemical stain positive for PMS2 marker, more than 40% nucleus of malignant cells are stained positive with negative internal controls. (IHC staining for PMS2 marker, 10x)

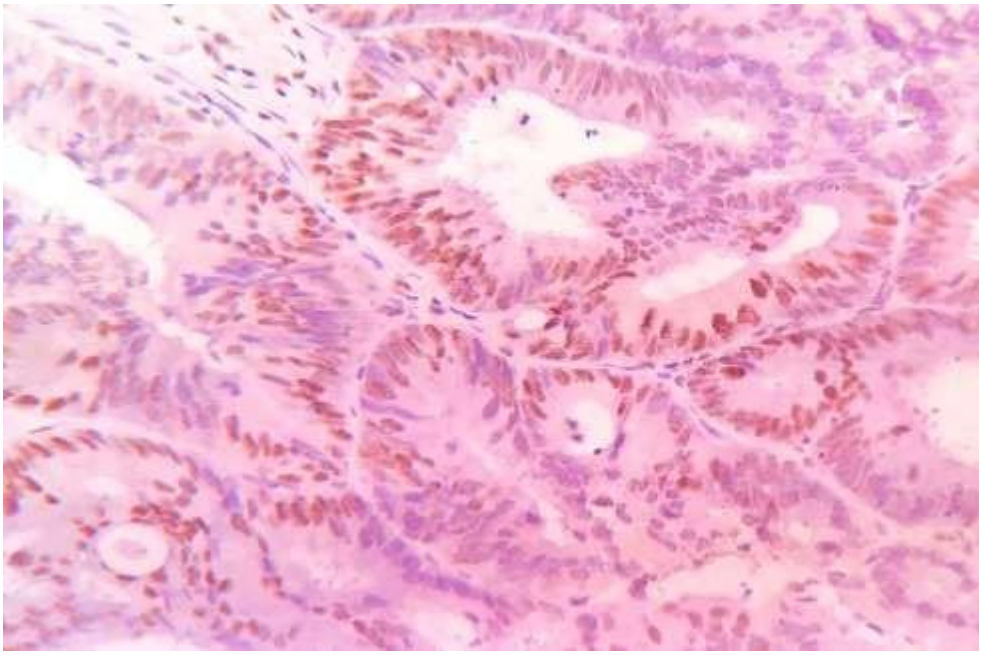

Fig-6B: Photomicrograph showing immunohistochemical stain positive for PMS2 marker, approx. 70% nucleus of malignant cells are stained positive with negative internal controls. (IHC staining for PMS2 marker, 40x)

All the assessed clinicopathological parameters categories are illustrated and correlated with the pattern of expression of the 4 markers of MSI in Table- 3 & Table-4.

Table-3: Expression pattern of MMR protein & clinicopathological characteristics of CRC(N=46)

Features		**No Loss Express--ion (n=31) (%)**	**Loss of expression of all markers (n=2) (%)**	**MLHI1 &PMS2 loss (n=8) (%)**	**MSH2 & MSH6 loss (n=2) (%)**	**Isolated PMS2 loss (n=1) (%)**	**Isolated MSH6 loss (n=2) (%)**
Gender	Male	20(64.5)	1(50)	5(62.5)	1(50)	1(100)	1(50)
	Female	11(35.5)	1(50)	3(37.5)	1(50)	00	1(50)
Age	<50 yrs	13(41.9)	0	3(37.5)	1(50)	00	00
	>50 yrs	18(58.1)	2(100)	5(62.5)	1(50)	1(100)	2(100)
Laterity	Right	13(41.9)	1(50)	6(75)	0	1(100)	2(100)
	Left	18(58.1)	1(50)	2(25)	2(100)	00	00
LVI*	Present	5(16)	1(50)	2(25)	1(50)	00	00
	Absent	26(84)	1(50)	6(75)	1(50)	1(100)	19(100)
PNI**	Present	2(6.5)	00	00	00	00	0
	Absent	29(93.5)	2(100)	8(100)	2(100)	1(100)	2(100)
Grade	I	1(3.2)	00	2(25)	00	00	00
	II	24(77.4)	1(50)	6(75)	1(50)	1(100)	2(100)
	III	6(19.4)	1(50)	00	1(50)	00	00
Variant	NOS[#]	26(83.9)	1(50)	7(87.5)	1(50)	1(100)	2(100)
	Muc[##]	4(12.9)	1(50)	1(12.5)	1(50)	00	00
	Sign[###]	1(3.2)	00	00	00	00	00
T stage	T1	1(3.2)	00	00	00	00	00
	T2	3(9.7)	00	1(12.5)	00	00	00
	T3	25(80.6)	1(50)	7(87.5)	2(100)	1(100)	2(100)
	T4	2(6.5)	1(50)	00	00	00	00

