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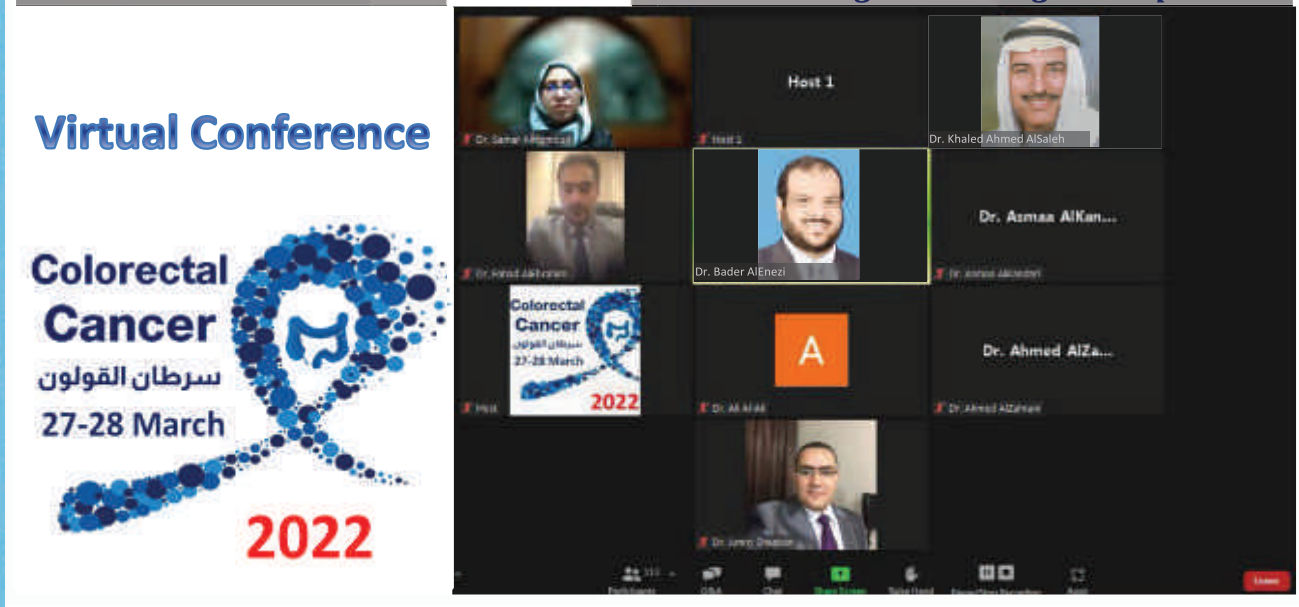

The Official Journal of the Gulf Federation For Cancer Control

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Original Article

Testing for Microsatellite Instability in Colorectal Cancer – a Comparative Evaluation of Immunohistochemical and Molecular Methods

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Abstract

Introduction: The majority of colorectal cancers (CRC) develop through the chromosomal instability pathway and approximately 15% display microsatellite instability (MSI) as a carcinogenic event. CRCs with microsatellite instability status have a characteristic phenotype. We aimed to assess the clinico–pathological and MSI profiles of sixty–one cases of CRC through immunohistochemical (IHC) staining for the mismatch repair(MMR) proteins and DNA based Polymerase Chain Reaction (PCR) assay for microsatellite markers.

Patients & Methods: Haematoxylin & Eosin stained sections of the tumor were evaluated for various histopathologic features. Immunohistochemistry was performed for the four MMR proteins, MLH1, MSH2, MSH6 and PMS2. NCI recommended panel of five nucleotide repeat markers was amplified from tumor DNA.

Results: The majority of the patients were males above fifty years of age. Around 61% of tumors were in the left–sided colon. Adenocarcinoma NOS (55, 90%) was the most common histological type. A total of 18 (29.5 %) cases showed dMMR by immunohistochemistry. Loss of PMS2 protein and combined loss of MSH2 & MSH6 were the most common findings in low and high MSI respectively. Of the 13 cases selected for PCR analysis, nine cases had high MSI (at least two markers unstable) and four cases had low MSI (one marker unstable) Results of PCR based DNA assay showed good concordance with IHC. No significant statistical association could be identified between the status of MSI by either methods and socio–demographic or clinical features.

Discussion: MSI constitutes 12%–20% and 6%–13% of CRCs in Western and Eastern countries respectively. In our series IHC staining revealed that 29.5% of cases showed dMMR. This was similar to other Indian studies which reported a prevalence of 22–27%. The combined loss of MSH2 & MSH6 (78%) was the most common type of dMMR. There was good concordance between IHC and PCR results. The issue of heterogenous or weak staining is a limiting factor in IHC interpretation and few cases of dMMR may be missed.

Conclusion: To conclude, IHC can be a very useful screening tool to detect microsatellite instability and triage cases of dMMR for MSI biomarker testing. The MSI status also serves as a prognostic and predictive tool.

