Histomorphology and Molecular Genetics in Different Intra-Tumoral Zones and Matched Metastatic Lymph Nodes of Colorectal Cancer with Heterogenous Mismatch Repair Status

Jing Zhang (iawguu@126.com)
Fudan University Shanghai Cancer Center
Xin Zhang
Zhongshan Hospital Fudan University
Qian Wang
Fudan University Shanghai Cancer Center
Yuyin Xu
Fudan University Shanghai Cancer Center
Qianlan Yao
Fudan University Shanghai Cancer Center
Dan Huang
Fudan University Shanghai Cancer Center
Weiqi Sheng
Fudan University Shanghai Cancer Center
Xiaoli Zhu
Fudan University Shanghai Cancer Center
Xiaoyan Zhou
Fudan University Shanghai Cancer Center
Qianming Bai
Fudan University Shanghai Cancer Center

Research

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Abstract

Objective: To better understand the clinicopathological characteristics and molecular alterations in different intra-tumoral components of colorectal cancer (CRC) with heterogeneity of mismatch repair (MMR) protein expression and microsatellite instability (MSI) status.

Methods: We identified 4 cases of CRC with heterogenous MMR protein expression and analyzed the histopathological features, MSI status and other molecular alterations in separately microdissected intra-tumoral zones and lymph node metastases by polymerase chain reaction (PCR) -based MSI testing, *MLH1* promoter methylation and targeted next-generation sequencing (NGS).

Results: Microsatellite instability-high (MSI-H**)** was identified in the MLH1/PMS2 deficient zones in Case 1-3, and in the MSH2/MSH6 deficient zone in Case 4, while MSS was in all the intra-tumoral zones and metastatic lymph nodes with proficient MMR (pMMR). Furthermore, heterogeneity of *MLH1* promoter methylation and/or other common driving gene mutations of CRC, such as KRAS, PIK3CA and so on, was identified in all the 4 CRCs. In addition, 75% (3/4) of cases showed heterogeneity of histomorphology in intra-tumoral components and metastatic lymph nodes (Case 1, 2, 4), and all the corresponding metastatic lymph nodes were moderate differentiation with MSS/pMMR (Case 2, 3).

Conclusions: The heterogeneous MSI status is highly correlated with histomorphological heterogeneity, which is also an important clue for the heterogeneity of drive gene mutations in CRC. These results suggest that it is essential to detect MMR protein expression and other gene mutations in metastases before treatment, especially for the CRCs with heterogenous MMR protein expression or histomorphology.

Background

Microsatellite instability (MSI), either due to inherited germline mutations of mismatch repair (MMR) genes or epigenetic inactivation of these genes, is found in approximately 15% of stage II to III colorectal cancers (CRCs)[1, 2]. The prognostic significance of MSI in CRC has been demonstrated in numerous studies. Decreased recurrence and metastasis and increased survival occur in CRCs with deficient MMR (dMMR) and/or high MSI (MSI-H), in comparison with those with proficient MMR (pMMR) and/or microsatellite stable (MSS)[3, 4]. Furthermore, MSI has been associated with resistance to 5-fluorouracil (5-FU)-based chemotherapy and sensitivity to immune checkpoint inhibitors (ICIs)[3, 5–7]. In addition, MSI testing is helpful for Lynch syndrome screening[8]. National Comprehensive Cancer Network (NCCN) guidelines recommend routine MMR testing for all patients with CRC[9]. Thus, it is crucial to give a precise evaluation of MMR status.

Although MSI is considered as an early event in CRC carcinogenesis, an analysis of paired primary tumors and metastases shows a high degree of concordance of genomic alterations including MSI[10, 11], intra- and inter-tumoral heterogeneity of MMR protein expression, and MSI status in sporadic CRCs have been reported in several recent studies[12–15]. Chapusot et al. demonstrated the presence of intra-tumoral heterogeneity of MSI in 8/100 right-sided sporadic CRCs[12]. Joost et al. collected 14 CRCs with heterogeneous immunochemical staining patterns that affected at least one of the MMR proteins (*MLH1, MSH2, MSH6, PMS2*), further analysis of MSI status and *MLH1* promoter methylation identified intra-tumoral heterogeneity of MSI in 3/14, *MLH1* methylation in 7/14 tumors[13]. Tachon et al. reported a case of CRC with intra-tumoral heterogeneous MLH1/PMS2 staining pattern confirmed by MSI testing, MSS in pMMR areas, and MSI in dMMR areas[14]. He et al. evaluated MSI

status in paired primary and metastatic CRC from 369 patients and found that 19.6% (9/46) had an MSS metastasis in patients with an MSI-H primary tumor (inter-tumoral heterogeneity), and the discrepancy was more likely to be limited to peritoneal or ovarian metastasis[15]. Therefore, heterogeneity of MMR protein expression and MSI status in CRC is rare but exists indeed. However, clinicopathology and molecular genetics in different intra-tumoral zones and matched metastatic lymph nodes of colorectal cancer with heterogenous MSI status are still not clear.

In this study, we collected 4 CRCs with heterogenous MMR protein expression and analyzed the clinicopathological and immunohistochemical characteristics. More importantly, we determined the MSI status, *MLH1* promoter methylation, and other molecular alterations in separately microdissected intratumoral components and matched metastatic lymph nodes with heterogenous MMR protein expression using PCR-based MSI testing and targeted NGS.