N stage	NO	14(45.2)	00	5(62.5)	1(50)	1(100)	2(100)
	N1	7(22.6)	00	2(25)	1(50)	00	00
	N2a	5(16.1)	1(50)	1(12.5)	00	00	00
	N2b	5(16.1)	1(50)	00	00	00	00

*(*LVI: Lymphovascular invasion; **PNI: Perineural Invasion; #NOS: Not otherwise specified; ##MUC: Mucinous and ###Sign: Signet ring)*

Among our study population, 31(67.4%) cases were observed without any loss of markers i.e. microsatellite stable. This pattern was observed predominantly in males with 20(64.5%) cases and older population. 18(58.1%) tumours were located in the left side. Lymphovascular invasion was absent in 26(84%) & perineural invasion was absent in 29(93.5%) cases. This pattern was predominantly consisting of Grade II tumours 24(77.4%) cases, T3 stages with 25(80.6%) cases and N0 stages with 14(45.2%) cases. Adenocarcinoma Not otherwise specific was the most common variant with 26(83.9%) cases. In our study, MMR protein loss was found in 15 (32.6%) patients out of 46 patients. We have observed 5(Five) patterns of MMR protein loss i.e. I) loss of all the four Markers (MLH1, MS2, MSH2 & MSH6) in 2(4.3%) cases, II) Combined loss of MLH1 & PMS2 in 8(17.6%) cases

III) Combined loss of MSH2 & MSH6 in 2(4.3%) cases, IV) Isolated loss of PMS 2 in 1(2.1%) case and V) Isolated loss of MSH 6 in 2(4.3%) cases. Among these most common MMR protein losses was combined loss of MLH1 & PMS2. Out of these 15 cases, 9(60%) cases were Male and 6(40%) cases were female patients. 4(26.6%) were younger than 50 years and the remaining 11(73.3%) were ≥ 50 years of age. MSI was more common in right sided tumour with 10(66.6%) cases. Among the MMR protein loss were mostly Grade II with 11(73.3%) cases and not otherwise specific variant with 12(80%) cases. 13(86.6%) cases were T3 stage and 9(60%) cases were in N0 stage. Perineural invasion was not seen in any case in our study. Overall in whole, total loss of MLH1 was present in 10(21%), loss of PMS2 in 11 (23.9%), loss of MSH2 in 4 (8.6%) and loss of MSH6 in 6(13%) patients.

Table-4: Expression pattern of MMR protein and histopathological characteristic of CRC (N=46)

Features		**No Loss Express--ion (n=31) (%)**	**Loss of express--ion of all markers (n=2) (%)**	**MLHI1 &PMS 2 loss (n=8) (%)**	**MSH2 & MSH6 loss (n=2) (%)**	**Isolated PMS2 Loss (n=1) (%)**	**Isolated MSH6 Loss (n=2) (%)**
Mucinous component	<10%	3(9.7)	00	1(12.5)	00	00	1(50)
	10-50%	2(6.5)	00	1(12.5)	00	00	00
	>50%	3(9.7)	1(50)	1(12.5)	1(50)	00	00
	Absent	23(74.2)	1(50)	5(62.5)	1(50)	1(100)	1(50)
Signet Ring	Present	4(12.9)	1(50)	1(12.5)	1(50)	00	00
	Absent	27(87.1)	1(50)	7(87.5)	1(50)	1(100)	2(100)
***TIL**	Absent	20(64.5)	00	1(12.5)	1(50)	00	1(50)
	Mild to mod**	8(25.8)	2(100)	4(50)	1(50)	1(100)	1(50)
	Marked	3(9.7)	00	3(37.5)	00	00	00
PTL***	Absent	25(80.6)	2(100)	5(62.5)	1(50)	1(100)	1(50)
	Mild to mod	5(16.1)	00	3(37.5)	1(50)	00	1(50)

	Marked	1(3.2)	00	00	00	00	00
Necrosis	Focal	21(67.7)	1(50)	5(62.5)	1(50)	1(100)	2(100)
	Wide[#]	7(22.6)	1(50)	1(12.5)	1(50)	0	0
	Absent	3(9.7)	00	2(25)	00	00	00

(*TIL: Tumour infiltrating lymphocyte, **Mod: Moderate, ***PTL: Peritumoral lymphocytic response, [#]Wide: Widespread)