Key words: Colorectal cancer, microsatellite instability, immunohistochemistry, Polymerase chain reaction

Introduction

Colorectal cancer has been acknowledged as a major cause of morbidity and mortality in both high–income and low–income nations. According to GLOBOCAN 2020, colorectal cancer (CRC) shows an incidence of 11% in both genders of all ages and mortality of around 9.4% [(1)]. The incidence of CRC varies widely across the globe, with a multifold increase in countries undergoing major developmental transitions. However, the mean fatality rate is seen to be higher in the less developed countries[(2)]. The disease may hence be considered a marker of socioeconomic development. Regular screening with

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colonoscopy and effective management protocols have succeeded in cutting the mortality rates in countries with high development indices. Alongside, rapid strides have been made in unravelling the genetic and molecular basis of CRC.

The two major genetic pathways implicated in the pathogenesis of CRC are chromosomal instability (CIN) and microsatellite instability (MSI). CIN is associated with high mutation rates in genes such as APC, KRAS, SMAD4, PI3KCA and TP53. Microsatellites are stretches of repetitive one to six base pairs of DNA occurring in different locations of the genome. The DNA mismatch repair system (MMR) recognizes any erroneous mispairs in the microsatellites during DNA synthesis. In normal cells, the mis–match repair systems maintains genomic stability by repairing the errors like insertions, deletions and base–base mispairs accumulated during DNA replication.MMR proteins like MLH1, MSH2, MSH3, MSH6, PMS2 and PSM1 form functional heterodimers or homodimers of different combinations to excise the mismatched nucleotides. Any mutations in the MMR system will eventually lead to the accumulation of the DNA error resulting in microsatellite instability. Thus, MSI is a form of genetic instability characterized by small deletions or insertions within repeated nucleotide units in DNA caused by alterations in the DNA mismatch repair (dMMR) system.

Although the majority of CRCs develop through the CIN pathway, approximately 15% of CRCs display MSI as a carcinogenic event. Approximately 2–3% of such MSI–associated CRC are attributed to germline mutation in an MMR gene (MLH1, MSH2, MSH6, PMS2) or Lynch syndrome/ Hereditary non polyposis colon cancer (HNPCC). These cancers, from affected HNPCC families exhibit genomic instability that can be detected as changes in the length of microsatellite sequences. A subset can be caused by epigenetic silencing of the MLH1 MMR gene or a combination of these factors. CRCs with dMMR/MSI status have a characteristic phenotype that includes a predilection for the right–sided colon, high–grade histology, mucinous differentiation and abundant tumor–infiltrating lymphocytes[21].Microsatellite instability also plays important prognostic and predictive roles in the management of colorectal carcinoma [3,4,20]. It is currently accepted that dMMR CRC behave well with reduced risk of recurrence and that stage II CRC does not respond to adjuvant 5–FU chemotherapy.[4,21].

There are both direct and indirect methods for MSI analysis; the direct method includes the Polymerase Chain Reaction (PCR) based detection of MSI repeats and the indirect method is immunohistochemical (IHC) based detection. Tumors with MSI can be identified by absent IHC staining or loss of expression of the MMR proteins namely MLH 1, MSH 2, MSH 6 & PMS2 or by molecular analysis for the microsatellite sequences. Loss of MMR protein expression by IHC has been shown to be highly concordant with DNA–based MSI testing with a good sensitivity and excellent specificity.

In this study, we aimed to assess clinicopathologic and MSI profiles of consecutive sixty–one cases of CRC. The MSI profile was assessed by both IHC staining for the MMR protein expression and DNA–based PCR and gel electrophoresis assay using a panel of microsatellite markers.

Patients and Methods

This was a hospital based retrospective study for a duration of three years wherein histologically proven colorectal carcinoma for whom surgical resection was performed at our centre was included. Patients treated with neoadjuvant chemotherapy were excluded. This was keeping in mind aberrant IHC staining in post neoadjuvant tumor tissue. Haematoxylin& Eosin stained sections of the tumor were evaluated specifically for all histopathologic features stressing on histologic subtype, grade, mucinous component and lymphovascular invasion. An additional point evaluated was the presence of intratumoral (stromal versus glandular) and peritumoral Crohn's like lymphocytic infiltrate which was scored as mild to marked.

Mismatch repair status by IHC

One of the formalin–fixed paraffin–embedded blocks which reflected the tumor features best was selected and immunohistochemistry for the four MMR proteins of MLH1, MSH2, MSH6 and PMS2 (concentrated antibody from Agilent Dako, United States; clones ES 05,FE11,EP49,EP 51 in dilutions of 1:40 and 1;50) was performed in LEICA BOND MAX automated IHC platform. A cut–off of 1% was fixed and less than 1% staining in the tumor nuclei, in the presence of positive internal control was taken as negative. Intact nuclear expression for all four markers was interpreted as Microsatellite stable(MSS). dMMR (deficient mismatch repair) was categorized as loss of one protein expression designated as Microsatellite instability– low (MSI–L) and loss of more than one protein expression was interpreted as Microsatellite instability–high(MSI–H) or). Three pathologists performed the independent scoring of the IHC slides.

