A pilot study on the detection of microsatellite instability using long mononucleotide repeats in solid tumors

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Abstract. Microsatellite instability (MSI) status is a prognostic biomarker for immunotherapy in certain types of cancers, such as colorectal cancers (CRCs) and endometrial cancers (ECs). Tumors that are categorized as having high MSI (MSI-H) express high levels of neoantigens for immune recognition. The typical MSI test measures the length of short mononucleotide repeats (SMR) poly(A) 21-27; however, a limitation of this test is the difficulty in determining the shift size, particularly in endometrial cancer. To investigate an MSI detection assay with improved performance, the present study analyzed the use of poly(A) 40-44 mononucleotide repeats to detect the MSI status of 100 patients with either CRC (n=50) or EC (n=50). Capillary electrophoresis was used to evaluate five long mononucleotide repeat (LMR) markers, including poly(A) 40-A, 40-B, 40-C, 40-D and 44. The concordance rate of the LMR-MSI assay compared with an immunohistochemistry MSI detection assay was 96.0 and 95.1% for CRCs and ECs respectively, with the detection limit of the LMR-MSI assay demonstrated to be 2.5% MSI-H in HCT116 colorectal carcinoma cell lines. The LMR-MSI assay yielded a 95.1% concordance rate in ECs compared with that in the SMR-MSI test (87.8%). The LMR-MSI test identified a significantly higher mean shift size (13 bp) in MSI-H tumors compared with the SMR-MSI test (10 bp), in both EC and CRC tissue samples. Together, the present study suggested that the LMR-MSI test could potentially be a sensitive and practical technology for molecular laboratory testing, particularly in the use of immunotherapy for patients with CRCs and ECs.

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Introduction

Microsatellites are short tandem repeats, formed of a set of 1-6 nucleotides, which repeat 3-60 times on DNA sequences (1). DNA slippage, repetitive elements prone to mutations during DNA replication (2), often occurs at short tandem repeats during replication, which leads to sequence insertion or deletion; this phenomenon is termed microsatellite instability (MSI) (3-5). The human DNA mismatch repair (MMR) system functions to repair replication errors. Specifically, the mutS homolog (MSH) 2-MSH6 heterodimer recognizes a DNA mismatch and the mutL homolog (MLH) 1-postmeiotic segregation increased 2 (PMS2) heterodimer repairs the DNA mismatch using proficient MMR (pMMR) systems (6). Deficient MMR (dMMR) leads to increased tumor mutational burden, with a heightened risk of neoplasia (7,8). Certain types of cancer are characterized by MSI-high/hypermutated (MSI-H), including 30% of primary endometrial cancer (EC) cases and 25% of colorectal cancer (CRC) cases (9-14).

Treatment options for patients with cancer typically include surgery, chemotherapy and immunotherapy, and the appropriate treatment is chosen based on tumor stage and characteristics (15,16). In the CheckMate 142 study including patients with metastatic CRC (mCRC) treated with nivolumab and low-dose ipilimumab, an overall survival rate of 65% and a disease control rate of 81% at 12 months were observed. In MSI-H or dMMR advanced EC, the response rates for the PD-1 inhibitors, dostarlimab and pembrolizumab, were 49 and 57%, respectively, while the PD-L1 inhibitors, avelumab and durvalumab, demonstrated response rates of 27 and 43%, respectively (17,18). In 2017, the U.S. Food and Drug Administration (FDA) approved pembrolizumab for the treatment of MSI-H or dMMR tumors, regardless of the location in the body the cancer originated. In July 2018, the

FDA approved the combination of nivolumab and low-dose ipilimumab in the treatment of patients with MSI-H/dMMR mCRC who have been previously treated with standard chemotherapy drugs (19). In March 2022, the FDA approved pembrolizumab as a single agent for patients with advanced endometrial carcinoma, that is patients with MSI-H or dMMR who have disease progression following prior systemic therapy in any setting and who are not candidates for curative surgery or radiation (20). Immunotherapy is an appropriate treatment option for patients with MSI-H/dMMR tumors, which are characterised by an increasing number of mutations and a higher number of neoantigens. $CD8^+$ T cells recognize these neoantigens, resulting in immune cell infiltration into tumors higher than microsatellite-stable (MSS) or pMMR tumors (21). However, patients with MSI-H/dMMR CRC tumors are reported to have a favorable prognosis and no benefit using 5-fluorouracil (5-FU), a first-line chemotherapy treatment for CRC (22). In tumor cells with pMMR, the MSH2-MSH6 heterodimers recognize 5-FU-induced DNA breaks and the excessive accumulation of DNA breaks induces cell apoptosis (23,24). In dMMR cells, the MSH2-MSH6 heterodimer loses the ability to recognize DNA errors, which leads to the evasion of cell apoptosis (25).

Personalized treatments for patients with dMMR and MSI-H should be considered. Traditionally, surgery remains a crucial component of treatment, especially for localized disease. Unresectable locally advanced or mCRC is treated with 5-FU-based chemotherapy. However, although immunotherapy strategies have been established, there remains a need to explore the choice between single-agent or dual checkpoint inhibitor treatment, as well as primary or secondary resistance.

The European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network's Clinical Practice Guidelines in Oncology recommended MMR and MSI detection using immunohistochemistry (IHC) staining or PCR tests, prior to the treatment of patients with CRC or EC (26,27). IHC for MMR and MSI testing detects the level of four MMR proteins: MLH1, MLH2, PMS2 and MSH6, to evaluate the pMMR or dMMR status in the tumor (28). Although there is a high degree of concordance between MSI-PCR and MMR-IHC tests, discordances of 3-5% between the two assays can occur via a number of mechanisms (29-31). MMR protein dysfunction causes an increase in missense mutations. However, in certain cases the epitope of a monoclonal antibody may be maintained, which can lead to intact MMR protein expression and false negative IHC results. Consequently, these MSI-PCR assays may yield MSI-H results. Next generation sequencing (NGS) analysis of an IHC and MSI-PCR inconclusive case revealed missense and frameshift mutations in the PMS2 and MSH6 genes, respectively (32-34). Therefore, the College of American Pathologists Guideline recommended using both IHC and PCR-based MSI tests for the detection of MSI status (35). The Bethesda panel is a set of markers used for MSI testing in patients with cancer. It was initially recommended by the National Cancer Institute in the United States. The panel includes two mononucleotide repeat markers (BAT-25 and BAT-26) and three dinucleotide repeat markers (D2S123, D5S346 and D17S250) (36). Dinucleotide repeats are reported to have a lower sensitivity in the detection of MSI status compared with mononucleotide repeats and therefore short mononucleotide repeats (SMRs) of 21-27 bp are often used to detect MSI status in patients with colorectal cancer (37). MSI status is determined using the number of positive markers: MSI-H refers to detection of ≥30% unstable microsatellite loci or ≥2 positive markers, low frequency of MSI (MSI-L) refers to detection of a singular positive marker and MSS refers to a lack of detected positive markers (38). However, the use of SMRs is currently insufficient for MSI analysis as equivocal (cases with ambiguous results or small shifts) were detected, especially in EC (39).

In a previous study, a shift of 1 nucleotide was observed in multiple markers in 76% of MSI-H EC cases, whereas only 12% of MSI-H CRC cases displayed a 1 nucleotide shift in one of five markers (40). The equivocal results were subtle (MSI-PCR with one or two nucleotides shifting) particularly in endometrial cancer. In such cases, patients may not receive an appropriate treatment regimen. A concurrent limitation is that DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples from patients is of low quality and is easily fragmented, although a number of companies are refining their products, such as the MSI Analysis System (Promega Corporation), to overcome this limitation (41-43). The present study suggested that long mononucleotide repeats (LMRs) of >40 bp have longer shift sizes, which could improve PCR-based MSI detection. LMR markers produce more pronounced fragment length changes, and therefore could improve the detection of MSI that may have otherwise gone undetected with the use of SMR markers alone. The present study aimed to investigate the LMR markers to improve PCR-based MSI test for patients with CRC and EC.

