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ORIGINAL ARTICLE

Screening for microsatellite instability in colorectal carcinoma: practical utility of immunohistochemistry and PCR with fragment analysis in a diagnostic histopathology setting

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Abstract

Since 2014, the National Comprehensive Cancer Network (NCCN) has recommended that colorectal carcinoma (CRC) be universally tested for high microsatellite instability (MSI-H) which is present in 15% of such cancers. Fidelity of resultant microsatellites during DNA replication is contingent upon an intact mismatch repair (MMR) system and lack of fidelity can result in tumourigenesis. Prior to commencing routine screening for MSI-H, we assessed two commonly used methods, immunohistochemical (IHC) determination of loss of MMR gene products viz MLH1, MSH2, MSH6 and PMS2 against PCR amplification and subsequent fragment analysis of microsatellite markers, BAT25, BAT26, D2S123, D5S346 and D17S250 (Bethesda markers) in 73 unselected primary CRC. 15.1% (11/73) were categorized as MSI-H while deficient MMR (dMMR) was detected in 16.4% (12/73). Of the dMMR, 66.7% (8/12) were classified MSI-H, while 33.3% (4/12) were microsatellite stable/low microsatellite instability (MSS/MSI-L). Of the proficient MMR (pMMR), 95.1% (58/61) were MSS/MSI-L and 4.9% (3/61) were MSI-H. The κ value of 0.639 (standard error =0.125; p = 0.000) indicated substantial agreement between detection of loss of DNA mismatch repair using immunohistochemistry and the detection of downstream microsatellite instability using PCR. After consideration of advantages and shortcomings of both methods, it is our opinion that the choice of preferred technique for MSI analysis would depend on the type of laboratory carrying out the testing.

Keywords: Microsatellite instability, DNA mismatch repair, PCR, fragment analysis, immuhohistochemistry, colorectal carcinoma

INTRODUCTION

Colorectal carcinoma (CRC) is the fourth leading cancer worldwide and results in over 700,000 deaths annually.[1] It ranks as the most common malignancy in Malaysian males and third most common in Malaysian females.{, 2008 #48}[2] Over the years, CRC is increasingly recognized as a biologically heterogenous disease. Stratification of the cancer by the commonly used TNM clinical staging may not be sufficient to prognosticate or predict response to treatment as patients demonstrate diverse intra-stage responses. Attempts have been made to subtype CRC using its genetic and epigenetic profiles and these include an earlier proposal by Jass[3] and recent ones e.g. The Cancer Genome Atlas (TCGA)[4] and others from different institutions.[5-8] Although there are similarities in these various classification systems, the Colorectal Cancer Subtyping Consortium's Consensus Molecular Subtyping (CMS) system which combined over 4000 samples from various genomic datasets seems to be one of the more robust in stratifying the biological groups of CRC.[9] Presently, some biomarkers stand out in their prognostic and predictive value. Among these biomarkers, microsatellite instability (MSI) is one, and the National Comprehensive Cancer Network (NCCN) has from 2014 recommended that it be universally tested in all colorectal cancers.[10]

Microsatellites are made up of repeats comprising of 1-6 nucleotides and are located in both coding and non-coding regions of the genome. Together they constitute about 3% of the

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human genome.[11] The mismatch repair (MMR) system ensures fidelity during DNA replication and maintains stability of the microsatellites. Besides repairing slippages in microsatellites, the MMR system is also involved in repairs of minor base pair mismatches as well as nucleotide changes caused by chemotherapy etc.[12] When there is failure of the MMR system, alterations occur in the microsatellites (microsatellite instability). These lead to "mutator" phenotypes that can affect genes e.g. PTEN, BAX, TGFBRII, EGFR etc, that have critical functions in cell signalling, apoptosis and proliferation.[13] A deficient mismatch repair system (dMMR) results in high microsatellite instability (MSI-H). MSI-H tumours constitute about 15% of all CRC, with autosomal-dominantly inherited cases (Lynch syndrome or hereditary non-polyposis colorectal cancer) forming 3% and sporadic cases, 12%.[14] The former condition which was first recognised by Aldred Warthin in 1913 as a "cancer family syndrome", was later re-named Lynch syndrome after Henry Lynch provided further evidence on the pattern of inheritance in 1984.[15,16] Majority (90%) of Lynch syndrome are caused by germline mutations in one of the MMR genes, namely mutL homolog 1 (MLH1), mutS homologs 2 (MSH2) and 6 (MSH6) or postmeiotic segregation increased 2 (PMS2).[17] In contrast, MSI-H which occurs in sporadic CRC is usually due to biallelic promoter hypermethylation that leads to inactivation of the MLH1 gene with a small number (~ 20%) being caused by mutations in the MMR genes.[18-21] Clinically, patients with MSI-H colorectal cancers, irrespective of germline status, have better prognosis in comparison with those who have proficient MMR (pMMR) and are microsatellite stable (MSS). This is despite MSI-H tumours being associated with unfavourable clinicopathological features e.g. poor differentiation and partial resistance to 5-fluorouracil-based adjuvant chemotherapy.[22-25]