Microsatellite instability by PCR

Three to four sections of 10–micron were taken from FFPE and deparaffinized using xylene and DNA isolation performed with Qiagen FFPE DNA isolation kit as per the manufacture's protocol. Using five markers of the reference NCI panel, recommended by the Bethesda guidelines,

namely the mononucleotides BAT–25 and BAT–26 as well as three dinucleotide markers D 2S123, D5S346 and D 17S250. MSI loci were amplified specifically from the genomic DNA. Briefly, the amplification condition of each locus is provided in Table 1.

All reactions contained approximately 300ng of template DNA in a total volume of 25µl with final reaction concentrations of 1× standard PCR buffer 200 mMdNTPs/1.5 mM Mg2+/0.2 mM of each primer/1 unit of *Taq* polymerase. The amplified product was run on SSCP PAGE. Samples showing shifts in band compared to the normal were taken as MSI. The tumor was classified as MSI–H (MSI–high) when two or more (≥30%) of the markers exhibited instability, MSI–L (MSI–low) if one (1%–29%) of the markers exhibited instability and microsatellite stable (MSS), if none of the markers were unstable.

Biostatistics

The data was analyzed using SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Bivariate analysis of clinical and histopathological features predicting MSI was done using the Chi–square test and Fischer's exact test wherever applicable. Logistic regression was performed for multivariate analysis to determine factors that are independently predictive of MSI. A p value <0.05 was considered statistically significant.

Results

A total of 61 cases were included in the study. The socio–demographic, clinical and pathologic features are listed in Table 2. More than half of the patients were males (33, 55%) and were above fifty years of age (36,59%). Almost three–quarters of the tumors were situated in the left half of the colon (41, 67%), predominantly in the sigmoid colon, rectosigmoid and rectum (Figure 1).

PRIMER NAME	Forward	Reverse	PCR CONDITION
D2S 123	5 – AAA CAG GAT GCC TGC CTT TA	5 – GGA CTT TCC ACC TAT GGG AC	95° – 45 Sec 55 °– 45 Sec 72 °– 45 Sec
BAT 25	5– TCG CCT CCA AGA ATG TAA GT	5 – TCT GCA TTT TAA CTA TGG CTC	95 °– 1 min 56 °– 45 sec 72°– 45 sec
BAT26	5 – TGA CTA CTT TTG ACT TCA GCC	5 – AAC CAT TCA ACA TTT TTA ACC C	95 °– 45sec 55 °– 1 min 72 °– 30 sec
D17S 250	5 – GGA AGA ATC AAA TAG ACA AT	5 – GCT GGC CAT ATA ATA TAT TTA AAC	95° – 45 sec 55 °– 45 sec 72° – 45 sec
D5S 346	5 – AGC AGA TAA GAC AGT ATT ACT AG	5 – ACT CAC TCT AGT GAT AAA TCG GG	95 °– 1 min 57° – 45 sec 72 °– 45 sec

Table 1: MSI PRIMERS and PCR conditions

Characteristics	n	%
Total	61	100
Gender		
Male	33	55
Female	28	45
Age group(years)		
< 50	25	41
>50	36	59
Family history of cancer	3	5
Other malignancy	2	3.2
Site		
(L) Colon	41	67
(R) Colon	16	26
Transverse colon	4	6.5
Colonic Polyps	9	14.75
Histology		
Adenocarcinoma NOS	55	90
Mucinous(>50% mucin)	4	6.5
Medullary	1	1.6
Signet ring cell	1	1.6
Grade of tumor		
High grade	5	8
Low grade	56	91
Lymphocytic response		
Intratumoral	32	52
PeritumoralCrohns like	28	46
Perineural invasion	4	6
Lymphovascular invasion	6	9
MSI by IHC	18	29.5
MSI L	9	50
MSI H	9	50
MSI by RT–PCR	13	21.3
MSI L	4	30.7
MSI H	9	69.3

Table 2: Sociodemographic, clinical, pathological and molecular characteristics of the study patients