Among the Microsatellite stable cases, Mucinous component was absent in 23(74.2%) cases and Signet ring component was absent in 27(87.1%) cases. Tumour infiltrating lymphocyte was not seen in 20(64.5%) cases. Peritumoral lymphocytic response was not seen in 25(80.6%) cases. Necrosis was focally present in 21(67.7%) cases and absent in 3(9.7%) cases. Among the microsatellite instable pattern, Mucinous component was absent in 32(69.5%) cases, <10% was present in 5(10.8%) cases, 10-50% were present in 3(6.5%) cases and lastly >50% were present in 6(13.2%) cases. Among MMR protein loss cases mucinous component was absent in 9(60%) cases and >50% component was present in 3(20%) cases. Signet ring component was absent in 12(80%) cases of MSI. There were no tumor-infiltrating lymphocytes in 23(50%) of total cases, while 17(36.9%) exhibited mild to moderate TILs and 6(13%) showed marked TILs. In tumors with loss of expression of all markers 100%(2/2) of the cases exhibited TILs. TILs were seen in 87.5% (07/8) of tumors with MLH1/PMS2 loss and 50% (1/2) of tumors with MSH2/MSH6 loss. Peritumoral lymphocytic response was not seen in of tumors with abnormal expression of all MMR proteins and isolated loss PMS2. PTL was seen in 37.5% (3/8) of tumors with MLH1/PMS2 loss, 50% (1/2) of tumors with MSH2/MSH6 loss and 50%(1/2) of tumors with isolated MSH6 loss. Focal necrosis was seen in 66.6%(10/15) cases of all MSI cases and necrosis was absent in 13.3%(2/15) cases of MSI.

IV. **Discussion**

The incidence of colorectal cancer is increasing in many developing countries, with westernization of lifestyle.[2] A higher risk of colorectal cancers was found in subjects consuming a diet poor in fiber and rich in meat.[5]

Our study includes 29(63%) number of males and 17(37%) number of females with a M:F ratio of 1.7:1 in this study compared to 1.2–1.5:1 in the western population reported by Bray F et al[1] with male preponderance. It was 2.1:1 in a study reported from south India by Peedikayil MC et al[77] and 3:1 in a study in north India reported by Kumar A et al.[43] Another study by Franklyn J et al[78] in south India which comprises of only right colon cancer, male to female ratio was 2.4:1. In yet another study by Kumari P et al[79] from Rajasthan (west India), the male to female ratio was 1.7:1.20. This shows an overall higher male to female ratio in India with large regional variations within India which could be due to a referral bias in terms of seeking treatment at a referral centre.

Total 17(37%) number of our patients were under 50 years which was exactly similar to a finding reported by Hashmi AA et al[45]. It was 46% in a study by Soliman NA et al[39] conducted in Egypt and another study by Yuan L et al[53] in China was 50.4%. It was 34.6% in another study in India conducted by Kumar A et al.[43] Comparatively only 10% cases were reported in USA by Bray F et al.[1] A large regional variation in the age distribution is seen in India with cases less than 50 years representing 20–50% of the total cases.[42,77,78,79] Colorectal cancer in India thus appears to be more frequent in younger patients compared to the west, with large regional variations which could be large proportion of young population in India.

According to the data from the SRS Statistical Report 2018, 66% of the population is in the age group 15–59 years.[82]

MMR protein loss of 15(32.60%) in the present study comparable to the finding in studies conducted by Hashmi AA et al[45] and Mojtahed A et al.[60] It was slightly higher than previously reported Indian studies. Earlier, studies from India by Dubey AP et al[48], Kumar A et al[43], Pandey V et al[81], Malhotra P et al[82] have reported MMR protein loss from 17.8–29%. Highest number with 66.9% cases was reported by Soliman NA et al[39] in Egypt. A study from the UK reported by Colling R et al[83] was of 21% while another reported by Lee-Kong SA et al[84] it as 19%. Most of studies from the west have reported MMR protein loss from 15–21%.[83-85] Yuan L et al[53] from China reported MMR loss in 26% patients. The frequency of loss of expression was found to be quite variable in different studies.

Frequency of expression pattern of MMR proteins:

No loss of expression of any MMR proteins (31 cases):

Total 31(67.4%) cases show no loss of expression of any MMR proteins i.e. they are microsatellite stable, similar with earlier studies conducted all over the world.[33, 39, 45,53, 60, 61,63,69,71] Male dominance is seen with 64.5% cases, Hashmi AA et al[45]found 57% and highest 71.4% was observed by Yuan L et al.[53]It comprises slightly older patients with 18(58.1%) of cases ≥ 50 years old.