Materials And Methods

Patients

Four cases with heterogenous MMR protein staining were retrieved from 500 cases of CRC resections in the pathology files in the Department of Pathology, Fudan University Shanghai Cancer Center during 2018 - 2020. All the cases were reviewed by two senior pathologists. Clinicopathologic information of these 4 cases was obtained from the medical records and/or discharge summary (Table1). Formalin-fixed paraffin- embedded sections, including the primary tumor tissues, metastatic lymph nodes, and matching normal tissue sections (3-4 μm thick) were collected. The present study was approved by our institutional ethics committee. All tests of samples in this study were carried out in ISO15189 certified laboratories.

Table 1
Clinicopathological characteristics of 4 CRCs with heterogenous MMR protein expression

Case No.	Sex /Age	Location	Size (cm)	Histologic Type	Differentiation	LN status	pTNM	Stage
1	F/53	Right colon	1.8	Adca	Moderate-Poor	0/31	T1N0M0	Ⅰ
2	F/67	Right colon	2.0	Adca	Moderate-Poor	2/29	T3N1bM0	ⅢB
3	M/50	Sigmoid colon	3.0	Adca	Moderate	3/22	T3N1bM0	ⅢB
4	M/68	Right colon	7.5	Adca, partly MA	Moderate-Poor	0/34	T3N0M0	ⅢA

CRC: colorectal cancer; MMR: mismatch repair; Adca: adenocarcinoma; MA: mucinous adenocarcinoma; LN: lymph node.

Hematoxylin and eosin (H&E)-stained sections of the resection specimens were reviewed. Several parameters, including tumor size, lesion location, the depth of tumor invasion, histological morphology, and grading were

recorded. CRC staging was performed according to the 8th American Joint Committee on Cancer (AJCC) cancer staging manual based on Tumor-Node-Metastasis (TNM) classification scheme.

Immunohistochemistry

Immunohistochemistry(IHC) for MMR proteins (*MLH1, PMS2, MSH2, and MSH6*), and other biomarkers including chromogranin A (*CgA*), synaptophysin (*Syn*), Caudal related homeodomain transcription 2 (*CDX2*), and Special AT-rich sequence-binding protein 2 (*SATB2*) was performed on 4 μm thick paraffin tissue sections using the following monoclonal antibodies: *MLH1* (Roche, USA), *PMS2* (Roche Diagnostics, USA), *MSH-2* (Roche, USA), *MSH6* (Roche, USA), *CgA* (MXB, China), *Syn*(DAKO, Denmark), *CDX2*(Roche, USA), and *SATB2*(ZSGB, China). Staining was performed on autostainer Benchmark XT/Ultra (Ventana Medical Systems, Tucson, AZ, USA) using OptiView universal DAB IHC detection and Amplification kit (Ventana), according to the manufacturer's instructions. The nuclear expression of all the 4 MMR markers (*MLH1, PMS2, MSH2*, and *MSH6*) was considered to be pMMR. The complete loss of nuclear expression of any of the 4 MMR markers in the neoplastic cells was considered as dMMR. The heterogeneity of MMR protein staining was defined as zonal or focal loss of MMR expression coexisting with other areas with diffuse expression[13, 16–18]. Appropriate positive and negative controls were included.

Microdissection And Dna Extraction

Intra-tumoral components with heterogenous MMR protein expression were separately microdissected by laser capture microdissection (LCM, Arcturus XT; Life Technologies, Mountain View, CA, USA) as previously described[19, 20]. Briefly, ten 6-μm-thick histologic sections were prepared from each selected block and adhered to a 1.4-μm membrane with metal frame slides (Applied Biosystems, Foster City, Germany). After drying and dewaxing routinely, these sections were fixed in 100% ice-cold ethanol for 10min, stained with hematoxylin for 1 min, and dehydrated in 100% ethanol for 30s and xylene for 5min. Target components with heterogenous MMR expression were isolated separately using an LCM system. Genomic DNA from different intra-tumoral components and corresponding metastatic lymph nodes with heterogenous MMR protein expression, as well as matching normal mucosa samples were extracted with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Pcr-based Msi Testing

Microsatellite instability status was evaluated by a fluorescent PCR-based assay with Promega MSI Analysis System (Promega Corporating, Madison, US), which included five mononucleotide markers (*BAT-25, BAT-26, NR-21, NR-24, and MONO-27*) and two pentanucleotide markers (Penta C, Penta D). The testing was conducted as previously described[21]. Briefly, Promega MSI testing was performed with a 2 ng DNA input for separately microdissected intra-tumoral components and metastatic lymph nodes with heterogenous MMR protein expression. PCR amplification was carried out in a GeneAmp PCR 9700 thermocycler (Applied Biosystems) and capillary electrophoresis was carried out in an ABI 3500xL automated DNA sequencer (Applied Biosystems). Data were analyzed with the GeneMapper Software Analyzer (Thermo Fisher Scientific, Waltham, MA). Compared with the normal mucosa control tissues, tumors were defined as MSI-high (MSI-H) when ≥ 2/5 of the mononucleotide

markers were altered, MSI-low (MSI-L) with only 1 marker altered, and microsatellite stable (MSS) with stability for all the five markers.