Materials and methods

Sample preparation. Data from 100 patients with either CRC or EC, who received surgical treatment or adjuvant chemotherapy between September 2017 and July 2022 at the National Cheng Kung University Hospital (NCKUH; Tainan, Taiwan) were retrospectively analyzed in the present study. All histopathological biopsies were independently graded by two pathologists, using the American Joint Committee on Cancer classification (44). Matched tumor (with at least 20% tumor content) and adjacent normal tissue samples were selected, which included tissue samples from 50 patients with CRC and 50 patients with EC. The protocols in the present study were approved by the NCKUH Institutional Review Board (approval nos. B-ER-109-15 and A-ER-108-311; Tainan, Taiwan) and all identifiable patient data were anonymized prior to analysis.

IHC. IHC was used to detect MMR protein expression to categorize tissue samples into either the dMMR or pMMR group. The thickness of the unstained sections was 5-μm per sample and the sections were prepared from FFPE tissue blocks. Deparaffinization and rehydration were performed using xylene and ethanol, respectively. The endogenous peroxidase was blocked using 3% hydrogen peroxide. Antigen retrieval was conducted using the Ph 9.0 Target Retrieval Solution (Dako; Agilent Technologies, Inc.). The sections were then incubated with primary antibodies for MLH1 (clone, M1; cat. no. 760-5091; diluted 1:1; Roche Tissue Diagnostics), PMS2

(clone, A16-4; cat. no. 760-5094; diluted 1:1; Roche Tissue Diagnostics), MSH2 (clone, G219-1129; cat. no. 760-5093; diluted 1:1; Roche Tissue Diagnostics) and MSH6 (clone, SP93; cat. no. 760-5092; diluted 1:1; Roche Tissue Diagnostics) at 4˚C overnight. Secondary antibodies were prepared for immediate use and were applied for 30 min at room temperature using EnVision+ System-HRP Labelled Polymer anti-rabbit (cat. no. K4003) and anti-mouse (cat. no. K4001) (Dako; Agilent Technologies, Inc.). Visualization of these proteins was achieved using the Liquid DAB+ Substrate Chromogen System (Dako; Agilent Technologies, Inc.). Sections were counterstained with hematoxylin for 5 min at room temperature. Stained tissue slides were visualized by light microscopy (OLYMPUS BX53) and analyzed using cellSens software (OLYMPUS cellSens Entry 3.1; https://www.olympus-lifescience.com/en/software/cellsens/).The results were evaluated by a pathologist who determined that the absence of nuclear staining in tumor cells and the presence of positive staining in surrounding stromal cells indicated a loss of signal. This was defined as a loss of MMR proteins, which is characteristic of dMMR tumors. Conversely, tumors that exhibited no loss of MMR proteins were classified as pMMR. Partial patient data (CRC nos. 47 and 50) were reviewed in our previous study (45) (Fig. S1).

DNA extraction. To extract DNA from tissue samples, 3-5 sections of FFPE tissue samples from a single patient were placed into a 1.5 ml tube and the QIAamp DNA FFPE Tissue Kit (cat. no. 56404; Qiagen GmbH) was used according to the manufacturer's instructions. DNA concentration was quantified using a NanoDrop® 2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Samples containing ≥30 ng of DNA were used in subsequent experiments.

MSI detection by SMR-MSI and LMR-MSI tests. In accordance with ESMO recommendations, five poly(A) SMRs (BAT-25, BAT-26, NR-21, NR-24 and NR-27) were selected for the SMR-MSI test based on data from our previous study (32,45). The LMR test was performed using patent protected markers for 40-A, 40-B, 40-C, 40-D and 44. The patent protected markers were used in the detection kit for MSI in biological samples. To select the LMR markers, the human reference genome (GRCh38; accession no. GCF_000001405.26) and the genome annotation from the National Centre for Biotechnology Information (NCBI) RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/) was obtained (46). The reference genome was parsed to identify ≥40 consecutive bases of adenines (or thymines) that represented LMRs. For each LMR, the closest flanking genes (based on the RefSeq annotation) were identified. The present study selected five LMRs that had flanking genes (*KIT*, KIT proto-oncogene receptor tyrosine kinase; *RAC1*, Rac family small GTPase 1; *KLF4*, krüppel-like factor 4; *INFAR1*, IFN-α and β receptor 1; and *FHIT*, fragile histidine triad diadenosine triphosphatase) involved in CRC and EC. The selected LMR sequences, together with the flanking sequences (80 bp), were used to design primers via the NCBI Primer-Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (47) and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the default parameters (Table SI).

The DNA extracted from the patient tissue samples were amplified using the Hot Start PCR Master Mix (Thermo Fisher Scientific, Inc.) and 10 pmol/μl forward and reverse primers. The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 30 sec, 54˚C for 45 sec and 72˚C for 1 min; and a final extension at 72˚C for 10 min, using a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.). Capillary electrophoresis analysis using the PCR products was performed using a QIAxcel DNA Screening Kit (Qiagen GmbH) according to the manufacturer's instructions, using a QIAxcel Advanced Instrument (Qiagen GmbH).

Tissue samples that presented MSI markers with either band shifts or smears were considered to be positive for MSI. For the SMR-MSI and LMR-MSI tests, classifications of MSI-H, MSI-L and MSS described the detection of ≥2, 1 and 0 positive markers, respectively.

Cell culture. The HCT116 human colon carcinoma cell line was cultured in McCoy's 5A (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 16600) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 26140079) and 1% streptomycin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15140-122) and cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Limit of detection (LoD) of LMR assay. The genomic DNA from MSI-H HCT116 cells, a CRC cell line harboring a heterozygous MLH1 c.755C>A mutation, was mixed with that of normal FFPE DNA to contain a total of 30 ng of DNA in each reaction. Genomic DNA was extracted from cells and FFPE using the QIAamp DNA Micro Kit (cat. no. 56304; Qiagen GmbH) and the QIAamp DNA FFPE Tissue Kit (cat. no. 56404; Qiagen GmbH), respectively. The samples contained final concentrations of 100.0, 50.0, 20.0, 10.0, 7.5, 5.0 and 2.5% HCT116 DNA for the LoD assay.

Concordance between SMR-MSI and LMR-MSI analyses. The concordance rate was calculated as the number of dMMR cases classed as MSI-H and pMMR cases classed as either MSS or MSI-L cases using the SMR-MSI or LMR-MSI tests, and the resulting number of cases was divided by the number of total cases. Sensitivity, or positive percentage agreement, was calculated as the number of MSI-H cases detected using the SMR or LMR-MSI tests divided by the number of dMMR cases detected using MMR-IHC. Specificity, or negative percentage agreement, was calculated as the number of MSI-L and MSS cases detected using the SMR or LMR-MSI tests divided by the number of pMMR cases detected using IHC.

Target NGS of MMR pathway genes. The Human Colorectal Cancer Focus Panel (cat. no. PHS-103Z; Qiagen GmbH) was used to perform NGS (Table SII) of CRC case nos. 48 and 49, which were classed as pMMR using the MMR-IHC assay but were classed as MSI-L using the SMR-MSI test and as MSI-H using the LMR-MSI test. The Human Breast Cancer Focus Panel (cat. no. PHS-102Z; Qiagen GmbH) was also performed on the EC case nos. 13 and 14, which were classed as MSI-H using the SMR and LMR-MSI tests, but classed as pMMR using the dMMR-IHC assay. The panel included the following

MMR pathway related genes: *MLH1, MLH3, MSH2, MSH3, PMS2* and *MSH6* (Table SII).

QIAamp DNA FFPE Kits (cat. no. 56404; Qiagen GmbH) were used for the purification of FFPE DNA for NGS sequencing. A total of 250 ng FFPE DNA was used for library construction. Amplicons were dual barcoded for sample identification. Construction of the DNA libraries was performed according to the manufacturer's instructions. Library QC was performed with QIAxcel DNA High Resolution Kit (cat. no. 929002; Qiagen GmbH) to check the correct size distribution of the library. The library was quantified using QIAseq Library Quant Assay Kit (cat. no. 333314; Qiagen GmbH). The loading concentration of the final library was 10 pM, measured using a Qubit fluorometer. The sequencing run was performed using the MiSeq Reagent Kit v2. (cat. no. MS-102-2002). Paired ends libraries (2X 150 bp) were sequenced using the Illumina MiSeq sequencer (Illumina, Inc.). Mean sequence depths of at least 800X were achieved for the tumor tissue.