Notwithstanding the availability of clinical and pathological parameters set out for identification of possible Lynch syndrome, e.g. Amsterdam II criteria (1999)[26] or the Revised Bethesda Guidelines (2004),[27] the lack of sensitivity of using these parameters alone has compelled the recommendation that MSI assessment be made a standard-of-care in CRC patients.[28] The recent exciting observation of MSI-H tumors faring well with immunomodulatory checkpoint inhibitors, further reinforces the need for such biomarking.[29]

Currently, unselected screening for mutations of the MMR genes is still expensive due to the heterogeneity of the MMR gene spectrum and not easily accessible. Furthermore, large numbers of the MSI-H occurring in sporadic CRC are caused by epigenetic silencing of the MLH-1 promoter rather than mutation of the MLH-1 gene. Presently, the two most common methods of screening for microsatellite instability are via (1) PCR amplification of microsatellite repeats using fluorescent primers and comparing the amplified fragments of microsatellite repeats between the tumour and normal tissue and (2) immunohistochemical detection of loss of protein products of the MMR genes. As a preliminary to establishing microsatellite instability screening for colorectal carcinomas at our institution we aimed to assess the immunohistochemical (IHC) determination of MMR gene products, a process that is more familiar and commonly employed in a routine histopathology laboratory, against PCR analysis followed by fragment analysis for microsatellite instability. In this study, IHC determination of MLH1, MSH2, MSH6 and PMS2 will be compared against PCR amplification of the original panel of microsatellite markers, recommended at the National Cancer Institute (NCI) Conference in Bethesda,[30] and often referred to as the "Bethesda markers"; the latter comprising of markers for two mononucleotide microsatellite repeats, BAT25 and BAT26 and three dinucleotide repeats, D2S123, D5S346 and D17S250. This study takes into cognizance that concordance of 68 to 80 plus percent, averaging at about 75%, between IHC detection of MMR proteins and PCR analysis of microsatellites in CRC and other cancers has been reported.[31] Nevertheless to the best of our knowledge there has been no published report of such a comparison in a Malaysian setting which we feel will serve as useful background information to local laboratories desiring to commence such testing.

MATERIALS AND METHODS

Cases

For this study, the CRC were from a cohort (comprising 40 consecutive right- and 40 consecutive left-sided cancers) previously analyzed for DNA mismatch repair proteins using immunohistochemistry by the authors.[32,33] These cases were diagnosed between 2005 till 2007 at the Department of Pathology, Faculty of Medicine, University of Malaya. Relevant clinical information was obtained from the surgical pathology examination request forms.

The study was approved by the Institutional Review Board (IRB) of the University of Malaya Medical Centre (MREC ID NO: 2016714-4022) and carried out in compliance with the Declaration of Helsinki.