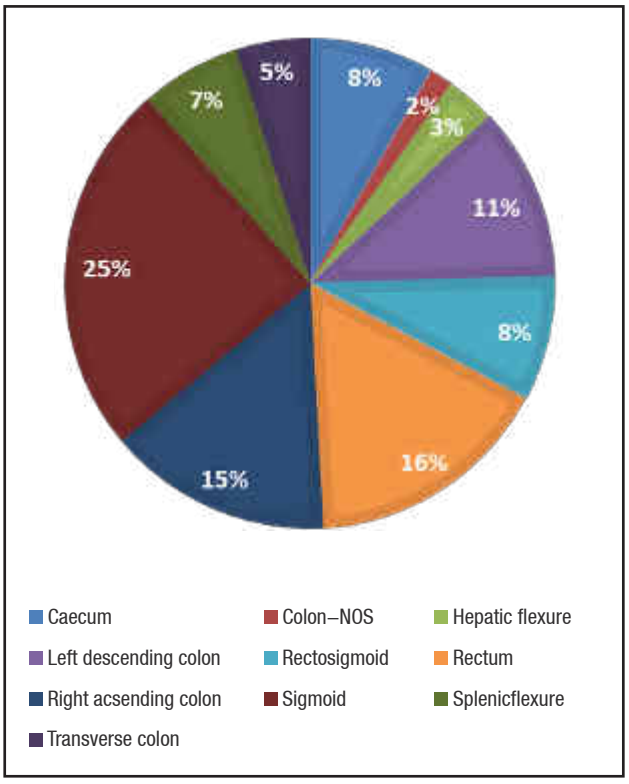

Figure 1: Site distribution of colorectal cancers

Polyps were seen in nine cases (13%) of which three had multiple polyposes. They were seen distributed in ascending, descending, transverse and sigmoid colon. The largest polyp was six cm in size. Histology of all polyps were tubulovillous adenomas with varying grades of dysplasia with the largest polyp measuring 6 cm showed focal Adenocarcinoma within it. Adenocarcinoma NOS (55,90%) was the commonest histological type. More than half were of low–grade histology (56, 91%). A total of 28 (46%) patients showed a peritumoral lymphocytic response.

Mismatch repair status by IHC

A total of 18 (29.5 %) cases showed dMMR by immunohistochemistry done for the four proteins, MLH1, MSH2,MSH6,PMS2. Of these nine were classified as MSI L (loss of single protein) and nine as MSI H (loss of more than one protein). Among MSI L, the majority showed loss of PMS2 protein (6, 67%) and among MSI H, the majority showed a combined loss of MSH2 & MSH6 (7,78%) {Figures 2,3} The pattern of IHC staining is highlighted in Figure 4

Microsatellite status by PCR

MSI analysis using PCR was performed for the 18 cases of dMMR detected by IHC. Out of 18 cases only 12 cases showed the PCR amplification of all five loci BAT 25, BAT 26, D 123, D 250, D 436. One case which had preserved protein expression on IHC was also analysed for MSI as a negative control. Of the 13 cases selected for analysis,

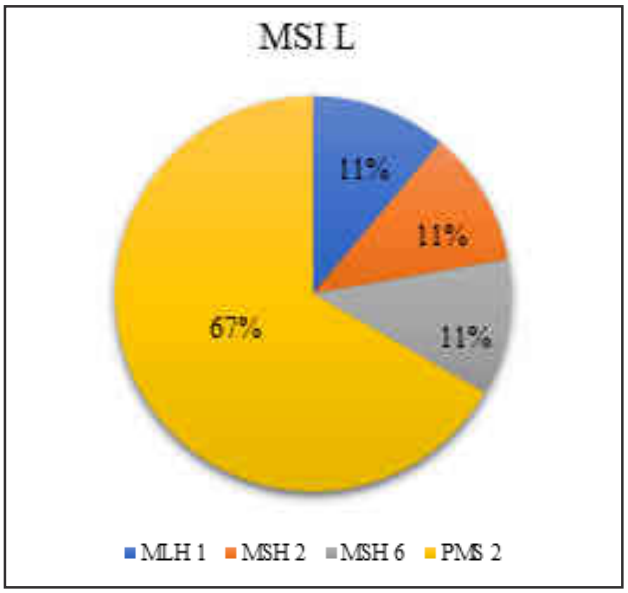

Figure 2: MSI L by immunohistochemistry (9,100%)

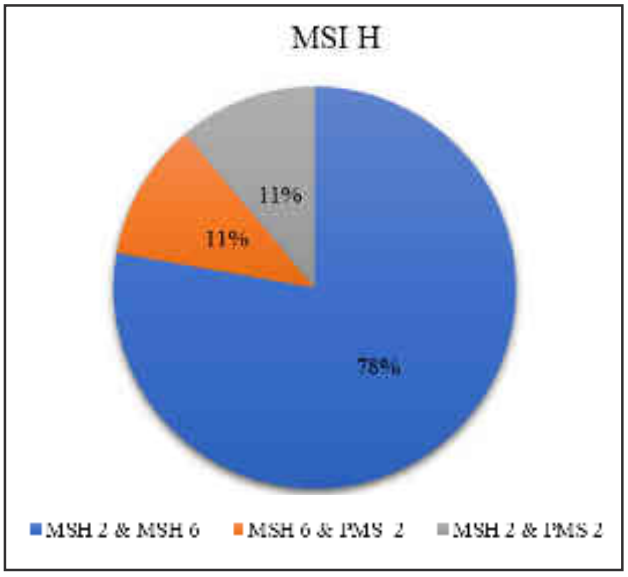

Figure 3: MSI H by immunohistochemistry (9,100%)

nine cases had high MSI (at least two markers unstable) and four cases had low MSI (one marker unstable) (Figure 5). The frequency distribution of the individual markers are represented in Figure 6. The representative gel image is seen in Figure 7.