Data analysis and interpretation was performed using the GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/us/analyze). Data analysis was focused on the MMR pathway and homologous recombination (HR)-related genes (the MMR pathway genes: *MLH1, MLH3, MSH2, MSH3, PMS2* and *MSH6*; the HR genes: *BRCA1, BRCA2, RAD51B, RAD51C, RAD51D* and *RAD54L*) using the QIAGEN Clinical Insights Interpret software (version 9.1.0.20230224; Qiagen GmbH) as it offered flexible and automatable interpretation workflows. The variants were classified as either pathogenic or likely pathogenic using the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/)

Statistical analysis. The sample size estimate was calculated using an online calculator (https://turkjemergmed.com/calculator) for statistical power calculations (48). The calculations were performed at 95% CI and the minimum sample size was calculated to be 97 cases for the present study. A two-tailed unpaired Student's t-test and Fisher's exact were performed using SPSS (version 17.0; IBM Corp.) to evaluate the shift sizes between the PCR-based MSI analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Clinicopathologic features of CRC and EC cases in the present study.

Clinicopathologic features	CRC (total, n=50)	EC (total, n=50)
Mean age at diagnosis, years ± SD	63.6±13.2	56.6±12.9
Sex, n (%)		
Male	32 (64)	0 (0)
Female	18 (36)	50 (100)
Histologic type, n (%)		
Adenocarcinoma	44 (88)	45 (90)
Mucinous adenocarcinoma	6 (12)	5 (10)
Histological features		
Well differentiated	7 (14)	11 (22)
Moderately differentiated	35 (70)	20 (40)
Poorly differentiated	8 (16)	19 (38)
TNM stage, n (%)		
0-I	3 (6)	10 (20)
II	15 (30)	17 (34)
III	28 (56)	19 (38)
IV	4 (8)	4 (8)
T stage, n (%)		
1	1 (2)	18 (36)
2	2 (4)	14 (28)
3	33 (66)	18 (36)
4	14 (28)	0 (0)
N stage, n (%)		
0	18 (36)	25 (50)
1	17 (34)	21 (42)
2	15 (30)	4 (8)
M stage, n (%)		
0	31 (62)	28 (56)
1	19 (38)	22 (44)

CRC, colorectal cancer; EC, endometrial cancers; TNM, tumor, node, metastasis.

Results

Study population and samples. To evaluate the LMR test for patients with CRC or EC, tissue sections were collected and independently validated by two board-certified pathologists. Patients with CRC had an age range of 36-89 years and a mean age of 64 years (Table I). Patients with EC had an age range of 29-89 years and a mean age of 57 years. Of the patients with CRC, 64% were male (32/50) and 36% were female (18/50). The mean percentage of tumor cells in CRC and EC tissue samples were 52.7% (range, 20-80%) and 54.4% (range, 10-90%), respectively.

Comparison of MMR-IHC, SMR-MSI and LMR-MSI assays. To examine LMR marker availability in patients with CRC or EC, DNA extracted from FFPE tissues were analyzed using MMR-IHC, SMR-MSI and LMR-MSI, the latter of which used LMR markers 40-A, 40-B, 40-C, 40-D and 44. Differences between tumor and normal allelic size of ≥3 bp were considered to represent a band shift; the presence of a number of newly generated bands in tumor tissues in comparison to normal tissues was considered to be indicative of band smears (Figs. 1 and S2). The LMR-MSI test LoD was evaluated by the detection of increasing ratios of MSI-positive DNA from HCT116 cells with patient DNA. The LoD was 2.5% for LMR markers 40-A, 40-B, 40-C, 40-D and 44 when using the QIAxcel high-resolution screening gel (Fig. S3).

In CRC cases, the concordance rates of the SMR and LMR-MSI assays were both 96.0% when compared with MMR-IHC (Table II). A large proportion of CRC cases showed high concordance across the MMR-IHC, SMR and LMR-MSI tests. Case nos. 1-26 were classed as dMMR with MSI-H using both SMR and LMR-MSI test results, while case nos. 27-46 were classed as pMMR with MSS using both SMR and LMR-MSI test results (Table III). Out of the present 50 CRC cases, only 4 cases had discrepancies across the MSI tests. This indicated that there was no overt tendency of false

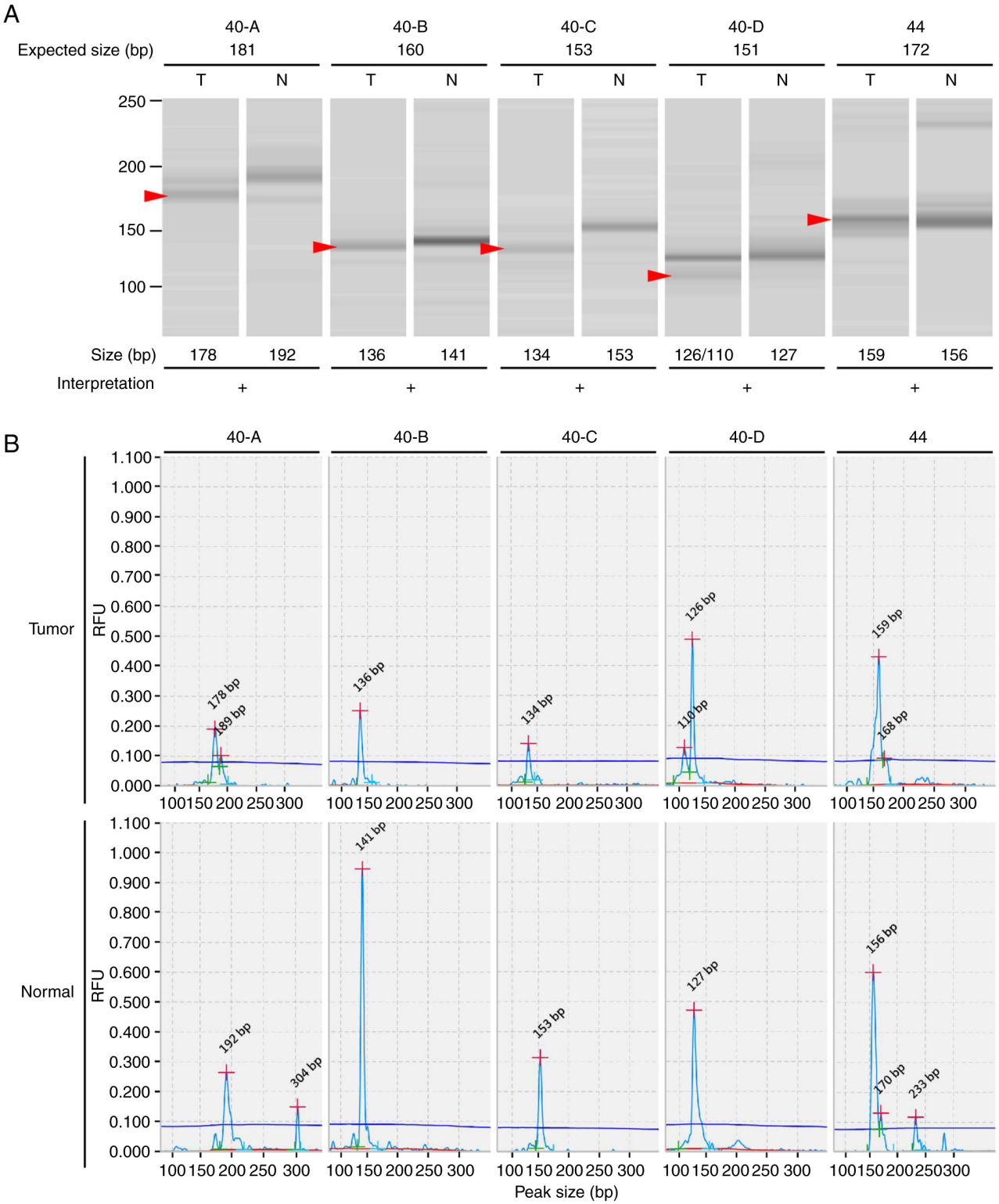

Figure 1. Representative shift peaks of LMR markers from CRC case no. 8. (A) Shift bands in the capillary electropherogram, produced using QIAxcel Advanced System (Qiagen GmbH). Red arrows indicate band shifts. (B) Shift peaks of tumor tissue and normal tissue were matched after analysis of capillary electrophoresis results. Red crosses indicate band shifts. RFU refers to the peak intensity in data. RFU, Relative Fluorescence Units; LMR, long mononucleotide repeats; T, tumor; N, normal; +, positive.

negative or positive results using the novel LMR-MSI test. Case nos. 47 and 50 were classed as dMMR with MSS and MSI-L using the SMR-MSI test, which represented potential false negatives, as the LMR-MSI test indicated the cases were MSI-H. Case nos. 48 and 49 were classed as pMMR using the MMR-IHC, MSI-L using the SMR-MSI test, but MSI-H using the LMR-MSI test, which represented a potential false positive using the LMR-MSI test. Tissue samples from the last two CRC cases, nos. 48 and 49, were used for NGS analysis.