Microsatellite analysis

The formalin-fixed, paraffin-embedded tissue blocks of the CRC cases used for the earlier analysis of MMR proteins were re-called. In addition, the further of the two colonic resection margins from the tumor of each of the 80 selected cases was checked to ensure that the colonic mucosa was free of dysplasia or malignancy. Once confirmed, this constituted the "normal" colonic control to be used in the analysis. Macrodissection to increase the proportion of tumor in the tumor sections, and normal colonic mucosa to stroma in the "normal" colonic tissue sections, was carried out by a pathologist (PLC), whenever necessary. Genomic DNA was extracted from four 5-µm-thick tissue sections of the CRCs and their paired normal colonic tissues respectively, using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol. The DNA quantity was determined using the Multiskan Go Microplate Spectrophotometer (Thermo Scientific, Vantaa, Finland). Only samples with a DNA concentration ≥ 5 ng/ μl and A260/A280 ratio ranging from 1.8-2.2 were subjected for further analysis. After successful extraction, the DNA was stored at -20˚C until microsatellite analysis. Polymerase chain reaction (PCR) amplification of the five microsatellite markers (BAT25, BAT26, D2S123, D5S346 and D17S250) was individually carried out for each marker on the CRC tissue and its paired normal colonic tissue in a Veriti 96-well thermal cycler (Applied Biosystems, Singapore). The five pairs of fluorescent FAM-labelled primers for each marker[34] were commercially synthesized (Applied Biosystems, California, USA). The PCR assay was carried out in a final volume of 20 µl containing 200 ng of genomic DNA, 1x PCR buffer (Thermo Scientific, Vilnius, Lithuania), 0.075 mmol/L dNTP Mix (Thermo Scientific, Vilnius, Lithuania), and 0.0375U/ μl *Taq* DNA polymerase (Thermo Scientific, Vilnius, Lithuania). Concentrations of $MgCl_2$ (Thermo Scientific, Vilnius, Lithuania) and primer pairs (Applied Biosystems, California, USA) were optimised for each marker. Ultrapure water derived from the Direct-Q® 3 UV Water Purification System (Merck Millipore, Molsheim, France) was added to make the total volume to 20 µl. The PCR conditions were initiation at 95˚C for 10 min, followed by 40 cycles of denaturation at 94˚C for 30 s, annealing at marker-specific temperature for 30 s and extension at 72˚C for 30 s, with a final extension at 72˚C for 7 min. The variables for each of the markers viz the primer sequences, primer pair concentration, $MgCl_2$ concentratios and annealing temperature are shown in Table 1. After a check for successful amplification of expected amplicons using 3.5% agarose gel electrophoresis, 0.5 μl of the PCR product, 0.5 μl GeneScan 500 LIZ Size Standard (Applied Biosystems, Warrington, UK) and 9 μl HiDi Formamide (Applied Biosystems, Warrington, UK) were added into the wells of a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, China) for denaturation of the DNA in a Thermomixer Comfort (Eppendorf, Hamburg, Germany) for 3 minutes at 95˚C and subsequently cooled down on ice for a few minutes. Fragment analysis was conducted via automated capillary electrophoresis in a 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA) and the data was analyzed using the GeneMapper 4.0 software (Applied Biosystems, Foster City, California, USA). The appearance of an allele shift of at least 3 base pairs in the tumor as compared to the corresponding normal tissue for any given marker was interpreted as showing microsatellite instability (Fig. 1).[35,36] The tumor was defined as exhibiting low microsatellite instability (MSI-L) if only one of the five markers showed instability, high microsatellite instability (MSI-H) if ≥2 markers displayed instability. If none of the 5 markers demonstrated instability, the tumor was categorized as microsatellite stable (MSS).[30]

DNA mismatch repair protein

The immunohistochemical staining had been carried out earlier.[32,33] In summary, 4 μm sections, cut from the formalin-fixed, paraffin-embedded (FFPE) tumor tissue block, were stained with monoclonal antibodies to MLH1 (1:100; BD Pharmingen, clone G168-728), MSH2 (1:800; BD Pharmingen, clone G219-1129), MSH6 (1:500; BD Transduction Laboratories, clone 44/MSH6) and PMS2 (1:100; BD Pharmingen, clone A16-4) on the Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, Arizona). Staining was detected by the ultraView universal DAB detection kit (Ventana Medical Systems Inc., Tucson, Arizona). Proficient MMR (pMMR) protein

TABLE 1: Forward (F) and reverse (R) primer sequences, primer pair concentration, magnesium chloride ($MgCl_2$) concentration and annealing temperature in the PCR amplification for the respective microsatellite marker