Methylation-specific Pcr Analysis

Extracted DNA from different intra-tumoral components and corresponding metastatic lymph nodes with heterogenous MMR protein expression was treated with bisulfite to convert unmethylated cytosines to uracil while leaving methylated cytosines unaltered. *MLH1* promoter methylation status was analyzed by means of a fluorescence-based, real-time methylation-specific PCR assay using the Human *MLH1* Gene Methylation Detection Kit (Gene Tech (Shanghai) Company Limited, China) according to the manufacturer's instructions. This kit analyzed the methylation of 5 CpG sites (AGAGCGGACAGCGATCTCTAACGCGCAAGCGCAT, chr3: 37, 034, 766-37,034,799, GRCh37/hg19). The PCR reactions (95℃ for 3 min, followed by 45 cycles of 95℃ for 15 sec and 60℃ for 60 sec) were performed on an ABI 7500 analyzer (Applied Biosystems, Foster City, CA). The Ct value of the sample and the external control must be less than or equal to 32. Positive results were defined as Ct (sample) - Ct(control) ≤7. Samples were run in duplicate, including positive and negative controls.

Dna-based Next Generation Sequencing

The ColonCore panel (Burning Rock Biotech, Guangzhou, China) is designed for simultaneous detection of MSI status and mutations in 41 CRC-related genes, including *KRAS, NRAS, BRAF, PIK3CA*, hereditary CRC genes, MMR genes, and other genes related to carcinogenesis and tumor development. The genes included in this panel are listed in Supplementary Table 1. All the detection was carried out according to the manufacturer's instructions as previously described using a NextSeq platform (Illumina Inc., San Diego, CA)[22, 23]. Briefly, DNA shearing was performed using Covaris M220 (Covaris, Inc., MA, US), followed by end repair, phosphorylation, and adaptor ligation. Fragment sizes ranging from 200 to 400 bp were selected using Agencourt AMPure beads (Beckman Coulter, CA, US) followed by hybridization with capture probes baits, hybrid selection with magnetic beads, and PCR amplification. Subsequently, Qubit® 3.0 and Agilent 2100 bioanalyzer (Agilent Technologies Inc., CA, US) was performed to assess the quality and size of the fragments. Indexed samples were sequenced on the Nextseq500 sequencer (Illumina, Inc., CA, US) with pair-end reads. The sequencing data were mapped to the human genome (hg19) using Burrows-Wheeler Aligner version 0.7.10. Local alignment optimization, variant calling, and annotation were performed using the Genome Analysis Toolkit version 3.2 and VarScan version 2.4.3.

The method of MSI phenotype detection in this ColonCore panel was a read-count-distribution-based approach. It took the coverage rate of a set of specific repeat lengths as the main feature of each microsatellite locus. The determination of MSI status was not required for the matched normal tissues. If the coverage rate was less than a given threshold, the locus was classified as unstable. The MSI status of a sample was determined by the percentage of unstable loci in the given sample. A tumor sample was considered MSI-H if more than 40% of the marker loci were length-instable, MSS if the percentage of length-instable loci was less than 15%, or MSI-L if the percentage was between 15–40%[22].

Results

MMR protein expression and microsatellite instability status

Four cases of CRC with heterogenous MMR protein expression were identified for in-depth analysis. Immunohistochemical staining was repeated to confirm the heterogenous MMR protein expression in all the 4 tumors (Table 2). Briefly, intra- tumoral heterogenous expression affected MLH1/PMS2 in Case 1-3, MSH2/MSH6 in Case 4. Interestingly, pMMR was demonstrated in all the metastatic lymph nodes in Case 2 and Case 3.

Table 2
MMR protein expression and MS status in separately microdissected intra-tumoral zones and matched MLN

Case No.	Tumor Zones	Histologic Type	Differentiation	MLH1	MSH2	MSH6	PMS2	MS status	MLH1 methylation
1	A	Adca	Moderate	+	+	+	+	MSS	-
	B	Adca	Poor	(-)	+	+	(-)	MSI-H	-
2	A	Adca	Moderate	+	+	+	+	MSS	-
	B	Adca	Poor	(-)	+	+	(-)	MSI-H	(+)
	MLN	Adca	Moderate	+	+	+	+	MSS	-
3	A	Adca	Moderate	+	+	+	+	MSS	-
	B	Adca	Moderate	(-)	+	+	(-)	MSI-H	-
	MLN	Adca	Moderate	+	+	+	+	MSS	-
4	A	Adca	Poor	+	+	+	+	MSS	-
	B	Adca, partly MA	Moderate	+	(-)	(-)	+	MSI-H	-

A: intra-tumoral Zone A; B: intra-tumoral Zone B; MLN: metastatic lymph nodes; MMR: mismatch repair; MS: microsatellite; MSS: microsatellite stability; MSI-H: microsatellite instability-high; Adca: adenocarcinoma; MA: mucinous adenocarcinoma.