Of the total 13 cases of dMMR/MSI detected by both IHC and RT–PCR, 100% concordance was noted in eight cases of MSI–H detected by both methods, i.e MMR deficient phenotype on IHC matched with MSI H phenotype on PCR (Table 3).Of the four cases of MSI L, the concordance was 80%, i.e three cases showed MSI L status by both methods and one case showed MSI H phenotype on PCR. One case of MSS by IHC (attempted as a negative control) showed MSI H on PCR. Table 3 gives the type of deficient protein and defective marker responsible for dMMR/MSI by IHC and PCR

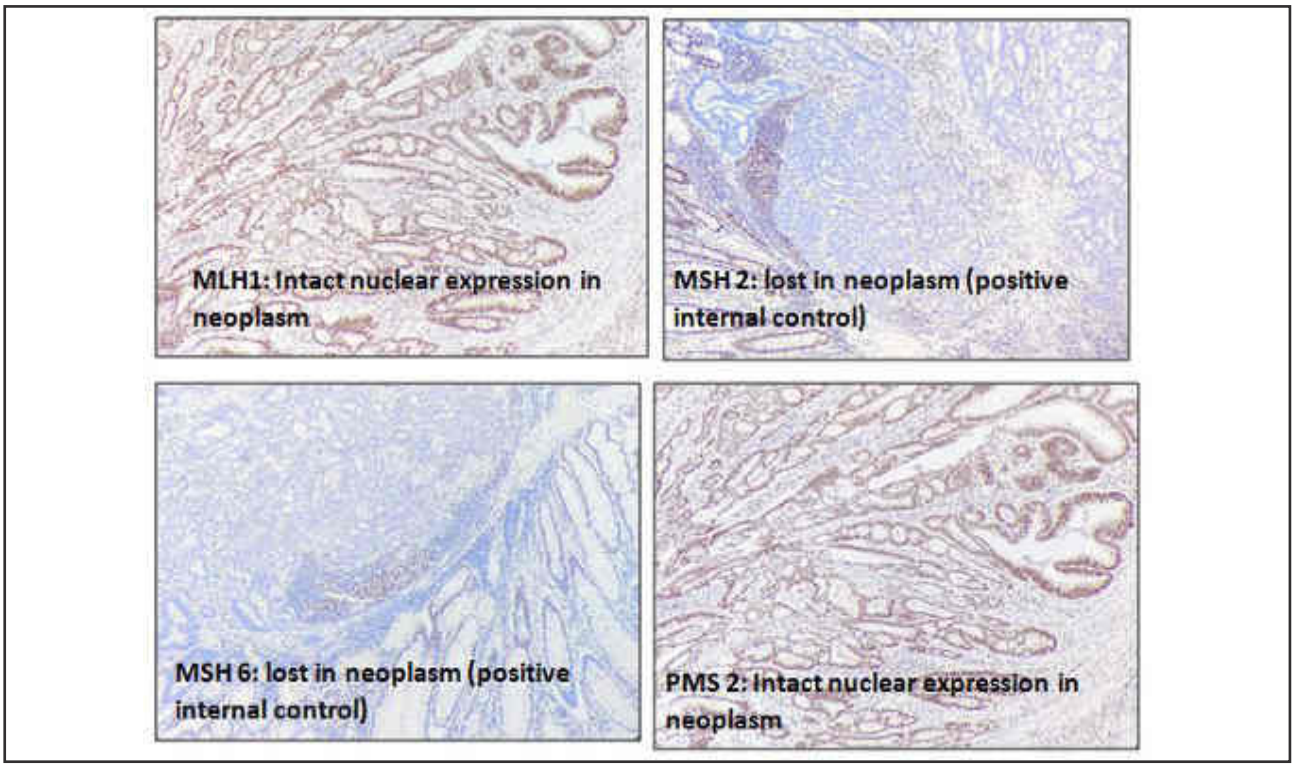

Figure 4: Immunohistochemical staining for MMR proteins (400X)