Marker	Primer sequences (5' to 3')	Primer pair concentration (µmol/L)	MgCl2 concentration (mmol/L)	Annealing temperature(˚C)
BAT25	F: TCGCCTCCAAGAATGTAAGT	0.5	1.5	60
	R: TCTGCATTTTAACTATGGCTC			
BAT26	F: TGACTACTTTTGACTTCAGCC	0.5	1.5	60
	R: AACCATTCAACATTTTTAACCC			
D2S123	F: GCCAGAGAAATTAGACACAGTG	0.75	0.75	55
	R: CTGACTTGGATACCATCTATCTA			
D5S346	F: TACTCACTCTAGTGATAAATCGG	0.5	0.75	60
	R: TTCAGGGAATTGAGAGTTACAG			
D17S250	F:AATAGACAATAAAAATATGTGTGTG	0.5	1.0	51
	R: TATATATTTAAACCATTTGAAAGTG			

expression meant unequivocal tumor nuclear immunostaining when the internal positive controls (lymphocytes, fibroblasts or normal enterocytes in the vicinity of the tumor) showed positive staining. In contrast, the tumor was categorized as having deficient MMR (dMMR) when there was no tumor nuclear staining (Fig. 2) for one or more of the MMR proteins despite immunoreactivity in the internal positive controls. Any case in which the internal controls failed were withdrawn.

Statistical analysis

Statistical analysis was performed using

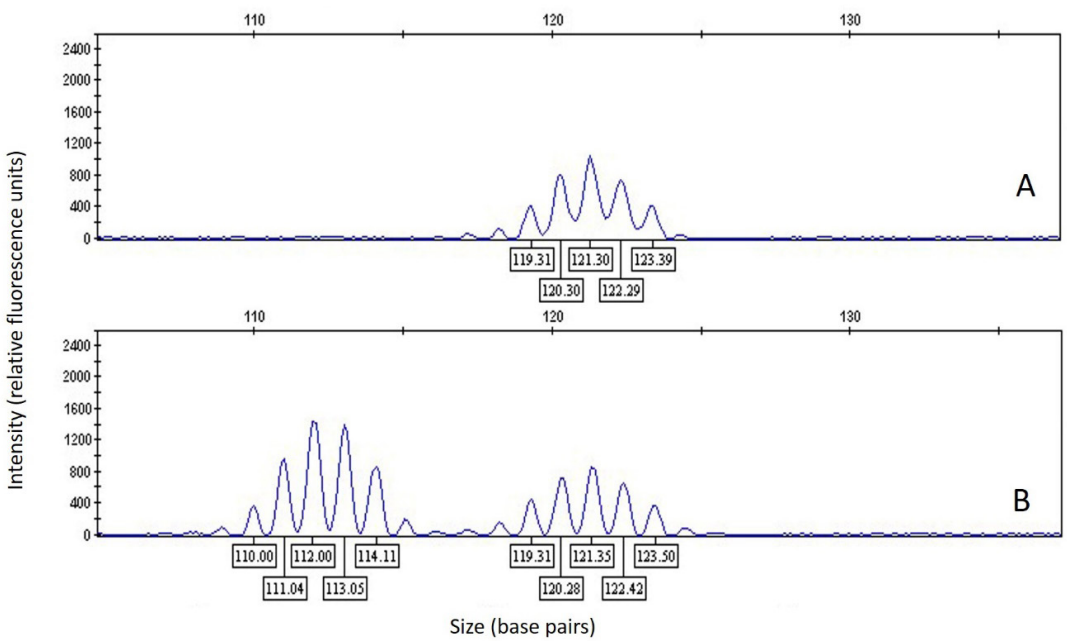

FIG. 1: Electropherograms of (A) the paired normal colon and (B) the colorectal carcinoma which demonstrates an allele shift of >3 base pairs in the tumour compared with the normal colon for BAT25.