In order to determine the authenticity of the heterogeneity of MMR protein expression in 4 cases of CRC, microsatellite testing was performed in separately microdissected intra-tumoral components and metastatic lymph nodes using Promega MSI Analysis System. As a result, all the 4 cases showed heterogeneity of MSI status, which was exactly matched with the heterogeneous zones of MMR protein expression. Briefly, MSI-H was identified in the MLH1/PMS2 deficient zones in Case 1-3, and in the *MSH2/MSH6* deficient zone in Case 4, while MSS was in all the intra-tumoral zones and metastatic lymph nodes with pMMR. Immunohistochemical staining of MMR proteins and MSI status in different intra-tumoral zones and metastatic lymph nodes of Case 2, 3, 4 were shown in Fig. 1, 2, 3, respectively.

Clinical And Histopathological Features

The clinical and histopathological features of the 4 CRCs with a heterogeneity of MMR protein expression and MSI status were summarized in Table 1. There were 2 female and 2 male patients, with ages at presentation ranging from 50 to 68 years (mean, 59.5 years). The tumor size was ranged from 1.8 to 7.5 cm. Most (3/4, 75%) of them were located in the right colon.

Histopathologically, intra-tumoral morphological heterogeneity was determined in 3 of the 4 cases, was exactly matched with the heterogeneous zones of MMR protein expression. Briefly, intra-tumoral Zone B with MSI-H/dMMR (*MLH1/PMS2* loss) in Case 1 and 2 were poorly differentiated, showing a solid or dense sieve growth pattern, while intra-tumoral Zone A and matched metastatic lymph nodes with MSS/pMMR were moderate differentiation with a glandular or cribriform-like pattern (Fig. 1, C-N). On the other hand, the matching was just opposite in Case 4, intra-tumoral Zone B with MSI-H/dMMR (MSH2/MSH6 loss) was moderate differentiation and partly showed mucinous adenocarcinoma (Adca), but poor differentiation in the Zone A with MSS/pMMR (Fig. 3, B-I). No morphological heterogeneity in primary tumor and metastatic lymph nodes was found in Case 3 (Fig. 2, C, D and E). It's worth noting that all the corresponding metastatic lymph nodes were moderate differentiation and determined to be MSS/pMMR (Fig. 1, E, H, K and N, Fig. 2, E, H, K and N). In addition, heterogeneous histomorphological components with moderate or poor differentiation in Case 1, 2, 4 were all positive for *CDX2* and *SATB2*, and all negative for *CgA* and *Syn* (data not shown).

Mlh1 Promoter Methylation And Other Molecular Features

To further explore the possible causes of heterogenous MMR expression, *MLH1* promoter methylation and targeted NGS were performed in separately microdissected intra-tumoral components and matched metastatic lymph nodes, respectively. As a result, NGS confirmed the heterogeneity of MSI status again, consistent with the above MSI-PCR results. Furthermore, significant differences in genetic mutations were identified intra-tumoral zones and matched metastatic lymph nodes with heterogenous MMR expression and MSI status, including the most common driving gene mutations related to CRCs, such as *KRAS* and *PIK3CA*, and so on. The gene mutations with pathogenic and likely pathogenic significance in different zones of all the 4 cases were listed in Table 3, and the gene mutations with uncertain significance were list in Supplementary Table 2.

Table 3
The gene mutations with pathogenic and likely pathogenic significance in different zones and matched MLN of CRC with heterogenous MMR expression by tageted NGS

Case No.	Tumor Zones	MS status	Gene	hgvsC	hgvsP	AF
1	A	MSS	MLH1	c.2041G>A	p.A681T	48.98%
	B	MSI-H	MLH1	c.2041G>A	p.A681T	80.25%
			KRAS	c.38G>A	p.G13D	12.25%
			PIK3CA	c.3140A>G	p.H1047R	12.27%
			PIK3CA	c.1258T>C	p.C420R	32.61%
			APC	c.4906dup	p.D1636fs	14.71%
			APC	c.4393_4394del	p.S1465fs	13.49%
			PTCH1	c.3606del	p.S1203fs	28.69%
2	A	MSS	APC	c.5799dup	p.P1934fs	47.32%
			TP53	c.994-1G>A	p.?	57.86%
	B	MSI-H	APC	c.5799dup	p.P1934fs	24.09%
			TP53	c.994-1G>A	p.?	23.29%
	MLN	MSS	APC	c.5799dup	p.P1934fs	18.06%
			TP53	c.994-1G>A	p.?	22.39%
3	A	MSS	MLH1	c.2035G>T	p.E679*	50.00%
			APC	c.4216C>T	p.Q1406*	46.30%
			PIK3CA	c.1633G>A	p.E545K	47.02%
			TP53	c.706T>G	p.Y236D	69.43%
	B	MSI-H	MLH1	c.2035G>T	p.E679*	42.36%
			PMS1	c.2480C>A	p.S827*	15.23%
			APC	c.4216C>T	p.Q1406*	34.58%
			PIK3CA	c.1633G>A	p.E545K	39.14%
			TP53	c.706T>G	p.Y236D	58.09%
	MLN	MSS	MLH1	c.2035G>T	p.E679*	10.95%
			APC	c.4216C>T	p.Q1406*	6.57%
			PIK3CA	c.1633G>A	p.E545K	14.25%
			TP53	c.706T>G	p.Y236D	22.47%
4	A	MSS	POLE	c.1307C>G	p.P436R	17.71%