Table II. Concordance rate of SMR-MSI and LMR-MSI assays compared with MMR-IHC in CRC and EC cases.

	CRC		EC	
Variable	SMR	LMR	SMR	LMR
Concordance rate, %	96.0	96.0	87.8	95.1

CRC, colorectal cancer; EC, endometrial cancer; SMR, short mononucleotide repeats; LMR, long mononucleotide repeats; IHC, immunohistochemistry; MMR, mismatch repair.

In the EC cases, 7 cases were excluded as MMR-IHC results were unavailable. The concordance rates of the SMR-MSI and LMR-MSI tests were 87.8 and 95.1% respectively, compared with MMR-IHC (Table II). For the EC cases with MMR-IHC results, a large proportion of cases demonstrated a high concordance across MMR-IHC, SMR-MSI and LMR-MSI tests. Case nos. 1-10 were classed as dMMR with MSI-H using both SMR-MSI and LMR-MSI tests, while case nos. 15-40 were classified as pMMR with MSS using both SMR-MSI and LMR-MSI tests (Table IV). In 41 EC cases with IHC results, 5 cases showed discrepancies between the three analyses. Case nos. 48-50 were classed as dMMR with MSS or MSI-L using the SMR-MSI test which represented a potential false negative, while the novel LMR-MSI tests indicated MSI-H for these cases. Case nos. 13 and 14 were classed as pMMR with MSI-H using both SMR-MSI and LMR-MSI tests. MMR proteins may be dysfunctional but retained structures recognized by antibodies, resulting in pMMR classification when using MMR-IHC detection (49). Tissue samples from EC case nos. 13 and 14 were used for NGS analysis.

LMR tests demonstrated larger shift sizes. The SMR-MSI test detected a small number of MSI-L cases, 3 CRC cases and 1 EC case (Fig. 2). The LMR-MSI test did not detect any MSI-L cases in the present patient cohorts. The cases detected as MSI-L by the SMR-MSI test were changed to an MSI-H classification following application of the LMR-MSI test (Tables III and IV). Of the 4 cases, 2 were classified as dMMR, while the other 2 cases were classified as pMMR. The LMR markers exhibited significantly larger shift sizes and a significantly higher mean shift size (13 bp) in MSI-H tumors compared with the SMR-MSI test (10 bp). The average shift sizes of LMR markers were 1.3-fold higher in both CRC ($P<0.0001$) and EC ($P=0.003$) (Fig. 3).

NGS. The 4 discrepant cases (CRC, case nos. 48 and 49; EC, case nos. 13 and 14) that were initially classed as pMMR but LMR-MSI tests indicated were MSI-H, were submitted for NGS. Of these, 3 of the cases did not demonstrate any pathological variations using the NGS panel. Only 1 EC case, no. 13, presented as MSI-H but with no loss of protein in the MMR-IHC, which represented pMMR. The NGS results demonstrated that the tumor sample of EC case no. 13 harbored one major somatic mutation: 1.25% alleles with a *MSH3* c.4091-2A>T mutation (accession no. NM_001040108.2), which occurs in the splice acceptor region, clinical significance was according to the ClinVar database (50). It was proposed that the *MSH3* c.4091-2A>T mutation could lead to premature translation termination and result in aberrant protein production of *MSH3*. This could potentially disrupt the normal function of the MMR and lead to MSI-H.

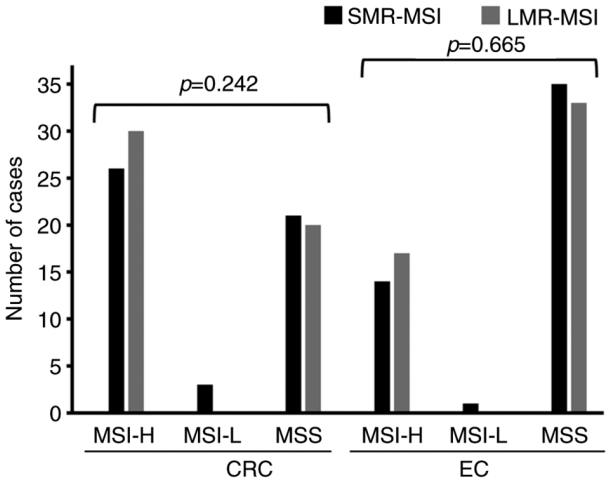

Figure 2. Number of cases in each MSI status group using the SMR and LMR-MSI tests. MSI status classification was based on the number of detected positive markers in both SMR and LMR tests. Each cancer group had 50 cases. The statistical analysis utilized a Fisher's exact test. MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stability; CRC, colorectal cancer; EC, endometrial cancer; SMR, short mononucleotide repeats; LMR, long mononucleotide repeats.

Discussion

The present study used LMR markers, including 40-A, 40-B, 40-C, 40-D and 44, to detect MSI status in patients with CRC and EC, using PCR amplification and capillary electrophoresis analysis. The LMR-MSI test showed high concordance rates compared with MMR-IHC analysis and traditional SMR-MSI tests. The present pilot study involved 100 patient cases, 50 of which were cases of patients with CRC and 50 were patients with EC. In total, 7 cases were excluded as MMR-IHC results were unavailable. Of the remaining 93 cases that had all three test results available, 82 were consistent across all three platforms. All 38 cases classed as dMMR/MSI-H cases using MMR-IHC/SMR-MSI assays were detected to be MSI-H cases using the LMR-MSI test. All 46 cases classed as pMMR/MSS cases using MMR-IHC/SMR-MSI assays, were detected to be MSS cases using the LMR-MSI test. The remaining 9 cases were categorized into two groups: i) MSI-L cases detected using SMR-MSI tests; and ii) cases with discrepancies between MMR-IHC and SMR-MSI tests.

LMR-MSI tests reduced the number of equivocal case generated compared with SMR-MSI tests. Cases classed as MSI-L using SMR-MSI tests were detected as MSI-H in 3 CRC cases and in 1 EC case, with use of the LMR-MSI assay. MSI-L is often misclassified as MSS and in such cases, the patients may potentially not receive the appropriate medication recommendations (51). In this study, 1 CRC case (no. 50) and 1 EC case (no. 50) were classed as MSI-L using SMR-MSI testing but were classed as dMMR using MMR-IHC and as

Table III. MMR and MSI status using MMR-ICH, SMR-MSI and LMR-MSI assays in 50 cases of patients with CRC.