Most of the tumours are located in left-side (58.1%) where highest percentage was observed by Yaun L et al[53] with 82.3%. Most of the cases of this pattern reveal no lymphovascular invasion (LVI) (84%) or perineural invasion (93.5%). According grading, most of the tumours were grade II (77.4%), followed by Grade III. The most common T stage was T3 with 25(80.6%) cases and followed by T3 with 3(9.7%) cases. Lymphnode involvement was seen most of the cases,

lymphnode was involved in 17 cases whereN1, N2a and N2b stages were 22.6%, 16.1% and 16.1% respectively. The most common variant of tumour was Adenocarcinoma NOS (83.9%) followed by Mucinous and Signet ring type of 12.9%, 3.2% respectively. Among this pattern, mucinous component was absent in 23(74.2%), signet ring component was absent in 27(87.1%). Tumour infiltrating lymphocyte were absent in majority of the cases (64.5%) and Peritumoral lymphocytic response (PTL) also not seen in most of the cases with 25 (80.6%) cases. Many cases show necrosis, mainly focal necrosis in 21(67.7%) of the cases followed by widespread necrosis with 7(22.6%) cases.

The above findings were comparatively similar to other studies mentioned earlier.[33,39, 45,53,60,61,63,69,71]

Loss of expression of at least one MMR proteins (15 cases):

In the 15 tumours with at least one MMR protein loss, five patterns were observed. The most frequent expression pattern was combined loss of MLH1 & PMS2 in 8(53.3%) cases, followed by loss of all the four Markers (MLH1, MS2, MSH2 & MSH6) in 2(13.3%) cases, combined loss of MSH2 & MSH6 in 2(13.3%) cases, isolated loss of PMS 2 in 1(6.6%) case and isolated loss of MSH 6 in 2(13.3%) cases. Similar patterns of expression were observed in many studies.[33,39,45,53,60,61,63,66,69,71]

Combined loss of expression of MLH1 and PMS2 proteins (8 cases):

This the most common pattern seen in 8(53.3%) cases with MMR Protein loss which is similar with other studies. [33,39,45,53,60,61,63,66,69,71]The highest cases with this pattern of expression were found by Hall G et al[61] with 86% of MSI and lowest was seen found by Soliman NA et al[39]with 37.6% of MSI cases.

Male dominance was seen with 62.5% of the cases was male. Most of MSI patients were ≥ 50 years old, 62.5% of them are. Majority of the lesions were situated in the right side, 6(75%) cases. Lymphovascular invasion was present only 2(25%) cases.

There was no perineural invasion in this pattern of cases. They were grouped in two grades, 75% of cases were grade II and remaining 25% cases were grade I. Stage T3(87.5%) was the most common stage followed by T2(12.5%). Lymph node involvement was not seen in most of the cases, involvement was seen in only 3 cases i.e. 2(25%) cases of N1 and 1(12.5%) case of N2a.

Predominantly Adenocarcinoma NOS variant with 7(87.5%) cases were seen, only 1(12.5%) case of mucinous carcinoma variant was seen. Mucinous component was absent in majority of the cases, only present in three cases with varying amount. Signet ring component was absent in 7(87.5%) cases.

Tumour infiltrating lymphocyte (TIL) was seen in most of the cases, mild to moderate amount was seen in 4(50%) cases and marked infiltration was seen in 3(37.5%) cases. Peri-tumoral lymphocytic response (PTL) was absent in majority of the cases, mild to moderate amount was seen in 3(37.5%) cases. 5(62.5%) cases show focal necrosis.

The above findings were comparatively similar to other studies mentioned earlier.[33,39,45,53,60,61,63,66,69,71]

Loss of expression of all MMR proteins (2 cases):

Total 2(13.3%) cases show this pattern and both of them were ≥ 50 years old. This pattern was seen in 20.5% of Pakistani population observed by Hashmi AA et al[45], 1.2% in Egyptian study conducted by Soliman NA et al[39] and 1% in Chinese study conducted by Wang Z et al.[35]

Lymphovascular invasion (LVI) was present in 1(50%) case and absent in another case. Perineural invasion (PNI) was not seen in both the cases. One tumour was graded as grade II (50%) & another was graded as Grade 3(50%). This pattern comprises one T3 (50%) & another T4 (50%) case.