Case No.	Tumor Zones	MS status	Gene	hgvsC	hgvsP	AF
			MSH2	c.256G>T	p.E86*	11.39%
			BRCA2	c.6155C>A	p.S2052*	12.61%
			ATM	c.8065G>T	p.E2689*	14.35%
			PIK3CA	c.333G>T	p.K111N	11.88%
			PIK3CA	c.112C>T	p.R38C	18.18%
			SMAD4	missense_variant	p.D355G	16.49%
			SMAD4	c.1082G>A	p.R361H	16.19%
	B	MSI-H	MSH2	c.2038C>T	p.R680*	31.80%
			MSH6	c.3261del	p.F1088fs	40.19%
			KRAS	c.34G>A	p.G12S	26.63%
			PIK3CA	c.3140A>G	p.H1047R	23.91%
			PTEN	c.800del	p.K267fs	46.79%
			PTEN	missense_variant	p.P246L	22.90%
			APC	c.4666dup	p.T1556fs	26.45%
			APC	c.1269G>A	p.W423*	23.00%

CRC: colorectal cancer; MMR: mismatch repair; NGS: next generation sequencing; A: intra-tumoral Zone A; B: intra-tumoral Zone B; MLN: metastatic lymph nodes; MS: microsatellite; MSS: microsatellite stability; MSI-H: microsatellite instability-high; AF: allele frequency.

In Case 1, germline likely pathogenic *MLH1* gene mutation (c.2041G>A; p.Ala681Thr) was identified in both intra-tumoral Zone A with MSS/pMMR and Zone B with MSI-H/dMMR. Another six somatic mutations were only identified in Zone B with MSI-H/dMMR, including *KRAS* (p.G13D), *PIK3CA* (p.H1047R and p.C420R), *APC* (p.D1636fs and p.S1465fs), and *PTCH1* (p.S1203fs). In Case 2, the same somatic gene mutations (*APC*, p.P1934fs and *TP53*, c.994-1G>A) were determined in different intra-tumoral zones and matched metastatic lymph nodes, however, *PMS2* (p.K651R) gene mutation with uncertain significance was only identified in the Zone B with MSI-H/dMMR. In Case 3, the same somatic gene mutations (*MLH1*, p.E679*, *APC*, p.Q1406*, *PIK3CA*, p.E545K, and *TP53*, c.994-1G>A) were determined in different intra-tumoral zones and matched metastatic lymph nodes, however, somatic *PMS1* (p.S827*) gene mutation was only identified in the Zone B with MSI-H/dMMR. In Case 4, there were significant differences in gene mutations between the zones with heterogenous MMR expression. *POLE* (p.P436R), *MSH2* (p.E86*), *BRCA2* (p.S2052*), *ATM* (p.E2689*), *PIK3CA* (p.R38C and p.K111N), and *SMAD4* (p.D355G and p.R361H) were identified in the Zone A with MSS/pMRR, while *MSH2* (p.R680*), *MSH6* (p.F1088fs), *KRAS* (p.G12S), *PIK3CA* (p.H1047R), *PTEN* (p.K267fs) and *APC* (p.T1556fs and p.W423*) were identified in the Zone B with MSI-H/dMMR.

MLH1 promoter methylation was just determined in Case 2 without MMR gene mutations, but not in Case 1, 3, and 4. Interestingly, heterogenous MMR protein staining for MLH1/PMS2 correlated with heterogenous *MLH1*

promoter methylation, i.e. intra-tumoral Zone B with loss of MLH1/PMS2 expression showed *MLH1* methylation, whereas intra-tumoral Zone A and matched metastatic lymph nodes with pMMR did not show *MLH1* methylation.

Discussion

Heterogenous MMR protein expression was a rare phenomenon in CRC. In this study, we identified 4 cases of CRC with heterogeneous MMR protein expression, and then analyzed the clinical and histopathological features of these cases. Furthermore, we determined the status of *MSI, MLH1* promoter methylation, and other molecular alterations in separately microdissected intra-tumoral components and matched metastatic lymph nodes with heterogenous MMR expression by PCR and targeted NGS. As a result, heterogeneity of histomorphology in intra-tumoral components and metastatic lymph nodes was determined in 75% (3/4) cases (Case 1, 2, 4). In addition, heterogeneity of *MLH1* promoter methylation and/or other common driving gene mutations of CRC, such as *KRAS, PIK3CA* and so on, was identified in all the 4 CRCs. These results indicated that heterogeneity of MMR protein expression and MSI status in CRC is rare, but exists indeed. These results also suggested that it might be necessary to detect biomarkers in different intra-tumoral components and corresponding metastases with heterogenous MMR expression for subsequent treatment.