CRC case no.	MMR-IHC status	MMR-IHC markers				SMR-MSI status	SMR-MSI markers					LMR-MSI status	LMR-MSI markers				
		MLH1	MSH2	PMS2	MSH6		BAT-25	BAT-26	NR-21	NR-24	NR-27		40-A	40-B	40-C	40-D	44
1	dMMR	+	-	+	-	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
2	dMMR	+	-	+	-	MSI-H	+	+	+	+	+	MSI-H	+	-	+	+	+
3	dMMR	+	-	+	-	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	+
4	dMMR	+	-	+	-	MSI-H	+	+	+	+	+	MSI-H	-	+	+	-	+
5	dMMR	-	-	-	-	MSI-H	+	+	+	+	+	MSI-H	+	+	+	-	-
6	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
7	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
8	dMMR	-	+	-	+	MSI-H	+[a]	+	+	+	+	MSI-H	+	+	+	+	+
9	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
10	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	-	+
11	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	-	+	+	+	+
12	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	-	+	+	+	+
13	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	-	+	+	+	+
14	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	-	+	+	-	+
15	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	-	+	+	-	+
16	dMMR	-	+	-	+	MSI-H	+	+	-	+	+	MSI-H	-	-	+	-	+
17	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	+
18	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	+
19	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
20	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	-	-
21	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	-	-	+
22	dMMR	+	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
23	dMMR	+	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	+	+
24	dMMR	+	+	-	+	MSI-H	+	+	+	-	+	MSI-H	+	+	+	-	+
25	dMMR	+	+	-	+	MSI-H	+[a]	+[a]	+[a]	+[a]	+[a]	MSI-H	+	-	+	-	+
26	dMMR	+	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	-
27	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
28	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
29	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
30	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
31	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
32	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-

Table III. Contniued.

CRC case no.	MMR-IHC status	MMR-IHC markers				SMR-MSI status	SMR-MSI markers					LMR-MSI status	LMR-MSI markers				
		MLH1	MSH2	PMS2	MSH6		BAT-25	BAT-26	NR-21	NR-24	NR-27		40-A	40-B	40-C	40-D	44
33	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
34	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
35	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
36	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
37	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
38	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
39	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
40	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
41	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
42	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
43	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
44	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
45	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
46	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
47	dMMR	+	+	-	+	MSS	-	-	-	-	-	MSI-H	-	-	-	+	+
48	pMMR	+	+	+	+	MSI-L	-	-	+	-	-	MSI-H	-	-	-	+	+
49	pMMR	+	+	+	+	MSI-L	-	-	-	+	-	MSI-H	+	-	-	+	+
50	dMMR	+	+	+	-	MSI-L	-	-	-	+	-	MSI-H	+	-	+	-	-

[a]MSI-test performed using QIAxcel high-resolution gel. CRC, colorectal cancer; MMR, mismatch repair system; dMMR, deficient mismatch repair; IHC, immunohistochemistry; pMMR, preserved mismatch repair; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stability.

Table IV. MMR and MSI status using different MMR-ICH, SMR-MSI and LMR-MSI assays in 50 cases of patients with EC.

EC case no.	MMR-IHC status	MMR-IHC markers				SMR-MSI status	SMR-MSI markers					LMR-MSI status	LMR-MSI markers				
		MLH1	MSH2	PMS2	MSH6		BAT-25	BAT-26	NR-21	NR-24	NR-27		40-A	40-B	40-C	40-D	44
1	dMMR	-	+	-	+	MSI-H	-	+[a]	-	+[a]	+[a]	MSI-H	+	+	+	-	+
2	dMMR	-	+	-	+	MSI-H	+	+	+	-	-	MSI-H	+	+	+	-	+
3	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	+
4	dMMR	-	+	-	+	MSI-H	+[a]	+[a]	+[a]	+[a]	+[a]	MSI-H	+	-	+	+	+
5	dMMR	-	+	-	+	MSI-H	+[a]	-	+[a]	+[a]	-	MSI-H	-	-	+	-	+
6	dMMR	-	+	-	-	MSI-H	+	+	+	-	+	MSI-H	+	-	+	-	+
7	dMMR	-	-	+	+	MSI-H	+	+	-	+	+	MSI-H	+	-	+	-	+
8	dMMR	+	-	+	-	MSI-H	+	+	+	+	+	MSI-H	+	-	+	-	+
9	dMMR	+	-	+	-	MSI-H	+	+	-	+	+	MSI-H	+	-	+	-	+
10	dMMR	+	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	+	+	-	+
11	dMMR	-	+	-	+	MSI-H	+	-	+	+	+	MSI-H	+	-	+	-	-
12	dMMR	-	+	-	+	MSI-H	+	+	+	+	+	MSI-H	+	-	-	-	+
13	pMMR	+	+	+	+	MSI-H	+[a]	+[a]	+[a]	+[a]	+[a]	MSI-H	+	+	+	-	-
14	pMMR	+	+	+	+	MSI-H	+	+	+	+	+	MSI-H	-	-	+	+	+
15	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
16	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
17	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
18	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
19	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
20	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
21	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
22	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
23	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
24	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
25	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
26	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
27	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
28	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
29	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
30	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
31	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-

Table IV. Continued.

EC case no.	MMR-IHC status	MMR-IHC markers				SMR-MSI status	SMR-MSI markers					LMR-MSI status	LMR-MSI markers				
		MLH1	MSH2	PMS2	MSH6		BAT-25	BAT-26	NR-21	NR-24	NR-27		40-A	40-B	40-C	40-D	44
32	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
33	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
34	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
35	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
36	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
37	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
38	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
39	pMMR	+	+	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
40	pMMR	NA	NA	+	+	MSS	-	-	-	-	-	MSS	-	-	-	-	-
41	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
42	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
43	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
44	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
45	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
46	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
47	NA	NA	NA	NA	NA	MSS	-	-	-	-	-	MSS	-	-	-	-	-
48	dMMR	+	+	+	-	MSS	-	-	-	-	-	MSI-H	+	+	-	-	+
49	dMMR	+	+	+	-	MSS	-	-	-	-	-	MSI-H	+	+	-	+	-
50	dMMR	+	+	+	-	MSI-L	-	-	-	+	-	MSI-H	+	+	+	+	+

[a]MSI-test performed using QIAxcel high-resolution gel. CRC, colorectal cancer; MMR, mismatch repair system; dMMR, deficient mismatch repair; pMMR, preserved mismatch repair; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stability; NA, not available.