Lymphnode involvement was seen in both the cases, one case was N2a stage & another was N2b stage. Adenocarcinoma NOS (50%) & adenocarcinoma mucinous (50%) were seen one case each. Mucinous component was seen in 1 (50%) case and absent in another case.

Signet ring component was also present in 1(50%) case and absent in another case. Tumour infiltrating lymphocyte (TIL) was mild to moderate in both cases (100%). Peritumoral lymphocytic response (PTL) was not

seen in both cases. Focal necrosis was seen in 1(50%) case and absent in another case.
The findings observed in our study were comparatively similar in most of the earlier studies both in Asian population or western population.[33,45,53,60,61,63,66,69,71] No dominance on the basis of gender or laterity was observed in our study but comparatively a higher number of males and left sided cases were observed by Hashmi AA et al.[39]

Combined loss of expression of MSH2 and MSH6 proteins (2 cases):

This pattern was seen only 2(13.3%) cases. It was the second most common pattern with the number of cases ranging from 11% to 28% in earlier various studies.[33,39,45,53,60,61,63,66,69,71] Lowest percentage was observed by Truninger K et al[71] and highest percentage was observed by Watson N et al.[66]No predominance based on gender which is exactly similar with the finding of Hashmi AA et al.[45] There was also no predominance based on age of the cases. Both the tumours were situated in the right side. Lymphovascular invasion was seen in 1(50%) case.

Perineural invasion was not seen in both the cases. One case was Grade-II and another case was Grade III. Bothe the cases were T3 stage. Lymphnode involvement was seen in 1(50%) case which was in N1 stage. In this pattern one case was Adenocarcinoma NOS variant & another was mucinous carcinoma variant. Mucinous component was present in one (50%) case with ≥ 50% mucinous component.

Signet ring component was present in 50% of the cases of this pattern. Tumour infiltrating lymphocyte (TIL) was absent in one case and mild to moderate was present in 1(50%) case. Peritumoral lymphocytic response (PTL) was present in one case. Necrosis was present in the both cases; focal necrosis was seen in one (50%) case and widespread necrosis in another case.

Isolated loss of expression of PMS2 (1 case):

Only 1(6.6%) case was seen with this type of pattern. Most of the earlier studies show percentage ranging from 1-13%.[33,39,45,53,60,61,63,66,69,71]The highest was reported by Shia J et al.[63] The patient was male and ≥ 50years old.

The tumour was right-sided. Both the lymphovascular invasion and perineural invasion were not seen. It was of adenocarcinoma NOS variant with Grade II. Stage was T3 without any lymphnode involvement. Mucinous or signet ring component were not seen it this tumour. Mild to moderate amount of tumour infiltrating lymphocyte (TIL) was seen with focal necrosis. Peritumoral lymphocytic response (PTL) was not observed in this case.

Isolated loss of expression of MSH6 (2 cases):

This pattern was seen in 2(13.3%) cases. In the literature lowest percentage was 1% observed by Hall G et al.[61] The highest percentage was 14% observed by Mojtahed A et al.[60]Among them 1 was male & another was female. Both of them were ≥ 50 years old and tumour were situated in right side. Both the lymphovascular invasion and perineural invasion were not seen in any case. Both the tumours were adenocarcinoma NOS variant, grade II & T3 stage without any lymphnode involvement.

Mucinous component was absent in one case and < 10% component was seen in one case. Signet ring component was not seen in any case. Tumour infiltrating lymphocyte (TIL) was absent in one case and mild to moderate amount was present in another case. Peritumoral lymphocytic response (PTL) was present in 1(50%) case and absent in another case. Focal necrosis was seen in both the cases.

The various findings observed in our study were comparatively similar with earlier findings observed in other studies.[33,39,45,53,60,61,63,66,69,71]

V. Conclusion

This study demonstrates high frequency of MMR protein loss in colorectal cancer of Manipuri population as compared to other Indian Studies. MMR protein loss was more common in males, older patients & right sided colon cancer. Focal necrosis and tumour infiltrating lymphocyte were present in majority of the tumours. Although they add a big burden on the pathologists, all clinicopathological and histological parameters should be assessed in all CRC for the sake of predicting MSI. Since it is not applicable to test all cases of CRC for MSI, selected cases only (according to clinicopathological predictors like a tumor is right sided and exhibits tumor-infiltrating lymphocytes) shall be proceeded to IHC for MLH1, MSH2, MSH6 & PMS2. IHC is easily available and inexpensive as part of the routine services in the department of pathology. Further research and larger studies are required to validate these findings and develop India specific criteria.

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