Histopathologically, heterogeneity of morphology in intra-tumoral components and metastatic lymph nodes was determined in 75% (3/4) of cases of CRC, which was exactly matched with the heterogeneous zones of MMR protein expression. Although the association between the heterogeneity of MMR protein expression and heterogenous histomorphology was formally proposed in our study, it was also found in previous publications[13, 16]. Joost et al. reported a case of CRC with retained expression for *PMS2* in a mucinous adenocarcinoma component and loss of *PMS2* expression in a non-mucinous component, and another case with clonal heterogeneity for *MSH6* in a poorly differentiated tumor component and homogenous expression in a well-differentiated tumor component[13]. Graham et al. reported several cases of CRC with retained expression for *MSH6* in the gland-forming areas of the adenocarcinoma and completely lost in areas of dedifferentiation[16]. Fadhil et al. reported an unusual tumor of mixed adeno-neuroendocrine carcinoma, in which the neuroendocrine component showed strong expression of all four MMR proteins, whereas the epithelial component was deficient in *MSH2* and *MSH6* expression[24]. These results indicated that histomorphological heterogeneity might be an important clue for the heterogeneity of MMR protein expression and MSI status, and detection of MSI status only in one histomorphological component for these tumors might lead to misdiagnosis[25].

It was worth noting that the consistency of MSI status between primary and metastatic lesions was still controversial[15, 26–28]. Some scholars believed that metastatic tissue can be used to screen for Lynch syndrome and dMMR, because all cases examined showed consistent IHC staining between the primary tumors and metastases[27]. However, other scholars hold that the MMR protein expression in the metastatic tissue is not entirely consistent with that in the primary tumor, and the incidence of MSI in metastatic tissue is low[15, 29, 30]. In our study, the metastatic lymph nodes in Case 2 and 3 all showed glandular and cribriform structures with pMMR. These findings suggested that the MSI status of the primary tumor could not be completely represented by the metastatic tissue, and provided strong evidence that the subclones with pMMR/MSS might be more prone to develop metastasis, consistent with previous publications[15, 31]. Therefore, it is essential to detect the MMR protein expression and MSI status of metastases before treatment with chemotherapy and/or immune checkpoint inhibitors[6, 7], especially for the CRCs with intra-tumoral heterogenous MSI status.

The NGS analysis not only offers further evidence of the true MSI heterogeneity in the 4 cases, but also provides insights into the tumor biology. Loss of MMR protein expression is mainly caused by several reasons: (1) germline mutations of MMR genes; (2) methylation of the *MLH1* gene promoter; (3) double somatic mutations of MMR genes[32, 33]. In Case 1, germline pathogenic missense mutation of *MLH1* (p.A681T)[34] was determined in both tumor zones with dMMR or pMMR. The heterogenous MMR protein expression, in this case, might be explained through allele frequency (AF) of MLH1 germline mutation, which suggests homozygous in Zone B with dMMR (AF=80.25%) and heterozygous in Zone A with pMMR (AF=48.98) (Table 3). Studies in vitro indicate that heterozygosity for *MLH1* mutations, unlike homozygosity, does not affect MMR protein expression[35]. In Case 2, there were no pathogenic mutations of MMR genes in different tumor zones and lymph node metastasis, whereas only one somatic missense mutation of *PMS2* (p.K651R) with uncertain significance was found in Zone B with MSI-H/dMMR. Thus, MLH1 promoter methylation was analyzed, as a result, intra-tumoral Zone B with loss of MLH1/PMS2 expression showed *MLH1* methylation, whereas intra-tumoral Zone A and matched metastatic lymph nodes with pMMR did not show *MLH1* methylation. Double somatic mutations of MMR genes, *MLH1* (p.E679*) and *PMS1* (p.S827*), which forms a heterodimer with MLH1, in Case 3, *MSH2* (p.R680*) and *MSH6* (p.F1088fs) in Case 4, were identified in the moderately differentiated components with dMMR, however, only one somatic mutation of MMR genes, *MLH1* (p.E679*) in Case 3 and *MSH2* (p.E86*) in Case 4, was identified in the components with pMMR. Unexpectedly, three different somatic mutations in *MSH2* and *MSH6* were identified in different intra-tumoral zones of Case 4. The most likely explanation for this rare event was that *MSH2* (p.E86*) mutation in the zone with pMMR might be caused by somatic *POLE* (p.P436R) mutation. Recently, germline and somatic mutations in the exonuclease domain of polymerase epsilon, catalytic subunit (*POLE*) gene have been reported in a small subset of microsatellite-stable and hypermutated colorectal carcinomas (CRCs), affecting the proofreading activity of the enzyme and leading to misincorporation of bases during DNA replication[36–38].

In addition, heterogeneity of mutations in oncogenes (*KRAS* and *PIK3CA*), tumor suppressor genes (*APC*, *PTEN*, and *SMAD4*), and homologous recombination repair genes (*BRCA2* and *ATM*) was determined in Case 1 and Case 4 with heterogeneous MMR protein expression and heterogeneous histomorphology, but not in Case 3 with homogeneous histomorphology, which provided strong evidence that these gene mutations also exist heterogeneity in CRC, as previous publication[39–42]. These results also indicated that histomorphological heterogeneity provided an effective clue for distinguishing the CRCs with heterogenous MSI status and other driver gene mutations.

In conclusion, heterogeneity of MMR protein expression and MSI status in CRC is rare but exists indeed. Our results provide strong evidence that the true heterogeneous MSI status is highly correlated with histomorphological heterogeneity, which is also an important clue for the heterogeneity of drive gene mutations in CRC, including *KRAS*, *APC*, *PTEN*, and *PIK3CA*. These results indicate that it is essential to detect the MMR protein expression and other gene mutations in metastases before the treatment with immune checkpoint inhibitors and/or other target drugs, especially for the CRC with heterogenous MMR protein expression. These results also emphasize the importance of histopathological morphology in the age of molecular pathology. However, because of limited cases in this study, heterogeneity of MMR protein expression and MSI status in CRC remains to be further research.