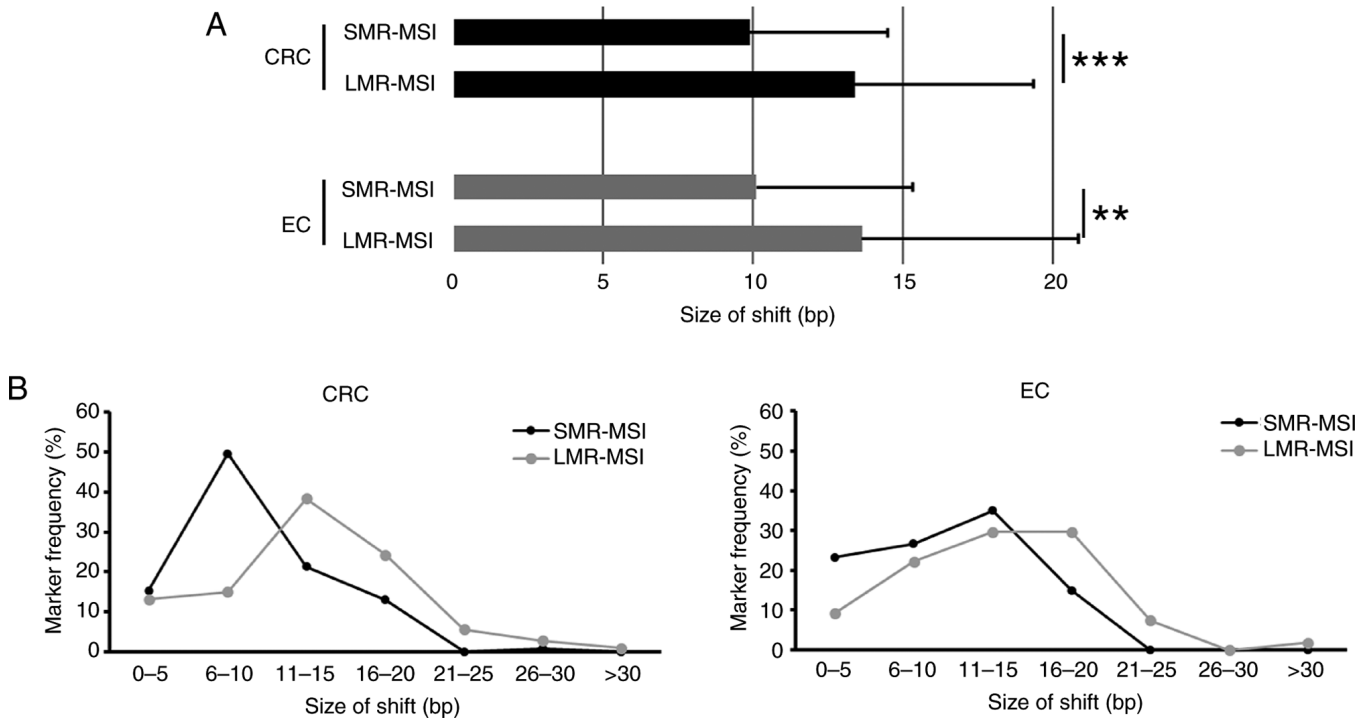

Figure 3. Shift sizes detected using the SMR and LMR markers. (A) Mean shift size detected by SMR and LMR tests. Error bars represent the standard deviations of the mean. Data were analyzed using a two-tailed unpaired Student's t-test, $^{**}P<0.005$; $^{***}P<0.0001$. (B) SMR and LMR marker frequency in different shift size categories in CRC and EC cases. CRC, colorectal cancer; EC, endometrial cancer; MSI, microsatellite instability; SMR, short mononucleotide repeats; LMR, long mononucleotide repeats.

MSI-H using the LMR-MSI test. Using the MMR-IHC and LMR-MSI results, these aforementioned cases were most likely dMMR cases. At present, the patients with tumors classed as dMMR cases used chemotherapy as the first-line therapy, despite the potential for long-term survival. In these cases, immune-checkpoint inhibitor (ICI) therapy could be considered as second-line therapy. In the case of CRC case no. 50, the patient commenced chemotherapy in November 2017, with the best treatment response recorded as 'stable disease' following 6 months of treatment. At the time-of-writing (May, 2024) the patient continues to attend regular follow-up appointments. In the case of EC case no. 50, the patient received chemotherapy in July 2018, with the best treatment response recorded as 'complete remission' following 18 months of treatment. The two aforementioned cases were treated with first-line chemotherapy, which resulted in long-term survival and a positive response to treatment. Jaffrelot *et al* (52) reviewed the occurrence of MMR-IHC-only loss of PMS2 or MSH6, which was present in 15% of dMMR tumors examined for all MMR IHC markers. The subgroups were found to be highly associated with MSI-H (81%), with 1 case detected to be MSI-L, similar to CRC case no. 50 and EC case no. 50 reported in the present study. The previous study further analyzed the NGS panel and found that the tumor mutational burden (TMB) of 20 cases (38%) was reported to be TMB-high or TMB-intermediate cases. Of the cases examined, 2 cases showed a complete response after immunotherapy.

The 2 MSI-L cases of the present study, CRC case nos. 48 and no. 49 were classed as pMMR using MMR-IHC but classed as MSI-H using the LMR-MSI assay. NGS of the 2 aforementioned tumor samples did not reveal any pathological mutations. The results suggested potential false positive results of the LMR-MSI test in the aforementioned CRC cases. However, although the NGS panel used was primarily designed for CRC samples and included several MMR-related genes, it may be far from fully comprehensive. Further investigation is required to determine the nature of the 2 aforementioned cases with pMMR, such as the methylation status of MLH1, MSH2, PMS2 and MSH6 promoter regions. Unfortunately, the 2 samples were unavailable for further investigation.

The 2 pMMR cases detected using MMR-IHC in EC cases of the present study were classed as MSI-H by both SMR and LMR-MSI tests. PCR-based MSI tests detect microsatellites at the DNA level and IHC analyzes MMR systems at the protein level using antibody specificity (4). Both methods have limitations, such as a low level of tumor tissue percentage (for example <10%), which can affect the tumor DNA concentration in the PCR-based MSI test, and the MMR-IHC test is limited in the detection of tumors containing functionally deleterious mutations but unaffected MMR protein expression levels (53). The two methods may be used in combination to provide patients with more comprehensive MSI test results. Of the discordant cases reported in the present study, EC case no. 13 exhibited pMMR using MMR-IHC, but was demonstrated to have a missense mutation in the *MSH3* gene using NGS.

Sole MSH6 deficiency may cause discrepancies between MMR-IHC and traditional SMR-MSI tests. MSH6 forms a heterodimer with MSH2 and is involved in DNA repair in MMR systems (7). Loss of MSH6 protein expression levels detected through IHC alone may not confirm MSI-H in patients. When the MSH6 protein is mutated the MSH2/MSH3 heterodimer still operates and DNA mismatch errors

are partially corrected. A number of patients with CRC no longer exhibit MSH6 expression following neoadjuvant therapy (54,55). Therefore, it would be necessary to use further methods, in addition to MMR-IHC, in such cases. In the present study, 4 cases exhibited MSH6 loss (3 EC cases and 1 CRC case), all without prior neoadjuvant therapy. The LMR-MSI test detected MSI-H in all 4 cases, while the SMR-MSI test indicated either MSS or MSI-L. Therefore, LMR-MSI test may have the potential to verify MSI status in cases with sole MSH6 loss without neoadjuvant therapy, particularly in patients with EC. CRC case no. 47 was classed as dMMR (PMS2 deficient alone) using the MMR-IHC testing but was classed as MSS using the SMR-MSI test. The LMR-MSI test indicated MSI-H in this case, which was consistent with MMR-IHC test results. The SMR-MSI test was repeated and demonstrated the same result of MSS status in the aforementioned CRC case. The reason that MSS was detected in the aforementioned case using the SMR-MSI test is currently unclear (56). However, a possible limitation is that the Bethesda panel (dinucleotides repeat markers) is less sensitive and specific than the LMR-MSI.

FFPE is beneficial for long-term tissue storage; however, FFPE-extracted DNA is occasionally fragmented and of low quality (41). The DNA extracted from FFPE tissue was sufficient for the LMR-MSI assay in the present study. The results of the present study have implications for the improvement of personalized treatment in certain types of cancer. Certain assay detection equipment, such as the MSI Analysis System (Promega Corporation), uses fluorescent signals for MSI analysis (57,58). A non-fluorescent targeted LMR-MSI test was used in the present study. The advantages of non-fluorescent systems are the low costs and no risk of ineffective fluorescent reagents due to improper storage or photobleaching (59). Idylla (Biocartis Group NV) have launched a non-fluorescent system that uses fully automated real-time PCR testing for MSI detection in FFPE tissues (60,61). NGS is also considered an MSI detection method; however, it is time-consuming with an operation time of 2 weeks and is relatively expensive compared with alternative MSI tests available (53). Each type of tumor requires screening in a number of databases during NGS data analysis (49,53).

The present study sought to evaluate the performance of the LMR-MSI assay by analyzing LMR markers in tumor and normal tissues using QIAxcel, a non-fluorescent system. The results demonstrated that the sensitivity of the LMR-MSI test was higher than that of the SMR-MSI test in both types of cancer. Notably, the LMR-MSI assay did not detect any cases of MSI-L in either the CRC or EC tissue samples. The present study demonstrated a possible benefit in the use of LMR-MSI testing compared with SMR-MSI tests for MSI analysis in patients with EC. The method presented a time-saving and non-fluorescent method for detecting MSI in FFPE tissues, as it mitigated the effects of using only SMRs in the detection of MSI status.

However, the present study had several limitations, such as a small sample size. Additionally, the patient cohorts with CRC or EC in the present study were selected based on 100 cases with MMR status, determined by MMR-IHC and SMR-MSI test results. Therefore, the present pilot study required a larger patient cohort size to confirm findings. An advantage in performance of the LMR-MSI assay is that microsatellites with longer homopolymer regions increases the size shift for the detection of dMMR/MSI-H cases in CRC and EC. The larger allele size shifts of the LMR markers compared with SMR markers provide increased confidence in shift interpretation. The discrepancies between the three tests are unclear and further evidence is needed to clarify these discrepancies.

The results of the present study were obtained by comparison of tumor tissues with normal tissues. In the future, the LMR-MSI test may be applied to a greater number of cases and other types of cancer, in particular stomach adenocarcinoma, which has a 22% detection rate of MSI-H according to The Cancer Genome Atlas database (62). The present study demonstrated a potential cost-effective and non-fluorescent method for the detection of MSI using LMR markers, which markedly reduced the number of equivocal MSI-L cases in patients with CRC and EC.