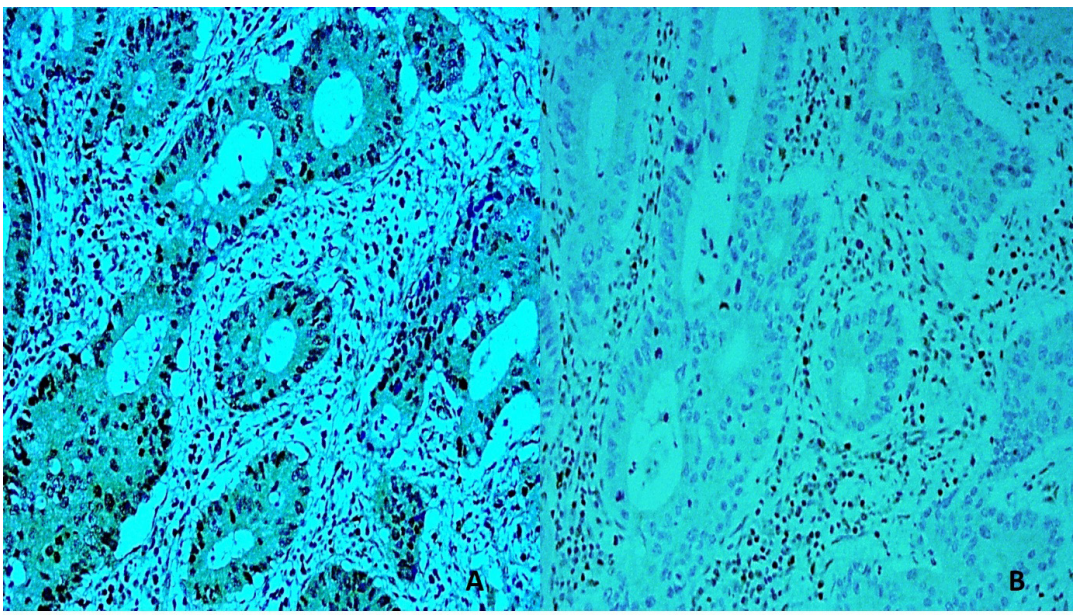

FIG. 2: Case of colorectal carcinoma which (A) demonstrated nuclear expression of MLH1 (x200) and (B) lack of expression of MSH2 (x200) and was interpreted as being deficient in mismatch repair. Note the immunopositivity in the surrounding lymphocytes which serve as internal controls in both (A) and (B).

SPSS version 24.0 (IBM, Chicago, Illinois, USA). Categorical variables would be tested by Chi-square or Fisher's exact test, while continuous variables would be tested with Independent Samples T-test for parametric data and Mann-Whitney U test for non-parametric data respectively. Cohen's kappa (κ) coefficient statistic would be used for studying agreement. The κ value will be interpreted as: almost perfect agreement (0.81-1.00); substantial agreement (0.61-0.80); moderate agreement (0.41-0.60); fair agreement (0.21-0.40), slight agreement (0.01-0.20) and poor agreement (< 0).[37,38] Statistical significance was set as $p < 0.05$.

RESULTS

Although 80 CRC which had been previously tested for DNA MMR status were initially recruited, only 73 cases could be satisfactorily analysed by PCR for microsatellite instability. The demographic data of the CRC cases finally enrolled in the study are in Table 2. The CRC on right and left appear to be fairly well-matched in age and gender (p>0.05). A difference in ethnic predominance between the right and left-sided CRCs was noted (p=0.02), with a preponderance of right-sided CRC among the Chinese with the reverse among the Malays.

TABLE 2: Demographic data of the colorectal carcinoma (CRC) cases (n=73) according to location of tumour

		Colorectal carcinoma		
		Right (n=37)	**Left (n-36)**	***p*-value**
Age	Range (years)	15-87	32-81	0.06
	Mean±SD (years)	64.2±17.6	60.2±11.9	
Gender	Male	18	24	0.12
	Female	19	12	
	M:F	1.0:1.1	1.0:0.5	
Ethnicity	Malay	3	13	0.02
	Chinese	23	14	
	Indian	9	7	
	Others	2	2	

TABLE 3: Cases of colorectal carcinoma (n-15) with either deficient DNA mismatch repair (dMMR) and/or high microsatellite instability (MSI-H) as determined by immunohistochemistry or polymerase chain reaction (PCR), respectively