Abbreviations

CRC

colorectal cancer
MMR
mismatch repair
MSI
microsatellite instability

Declarations

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Authors' contributions

JZ and XZ: writing and data analysis. QW and YX: sample collection, detection and quality control. QY and DH: sample bioinformatics analysis and clinical annotation. XZ and WS: pathology assessment and article revision. XZ and QB: concept and design. All authors read and approved the final paper.

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Availability of data and materials

The analyzed data during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by Fudan University Shanghai Cancer Center institutional ethics committee (registration number 1812195-3).

Consent for publication

´Not applicable´.

Competing interests

The authors declare that they have no conflicts of interest.

Author details

[1]Department of Pathology, Fudan University Shanghai Cancer Center;

[2]Department of Oncology, Shanghai Medical College, Fudan University;

[3]Institute of Pathology, Fudan University, Shanghai, China, 200032;

[4]Department of Pathology, Fudan University Zhongshan Hospital, Shanghai, China, 200032

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Figures

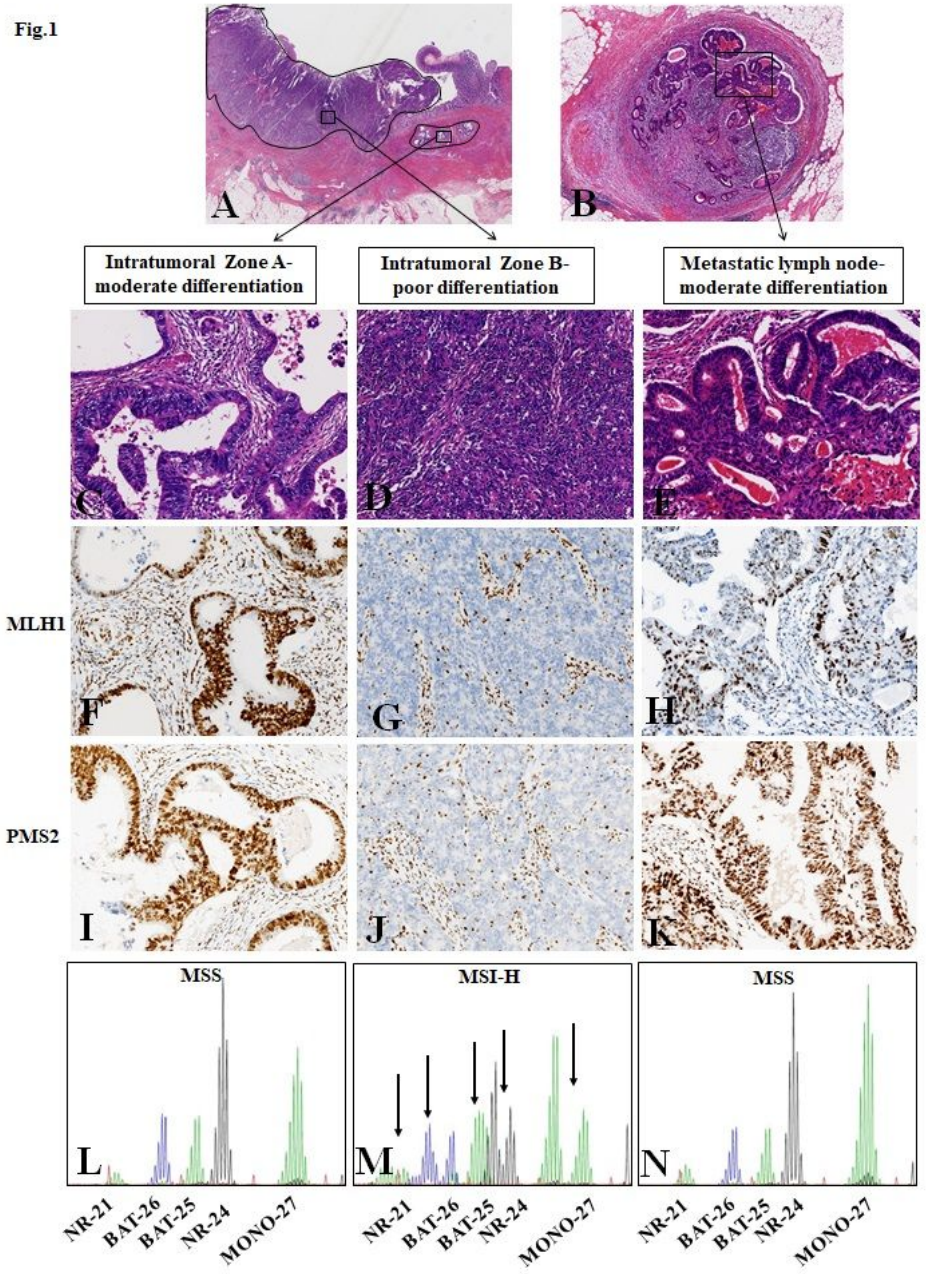

Figure 1

Histopathological morphology, immunohistochemical staining of MMR protein expression (including MLH1 and PMS2) and the status of microsatellite instability (MSI) in different intra-tumoral zones (Zone A and Zone B) and metastatic lymph nodes in CRC with heterogenous MMR protein expression (Case 2). Histopathological morphology of primary CRC and matched metastatic lymph node at low magnification(A-B). At high magnification, intra-tumoral Zone A (C, F, I, and L) and matched metastatic lymph nodes (E, H, K, and N) with pMMR/MSS were moderate differentiation with a glandular or cribriform-like pattern; Intra-tumoral Zone B (D, G, J, and M) with dMMR (MLH1/PMS2 loss) and MSI-H was poorly differentiated, showing a solid or dense sieve growth pattern.