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Availability of data and materials

The data generated in the present study may be found in the National Centre for Biotechnology Information (NCBI) database under at the following URLs: CRC case no. 48, https://trace.ncbi.nlm.nih.gov/Traces/index.html?view=run_browser&acc=SRR23096330&display=metadata; CRC case no. 49, https://trace.ncbi.nlm.nih.gov/Traces/index.html?view=run_browser&acc=SRR23096329&display=metadata; EC case no. 13, https://trace.ncbi.nlm.nih.gov/Traces/index.html?view=run_browser&acc=SRR23096328&display=metadata; and EC case no.14, https://trace.ncbi.nlm.nih.gov/Traces/index.html?view=run_browser&acc=SRR23096327&display=metadata.

Authors' contributions

YiC, CH, WH and TL conceived and designed the experiments. YiC and WC confirm the authenticity of all the raw data. YiC, PC and WC performed the experiments. YiC, PC, TL and YaC analyzed the data. YiC, CH, TL, YaC and PC wrote the manuscript. CL and NC interpreted MMR-IHC data. CL interpreted the PCR-based MSI tests. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the National Cheng Kung University Hospital

(Tainan, Taiwan). The study design involved the retrospective collection of data from patients with either CRC or EC diagnosed at the National Cheng Kung University Hospital (Tainan, Taiwan) between September 2017 and July 2022. The MMR status was analyzed following an approved protocol (approval nos. B-ER-109-152 and A-ER-108-311; Tainan, Taiwan). Written informed consent was obtained from the participating patients with discrepant cases between pMMR-IHC and MSI-PCR assays. Written informed consent was obtained from the relevant patients for use of their data for NGS analysis. Written informed consent was waived for analyses that would not influence treatment decisions. All patient tissues and data were anonymized prior to analysis.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Richard GF, Kerrest A and Dujon B: Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. Microbiol Mol Biol Rev 72: 686-727, 2008.
2. Lynch HT, Shaw MW, Magnuson CW, Larsen AL and Krush AJ: Hereditary factors in cancer. Study of two large midwestern kindreds. Arch Intern Med 117: 206-212, 1966
3. Dean DA, Wadl PA, Hadziabdic D, Wang X and Trigiano RN: Analyzing microsatellites using the QIAxcel system. Methods Mol Biol 1006: 223-243, 2013.
4. Li K, Luo H, Huang L, Luo H and Zhu X: Microsatellite instability: A review of what the oncologist should know. Cancer Cell Int 20: 16, 2020.
5. Fan H and Chu JY: A brief review of short tandem repeat mutation. Genomics Proteomics Bioinformatics 5: 7-14, 2007.
6. Roudko V, Cimen Bozkus C, Greenbaum B, Lucas A, Samstein R and Bhardwaj N: Lynch Syndrome and MSI-H Cancers: From Mechanisms to 'Off-The-Shelf' Cancer Vaccines. Front Immunol 12: 757804, 2021.
7. Reyes GX, Schmidt TT, Kolodner RD and Hombauer H: New insights into the mechanism of DNA mismatch repair. Chromosoma 124: 443-462, 2015.
8. Tamura K, Kaneda M, Futagawa M, Takeshita M, Kim S, Nakama M, Kawashita N and Tatsumi-Miyajima J: Genetic and genomic basis of the mismatch repair system involved in Lynch syndrome. Int J Clin Oncol 24: 999-1011, 2019.
9. Hause RJ, Pritchard CC, Shendure J and Salipante SJ: Classification and characterization of microsatellite instability across 18 cancer types. Nat Med 22: 1342-1350, 2016.
10. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, *et al*: PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 372: 2509-2520, 2015.
11. McMeekin DS, Tritchler DL, Cohn DE, Mutch DG, Lankes HA, Geller MA, Powell MA, Backes FJ, Landrum LM, Zaino R, *et al*: Clinicopathologic significance of mismatch repair defects in endometrial cancer: An NRG Oncology/Gynecologic oncology group study. J Clin Oncol 34: 3062-3068, 2016.
12. Bonneville R, Krook MA, Kautto EA, Miya J, Wing MR, Chen HZ, Reeser JW, Yu L and Roychowdhury S: Landscape of microsatellite instability across 39 cancer types. JCO Precis Oncol 2017: PO.17.00073, 2017.
13. Green AK, Feinberg J and Makker V: A Review of immune checkpoint blockade therapy in endometrial cancer. Am Soc Clin Oncol Educ Book 40: 1-7, 2020.
14. Boland CR and Goel A: Microsatellite instability in colorectal cancer. Gastroenterology 138: 2073-2087.e3, 2010.
15. Petrelli F, Ghidini M, Cabiddu M, Pezzica E, Corti D, Turati L, Costanzo A, Varricchio A, Ghidini A, Barni S, *et al*: Microsatellite instability and survival in stage ii colorectal cancer: A systematic review and meta-analysis. Anticancer Res 39: 6431-6441, 2019.
16. Lin A, Zhang J and Luo P: Crosstalk Between the MSI status and tumor microenvironment in colorectal cancer. Front Immunol 11: 2039, 2020.
17. Makker V, Colombo N, Casado Herraez A, Santin AD, Colomba E, Miller DS, Fujiwara K, Pignata S, Baron-Hay S, Ray-Coquard I, *et al*: Lenvatinib plus Pembrolizumab for advanced endometrial cancer. N Engl J Med 386: 437-448, 2022.
18. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, *et al*: Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357: 409-413, 2017.
19. Lenz HJ, Van Cutsem E, Luisa Limon M, Wong KYM, Hendlisz A, Aglietta M, Garcia-Alfonso P, Neyns B, Luppi G, Cardin DB, *et al*: First-Line nivolumab plus Low-Dose ipilimumab for microsatellite Instability-High/Mismatch repair-deficient metastatic colorectal cancer: The phase II CheckMate 142 study. J Clin Oncol 40: 161-170, 2022.
20. Green AK, Feinberg J and Makker V: A review of immune checkpoint blockade therapy in endometrial cancer. Am Soc Clin Oncol Educ Book 40: 1-7, 2020.
21. Narayanan S, Kawaguchi T, Peng X, Qi Q, Liu S, Yan L and Takabe K: Tumor infiltrating lymphocytes and macrophages improve survival in microsatellite unstable colorectal cancer. Sci Rep 9: 13455, 2019.
22. Diao Z, Han Y, Chen Y, Zhang R and Li J: The clinical utility of microsatellite instability in colorectal cancer. Crit Rev Oncol Hematol 157: 103171, 2021.
23. Pal A, Greenblatt HM and Levy Y: Prerecognition diffusion mechanism of human DNA mismatch repair proteins along DNA: Msh2-Msh3 versus Msh2-Msh6. Biochemistry 59: 4822-4832, 2020.
24. Marsischky GT, Filosi N, Kane MF and Kolodner R: Redundancy of saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair. Genes Dev 10: 407-420, 1996.
25. Hewish M, Lord CJ, Martin SA, Cunningham D and Ashworth A: Mismatch repair deficient colorectal cancer in the era of personalized treatment. Nat Rev Clin Oncol 7: 197-208, 2010.
26. Luchini C, Bibeau F, Ligtenberg MJL, Singh N, Nottegar A, Bosse T, Miller R, Riaz N, Douillard JY, Andre F, *et al*: ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumour mutational burden: A systematic review-based approach. Ann Oncol 30: 1232-1243, 2019.
27. Benson AB, Venook AP, Al-Hawary MM, Arain MA, Chen YJ, Ciombor KK, Cohen S, Cooper HS, Deming D, Farkas L, *et al*: Colon cancer, version 2.2021, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 19: 329-359, 2021.
28. Funkhouser WK Jr, Lubin IM, Monzon FA, Zehnbauer BA, Evans JP, Ogino S and Nowak JA: Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: A report of the association for molecular pathology. J Mol Diagn 14: 91-103, 2012.
29. Cicek MS, Lindor NM, Gallinger S, Bapat B, Hopper JL, Jenkins MA, Young J, Buchanan D, Walsh MD, Le Marchand L, *et al*: Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the colon cancer family registry. J Mol Diagn 13: 271-281, 2011.
30. Hechtman JF, Rana S, Middha S, Stadler ZK, Latham A, Benayed R, Soslow R, Ladanyi M, Yaeger R, Zehir A, *et al*: Retained mismatch repair protein expression occurs in approximately 6% of microsatellite instability-high cancers and is associated with missense mutations in mismatch repair genes. Mod Pathol 33: 871-879, 2020.
31. Cohen R, Hain E, Buhard O, Guilloux A, Bardier A, Kaci R, Bertheau P, Renaud F, Bibeau F, Flejou JF, *et al*: Association of primary resistance to immune checkpoint inhibitors in metastatic colorectal cancer with misdiagnosis of microsatellite instability or mismatch repair deficiency status. JAMA Oncol 5: 551-555, 2019.
32. Huang W, Ho CL, Lee CT, Chen WL, Yang SC, Chow NH and Chen YL: High concordance rate of capillary electrophoresis workflow for microsatellite instability analysis and mismatch repair (MMR) immunostaining in colorectal carcinoma. PLoS One 18: e0284227, 2023