Case	Tumor location	DNA mismatch repair status	MMR protein lost	Microsatellite instability status	Unstable marker
1	Right	dMMR	MLH1, PMS2	MSI-H	D2S123, D5S346, D17S250
2	Right	dMMR	MSH2, MSH6	MSS	
3	Right	dMMR	MLH1, PMS2	MSI-H	BAT25, BAT26, D17S250
4	Right	dMMR	MLH1, PMS2	MSI-H	BAT25, BAT26
5	Right	pMMR		MSI-H	BAT26, D5S346
6	Right	dMMR	MLH1, PMS2	MSI-H	BAT25, BAT26, D2S123, D5S346, D17S250
7	Right	dMMR	PMS2	MSI-H	BAT26, D2S123, D17S250
8	Right	dMMR	MLH1, PMS2	MSI-H	BAT25, BAT26
9	Right	dMMR	MLH1, PMS2	MSS	
10	Right	dMMR	MLH1, PMS2	MSS	
11	Right	dMMR	MSH2, MSH6	MSI-H	BAT25, D5S346
12	Right	dMMR	MLH1, PMS2	MSI-H	BAT26, D2S123, D5S346, D17S250
13	Right	dMMR	MSH6	MSI-L	BAT25
14	Left	pMMR		MSI-H	D2S123, D17S250
15	Left	pMMR		MSI-H	D2S123, D5S346

MSS: microsatellite stahle; MSI-L: low microsatellite instability; pMMR: proficient mismatch repair

Using PCR with subsequent fragment analysis, 11 of the 73 CRC (15.1%) were categorized as MSI-H, 12 (16.4%) MSI-L and 50 (68.5%) MSS. The number of microsatellite markers which were unstable in the 11 MSI-H cases ranged from 2-5 per case. Instability of the mononucleotide BAT25 was detected in 6 and BAT26 in 7 cases. Instability of the dinucleotide repeats, D2S123, D5S346 and D17S250, were each respectively detected in 6 cases. In contrast, using IHC, dMMR was detected in 12 of the 73 CRC (16.4%), with 8 demonstrating a combined loss of MLH1 and PMS2, 2 a combined loss of MSH2 and MSH6 and one case each with loss of PMS2 and MSH6 only. While all the dMMR cases were confined to right-sided CRCs, MSI-H was detected in 2 left-sided CRC and 9 right-sided cases. Table 3 shows the CRC with either dMMR and/or MSI-H.

Table 4 tabulates the DNA mismatch repair status by IHC versus microsatellite instability by PCR of the right-sided and left-sided CRC. Taking into consideration the lack of clinically evident differences between CRC with MSI-L and MSS,[11,21] cases with MSI-L and MSS were further grouped together. Of the 12 dMMR cases, 8 (66.7%) were classified MSI-H, while 4 (33.3%) were MSS/MSI-L (3 MSS and 1 MSI-L). Of the 61 pMMR cases, 58 (95.1%) were MSS/MSI-L (47 MSS and 11 MSI-L) while 3 (4.9%) were MSI-H. The κ value of 0.639 (standard error =0.125; p = 0.000) indicated substantial agreement between detection of DNA mismatch repair using IHC and the downstream microsatellite instability using PCR.

DISCUSSION

In a cohort of 37 right-sided and 36 left-sided CRC, unselected for Lynch syndrome, the right and left-sided CRC were fairly well-matched for age and gender. Nevertheless, there seemed to be a preponderance of right-sided tumours amongst Chinese, with a reverse pattern amongst the Malays. Furthermore, the Chinese constituted 50.7% of the total cases which is in line with the acknowledged prevalence of CRC among the Chinese in Malaysia.[2]

In this study, MSI-H was detected by PCR

TABLE 4: DNA mismatch repair status determined by immunohistochemistry (IHC) as compared with microsatellite instability determined by polymerase chain reaction (PCR) of the right- and left-sided colorectal carcinoma