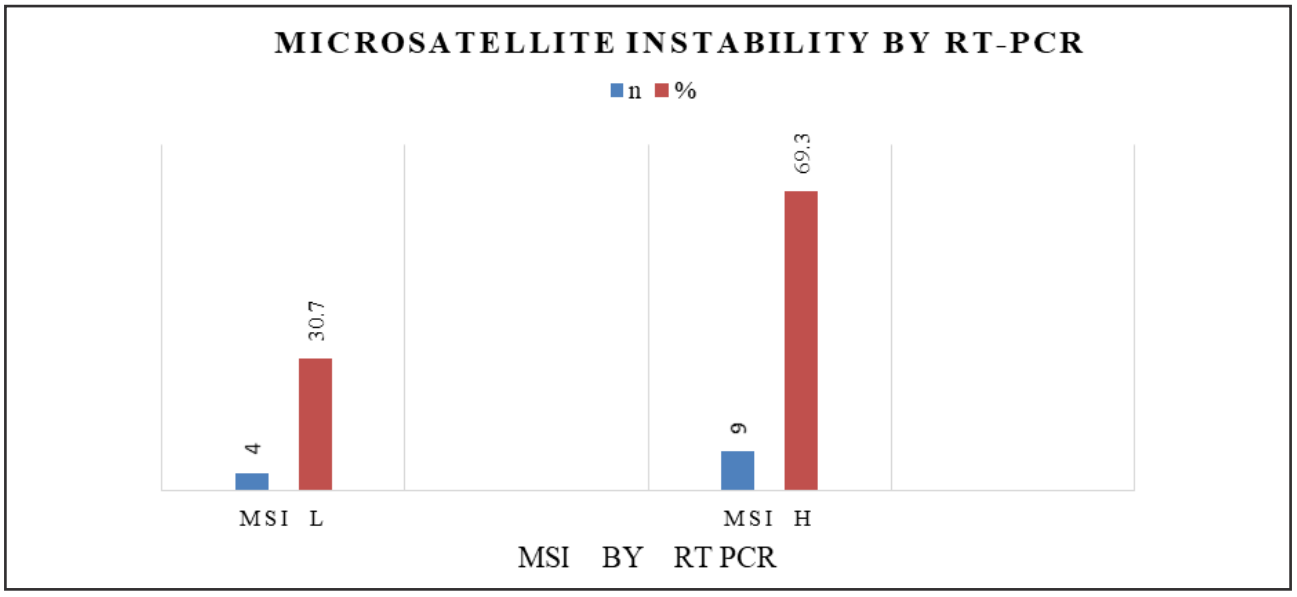

Figure 5: Microsatellite instability by RT–PCR

Association of MSI with clinical and histopathologic features

Out of 18 MSI positive cases by either methods, the majority were males (11,61%).The frequency of MSI H was more in tumors of (L) colon (11, 61%) as compared to tumors of (R) colon(6,33%). No significant statistical association was identified between the status of MSI by both methods and socio–demographic or clinical features like age, family history of cancer or other associated malignancy. Two out of four cases (50%) of mucinous carcinoma in this cohort showed dMMR for the combined loss of MSH2 & MSH6. The single case each of signet ring cell carcinoma and medullary carcinoma did not show dMMR. Tumor characteristics like the site of the tumor, presence of polyps, histology, grade of tumor or lymphocytic response did not have a significant statistical association with MSI.

A positive correlation between the two methods of IHC and PCR for MSI detection was obtained (Spearman correlation of 0.853 with $p< 0.001$).