33. Murphy KM, Zhang S, Geiger T, Hafez MJ, Bacher J, Berg KD and Eshleman JR: Comparison of the microsatellite instability analysis system and the Bethesda panel for the determination of microsatellite instability in colorectal cancers. J Mol Diagn 8: 305-311, 2006.
34. Vikas P, Messersmith H, Compton C, Sholl L, Broaddus RR, Davis A, Estevez-Diz M, Garje R, Konstantinopoulos PA, Leiser A, *et al*: Mismatch repair and microsatellite instability testing for immune checkpoint inhibitor therapy: ASCO endorsement of college of american pathologists guideline. J Clin Oncol 41: 1943-1948, 2023.
35. Taieb J, Svrcek M, Cohen R, Basile D, Tougeron D and Phelip JM: Deficient mismatch repair/microsatellite unstable colorectal cancer: Diagnosis, prognosis and treatment. Eur J Cancer 175: 136-157, 2022.
36. Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, *et al*: A National cancer institute workshop on hereditary nonpolyposis colorectal cancer syndrome: Meeting highlights and Bethesda guidelines. J Natl Cancer Inst 89: 1758-1762, 1997.
37. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, *et al*: Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 96: 261-268, 2004.
38. Bacher JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, Megid WM and Thibodeau SN: Development of a fluorescent multiplex assay for detection of MSI-High tumors. Dis Markers 20: 237-250, 2004.
39. Goel A, Nagasaka T, Hamelin R and Boland CR: An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. PLoS One 5: e9393, 2010.
40. Wang Y, Shi C, Eisenberg R and Vnencak-Jones CL: Differences in microsatellite instability profiles between endometrioid and colorectal cancers: A potential cause for False-Negative results? J Mol Diagn 19: 57-64, 2017.
41. Do H and Dobrovic A: Sequence artifacts in DNA from formalin-fixed tissues: Causes and strategies for minimization. Clin Chem 61: 64-71, 2015.
42. McDonough SJ, Bhagwate A, Sun Z, Wang C, Zschunke M, Gorman JA, Kopp KJ and Cunningham JM: Use of FFPE-derived DNA in next generation sequencing: DNA extraction methods. PLoS One 14: e0211400, 2019.
43. Wu S, Liu X, Wang J, Zhou W, Guan M, Liu Y, Pang J, Lu T, Zhou L, Shi X, *et al*: DNA Mismatch repair deficiency detection in colorectal cancer by a new microsatellite instability analysis system. Interdiscip Sci 12: 145-154, 2020.
44. Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR and Winchester DP: The eighth edition AJCC cancer staging manual: Continuing to build a bridge from a population-based to a more 'personalized' approach to cancer staging. CA Cancer J Clin 67: 93-99, 2017
45. Lee CT, Chow NH, Chen YL, Ho CL, Yeh YM, Lin SC, Lin PC, Lin BW, Chu CA, Tsai HW, *et al*: Clinicopathological features of mismatch repair protein expression patterns in colorectal cancer. Pathol Res Pract 217: 153288, 2021.
46. O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D, *et al*: Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 44: D733-D745, 2016.
47. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL: Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13: 134, 2012.
48. Akoglu H: User's guide to sample size estimation in diagnostic accuracy studies. Turk J Emerg Med 22: 177-185, 2022.
49. Yu F, Makrigiorgos A, Leong KW and Makrigiorgos GM: Sensitive detection of microsatellite instability in tissues and liquid biopsies: Recent developments and updates. Comput Struct Biotechnol J 19: 4931-4940, 2021.
50. Liu HX, Zhou XL, Liu T, Werelius B, Lindmark G, Dahl N and Lindblom A: The role of hMLH3 in familial colorectal cancer. Cancer Res 63: 1894-1899, 2003.
51. Kawakami H, Zaanan A and Sinicrope FA: Microsatellite instability testing and its role in the management of colorectal cancer. Curr Treat Options Oncol 16: 30, 2015.
52. Jaffrelot M, Farés N, Brunac AC, Laurenty AP, Danjoux M, Grand D, Icher S, Meilleroux J, Mery E, Buscail E, *et al*: An unusual phenotype occurs in 15% of mismatch repair-deficient tumors and is associated with non-colorectal cancers and genetic syndromes. Mod Pathol 35: 427-437, 2022.
53. Wang C, Zhang L, Vakiani E and Shia J: Detecting mismatch repair deficiency in solid neoplasms: Immunohistochemistry, microsatellite instability, or both? Mod Pathol 35: 1515-1528, 2022.
54. Bao F, Panarelli NC, Rennert H, Sherr DL and Yantiss RK: Neoadjuvant therapy induces loss of MSH6 expression in colorectal carcinoma. Am J Surg Pathol 34: 1798-1804, 2010.
55. Kuan SF, Ren B, Brand R, Dudley B and Pai RK: Neoadjuvant therapy in microsatellite-stable colorectal carcinoma induces concomitant loss of MSH6 and Ki-67 expression. Hum Pathol 63: 33-39, 2017.
56. Guyot D'Asnières De Salins A, Tachon G, Cohen R, Karayan-Tapon L, Junca A, Frouin E, Godet J, Evrard C, Randrian V, Duval A, *et al*: Discordance between immunochemistry of mismatch repair proteins and molecular testing of microsatellite instability in colorectal cancer. ESMO Open 6: 100120, 2021
57. Bacher JW, Sievers CK, Albrecht DM, Grimes IC, Weiss JM, Matkowskyj KA, Agni RM, Vyazunova I, Clipson L, Storts DR, *et al*: Improved detection of microsatellite instability in early colorectal lesions. PLoS One 10: e0132727, 2015.
58. Lin JH, Chen S, Pallavajjala A, Guedes LB, Lotan TL, Bacher JW and Eshleman JR: Validation of long mononucleotide repeat markers for detection of microsatellite instability. J Mol Diagn 24: 144-157, 2022.
59. Turner EH, Dickerson JA, Ramsay LM, Swearingen KE, Wojcik R and Dovichi NJ: Reaction of fluorogenic reagents with proteins III. Spectroscopic and electrophoretic behavior of proteins labeled with Chromeo P503. J Chromatogr A 1194: 253-256, 2008.
60. Zwaenepoel K, Holmgaard Duelund J, De Winne K, Maes V, Weyn C, Lambin S, Dendooven R, Broeckx G, Steiniche T and Pauwels P: Clinical performance of the idylla MSI test for a rapid assessment of the DNA microsatellite status in human colorectal cancer. J Mol Diagn 22: 386-395, 2020.
61. Siemanowski J, Schomig-Markiefka B, Buhl T, Haak A, Siebolts U, Dietmaier W, Arens N, Pauly N, Ataseven B, Buttner R, *et al*: Managing difficulties of microsatellite instability testing in endometrial Cancer-Limitations and advantages of four different PCR-Based approaches. Cancers (Basel) 13: 1268, 2021.
62. Cancer Genome Atlas Research Network: Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513: 202-209, 2014.