	Colorectal carcinoma			
	Right		Left	
	pMMR	dMMR	pMMR	dMMR
MSI-H	1	8	2	0
MSI-L	3	1	8	0
MSS	21	3	26	0
Total	25	12	36	0

pMMR: proficient mismatch repair; dMMR: deficient mismatch repair; MSI-H: high microsatellite instability; MSI-L: low microsatellite instability; MSS: microsatellite stable

in 15.1% and dMMR by IHC in 16.4% of the 73 CRCs with both the rates being comparable to the 10-20% generally reported.[39-41] There was substantial agreement ($\kappa = 0.639$) between the two methods in determining microsatellite instability. While the results indicate that both methods are able to detect the problem at hand in the rate range that most studies have reported, endorsing the NCCN recommendation that either test can be chosen for use by the laboratory,[42] it is appropriate at this juncture (1) to understand the "measurands" of both methods and (2) to be aware of the advantages and shortcomings of each method, when making a choice of tests. Put simply, microsatellite instability results from deficiency of MMR proteins, the latter being due to defects in the MMR genes. Thus, PCR amplification identification of microsatellite alteration and immunohistochemical detection of loss of MMR proteins measure two related but different parameters. Inevitably, these two methods are both, at best, surrogates for comprehensive mutational screening of the mismatch repair genes; the latter being currently expensive, laborious and not readily available.[43] It is also important to note that under certain circumstances e.g. in sporadic CRC when hypermethylation of the 5'CpG island of the MLH1 promoter leads to epigenetic silencing of MLH1 function, there is loss of MLH1 protein production without actual mutation of the MLH1 gene.

In considering the use of PCR and subsequent fragment analysis for identification of microsatellite instability, it noteworthy that there are many panels of markers available, including that used in this study. While this method is considered to be satisfactorily reproducible,[44] the utilization of several combinations of microsatellite markers for testing,[30,44,45] will unavoidably result in variations in sensitivity and specificity. Furthermore, analysis of long alleles made up of few nucleotide repeats tends to result in PCR-induced errors and stutter peaks in the electropherograms due to the instability of such alleles. It has to be cautioned that these stutters can make interpretation of the electropherograms tricky.[46] In addition, the necessity for accompanying normal tissue to be co-tested to determine the presence of microsatellite alteration in the tumour can be a setback when the only tissue available for testing is tumour tissue.

For IHC detection of the gene products of the DNA mismatch repair system, different combinations of antibodies have also been used, with some studies focusing on MLH1 and MSH2,[47-50] while others use MLH1, MSH2, MSH6 and PMS2.[51-53] Notwithstanding, majority cover the loss of MLH1 and MSH2 which together result in 85% of MSI-H cases.[31] Nonetheless, apart from the usual MLH1, MSH2, MSH6 and PMS2, mutations in other genes e.g. MSH3, PMS1 or epithelial cell adhesion molecule (EpCAM) can also lead to dMMR and MSI-H.[54] Unlike PCR detection of microsatellite alteration, one outstanding advantage of using IHC to identify the MMR gene product is the ability to identify the target gene and this aids in future mutational confirmation. Among the shortcomings of using IHC, would be the heterogenous staining that can make interpretation challenging in small biopsies. Suboptimal quality of the tissue that results in loss of mandatory internal positive controls is another consideration. Although claims have been made that IHC requires high skill in its interpretation,[44] counter claims have also been made that IHC is a routine examination in histopathology laboratories.[43]

Setting aside the above possible technical glitches, both methods are known to produce false negative results under certain circumstances, even in technically ideal situations. For one, due to a redundancy of MSH6 with MSH3 which can compensate for the MSH6 dysfunction, PCR may not detect any microsatellite instability or sometimes only MSI-L in cases with MSH6 gene mutation detected by IHC as loss of MSH6 protein.[55] We surmise this as the situation in one of our right-sided colonic tumours which demonstrated alteration in BAT25 alone, hence interpreted as MSI-L, but demonstrated loss of MSH6 nuclear expression on IHC and interpreted as dMMR. Vice versa, due to non-functioning but antibody-binding MMR proteins there can be situations when MSI-H detected by PCR in a tumour appears to be MMR proficient on IHC staining. These non-functioning proteins have been attributed to missense mutations which unlike truncating MMR mutations appear not to lead to loss of staining.[43]

In summary, we are of the opinion that both methods can be used in determining microsatellite instability in colorectal carcinomas considering the substantial agreement of the two methods. The preference of one method over the other would depend on the expertise and type of laboratory assigned to run the test i.e. one specialized in molecular diagnostics or one specialized in histopathology. Nonetheless, it is prudent to acknowledge and understand the advantages and shortcomings of each method no matter which is finally used.

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