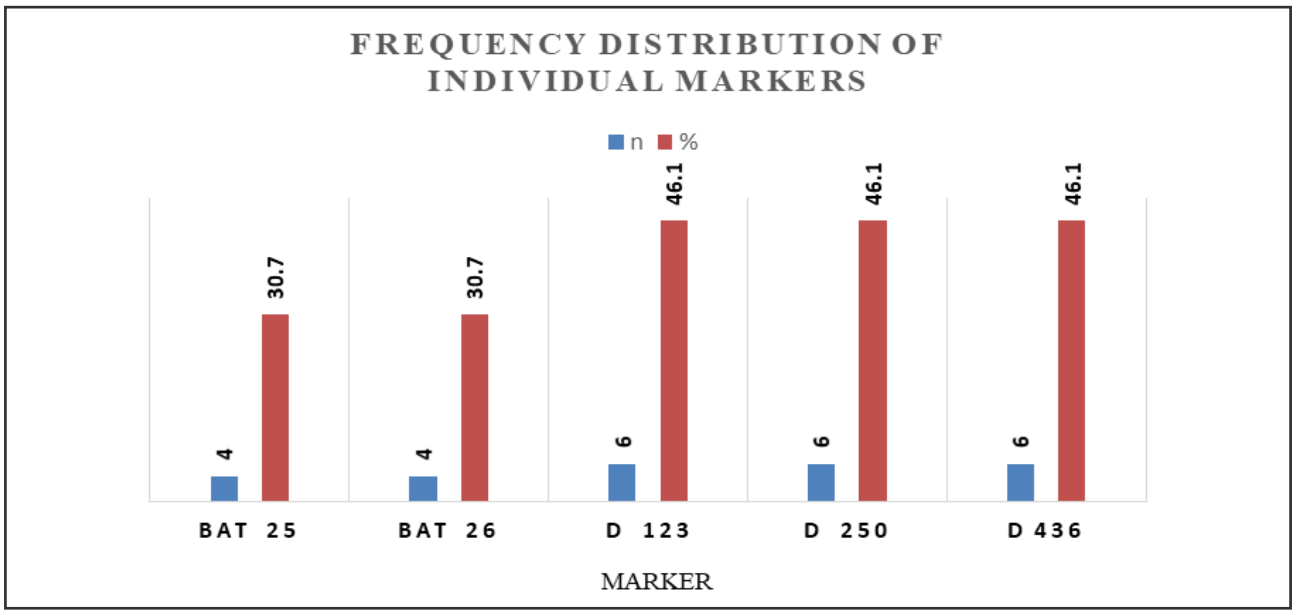

Figure 6: Frequency distribution of individual loci

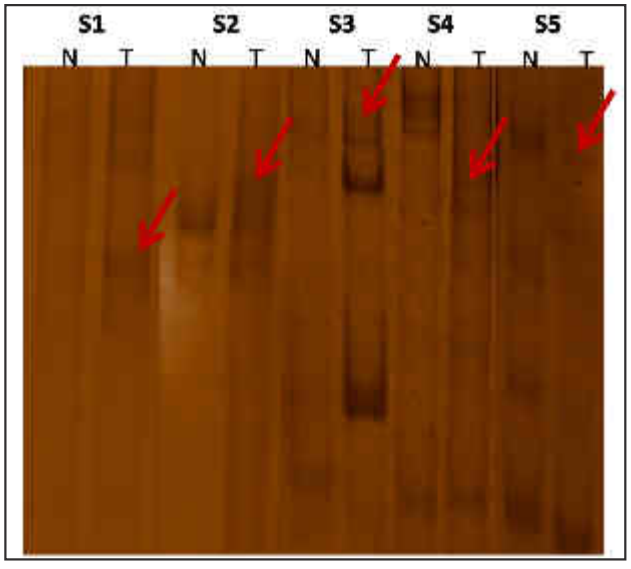

Figure 7: Electrophoresis gel image (BIORAD gel doc system)

Discussion

MSI pathway has a role in the carcinogenesis of 15% of sporadic colorectal cancers and 2–3% of Lynch syndrome. MSI status also has important therapeutic and prognostic implications. The human system has seven MMR genes and their encoded proteins namely MLH1,MLH3, MSH2, MSH3,MSH6, PMS1and PMS2 which play a role in the repair of DNA mismatches. Defective MMR genes result in the production of dysfunctional proteins or loss of proteins (dMMR). The defects can be sporadic, inherited germline mutation or epigenetic inactivation (MLH1). MSI tumors in Lynch syndrome are caused by germline mutations in MLH 1, MSH 2, MSH 6 or PMS2. MSI analysis is possible by two methodologies, immunohistochemical assays for MMR and PCR–based analysis for MSI.

The literature showed that MSI constitutes 12%–20% and 6%–13% of CRCs in Western and Eastern countries, respectively[5].In our series of 61 consecutive cases of CRC, IHC staining revealed that 29.5% of cases showed loss of expression of one or more MMR proteins. This was similar to a study by SS Nayak et al where they detected a loss of MMR IHC in 22.94% [6]. Pandey et al reported a lesser prevalence (10%) of MSI [7]. Another Indian study by Paulose et al found 27.1% of MSI CRC in their patients [8]. In a study by Chen M et al,dMMR accounted for 12.13% of all cases[9]. A study on young Jordanian CRC patients revealed dMMR in 19%[10]. A study by Soliman et al recorded significant cases (67%) of MSI in the Egyptian population [11].

In our study, combined loss of MSH2 & MSH6 (78%) was the most common type of dMMR detected by IHC followed by solitary loss of PMS2 (67%). The other studies showed a predominance of dual loss of MLH1–PMS2 [6,8,11,18].Loss of expression of PMS2 protein was the most common in the study on Chinese and young Jordanian patients. A 29% prevalence of combined loss of MSH2 and MSH 6 was noted in Soliman'sstudy.In their study, Pandey et al had found loss of expression of hMLH1 protein in the majority of cases. It can be seen that loss of MLH1 protein was extremely rare in our study and only one case was detected on IHC, in the right–sided colon of a male in the age group 60–69 years.

Comparison of IHC and PCR for MSI detection

Detection of dMMR by IHC and MSI by PCR provide different information on tumor samples. IHC measures the expression of MMR proteins whereas PCR detects defective function of the MMR system. MSI testing by PCR

Sl no:	dMMR by IHC	Type of protein	dMMR by RT PCR	Type of locus
1	MSI L	MSH6 loss	MSI L	D 346
2	MSI L	PMS2 loss	MSI L	D 346
3	MSI L	PMS2 loss	MSI L	D 250
4	MSI H	MSH 2 loss MSH 6 loss	MSI H	D 123 D 250 D 346
5	MSI H	MSH 6 loss PMS2 loss	MSI H	BAT 26 D 123
6	MSI H	MSH 2 loss MSH 6 loss	MSI H	BAT 25 BAT 26 D 250
7	MSI H	MSH 2 loss MSH 6 loss	MSI H	BAT 25 D 123 D 250
8	MSI H	MSH 2 loss MSH 6 loss	MSI H	BAT 26 D 346
9	MSI H	MSH 2 loss MSH 6 loss	MSI H	BAT 25 D 123
10	MSI H	MSH 2 loss PMS2 loss	MSI H	D 250 D 346
11	MSI H	MSH2 loss MSH6 loss	MSI H	BAT 25 BAT 26 D 123 D 250
12	MSI L	PMS2 loss	MSI H	D 123 D 250
13	MSS	All positive	MSI L	D 346

Table 3: Types of dMMR by IHC and PCR detection methods

and IHC can be used complimentarily, and loss of MMR protein expression by IHC is highly concordant with DNA–based MSI testing with good sensitivity and excellent specificity. By IHC, MSI L was defined as loss of a single protein and MSI H as loss of more than one protein. By PCR, high MSI was defined as instability of least two markers and low MSI as one unstable marker.

ML Chen et al reported that the coincidence rate of the two methods for detecting microsatellite status was 91.92%. Thus IHC and the PCR methods displayed high consistency in microsatellite status [9].One large study had shown that the predictive value of IHC for MSS/MSI–L was 96.4% and for MSI–H phenotype was 100% [11].

The National Cancer Institute Workshop advocates the use of five microsatellite markers that includes two mononucleotides, BAT 25 and 26 and three dinucleotides D 2S123, D 5S346 and D 17S250 to determine MSI. Interpretation involves a comparison with normal DNA from each patient[5,12]. We performed PCR–based DNA assay using this five nucleotide marker panel on 13 cases and the results showed good concordance with IHC [Table 3]. Two cases of discordance were both of cases read as MSS/MSI L in IHC and reported as MSI H on PCR.

Both IHC and PCR are used in laboratories to detect the microsatellite status of colorectal carcinoma. Co–testing can improve the sensitivity of tumor characterisation to over 99%. Dieumegard et al and Cawkwell et al have found 100% correspondence between tumor MSI results and tumor IHC[13,14].Other studies also showed large levels of concordance upto 97%. However certain studies have shown less than 100% correspondence between the two methods. Our results also showed a positive correlation between the two methods with a significant value of $p< 0.001$.

IHC as a screening tool for the detection of MMR proteins has been a vastly studied field of interest. IHC is a simple, easy to perform and less time consuming procedure that can be performed in most histopathology laboratories. The advantages of IHC are that it is rapid, cost–effective and can direct specific gene testing. Tumors identified by pathologists as showing features suggestive of MSI based on the Bethesda guidelines like the right–sided colon, high–grade histology, mucinous differentiation and abundant tumor–infiltrating lymphocytes can be screened for dMMR**.** The use of four antibody panels of MLH1,MSLH2,MSH6,PMS2 increases the sensitivity of IHC as an optimal first–line screening tool[15,22]. Detection of loss of protein product in IHC helps to triage the case for DNA assay. However the disadvantages are that the sensitivity of IHC is highly dependant on factors like quality of fixation, processing, type of antibody and technical modality of manual versus automated platform used. The issue of heterogeneous staining, weak staining can be other limiting factors in IHC interpretation and few cases of dMMR may be missed. This highlights the point that some cases of microsatellite instability may be missed on IHC and a plan for MSI testing must consider this point. In our study two cases of MSS by IHC were found to be MSI–H on PCR. On analysis, it was found that both cases had weak IHC expression of the proteins which was as low as less than 10% which was misread as preserved staining This implies that a case displaying a focal, heterogeneous or weak staining pattern is potentially a mutation carrier and requires further testing by MSI [14,19].

Bartley AN et al summarised that concordance between IHC and MSI was high for tumors that are microsatellite stable. A greater frequency of test discordance was identified in the tumors that were MSI–high [16]. Similar results were described by Lindor M et al who concluded

that the predictive value of normal IHC for an MSS/MSI–L phenotype was 96.7% and abnormal IHC was 100% for an MSI–H phenotype[17].

Thus both methodologies can go hand in hand in improving efficiency of MSI detection in colorectal cancers. Laboratories without an infrastructure for molecular assays can rely on IHC as a dependable tool of screening as well as diagnosis.

Conclusion

To conclude, IHC can be a very useful screening tool to detect microsatellite instability and triage cases of dMMR for MSI biomarker testing. The MSI status also serves as a prognostic and predictive tool.

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Funding and Conflict of Interest

This study has been reviewed and approved by the Institutional Ethics Committee (Ref.no: 1617/IRB–IEC/13/MCC/21–07–16).This study was funded by an Intramural grant from the institution towards histopathology and molecular lab experiments. All authors declare no conflict of interest in the project.

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