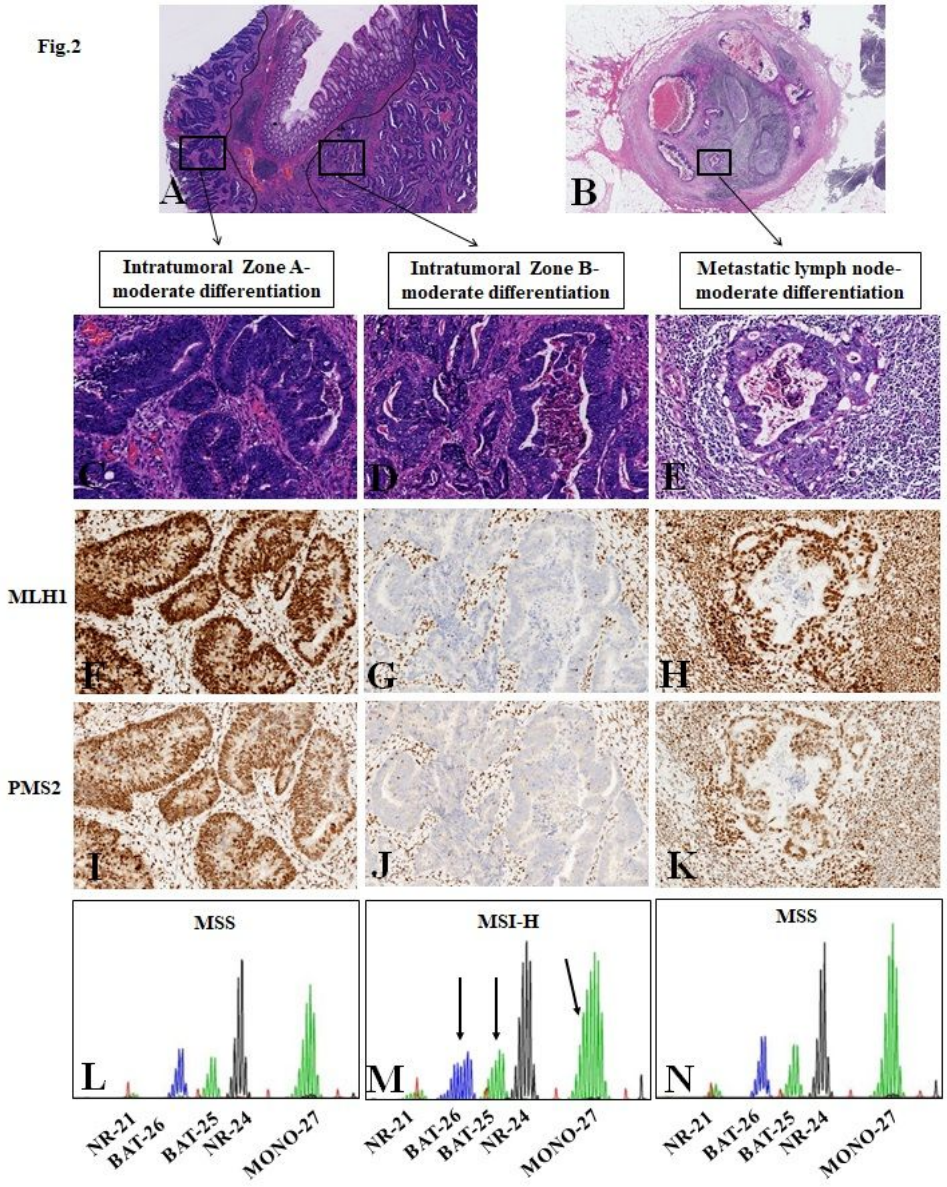

Figure 2

Histopathological morphology, immunohistochemical staining of MMR protein expression (including MLH1 and PMS2) and the status of microsatellite instability (MSI) in different intra-tumoral zones (Zone A and Zone B) and metastatic lymph nodes in CRC with heterogenous MMR protein expression (Case 3). Histopathological morphology of primary CRC and matched metastatic lymph nodes at low magnification(A-B). At high magnification, intratumoral Zone A (C), intratumoral Zone B (D), and matched metastatic lymph nodes (E) all showed moderate differentiation with a glandular or cribriform-like pattern; Intra-tumoral Zone A (F, I, L) and matched metastatic lymph nodes (H, K, N) were identified as pMMR and MSS; Intratumoral Zone B (G, J, M) was identified as dMMR (MLH1/PMS2 loss) and MSI-H.

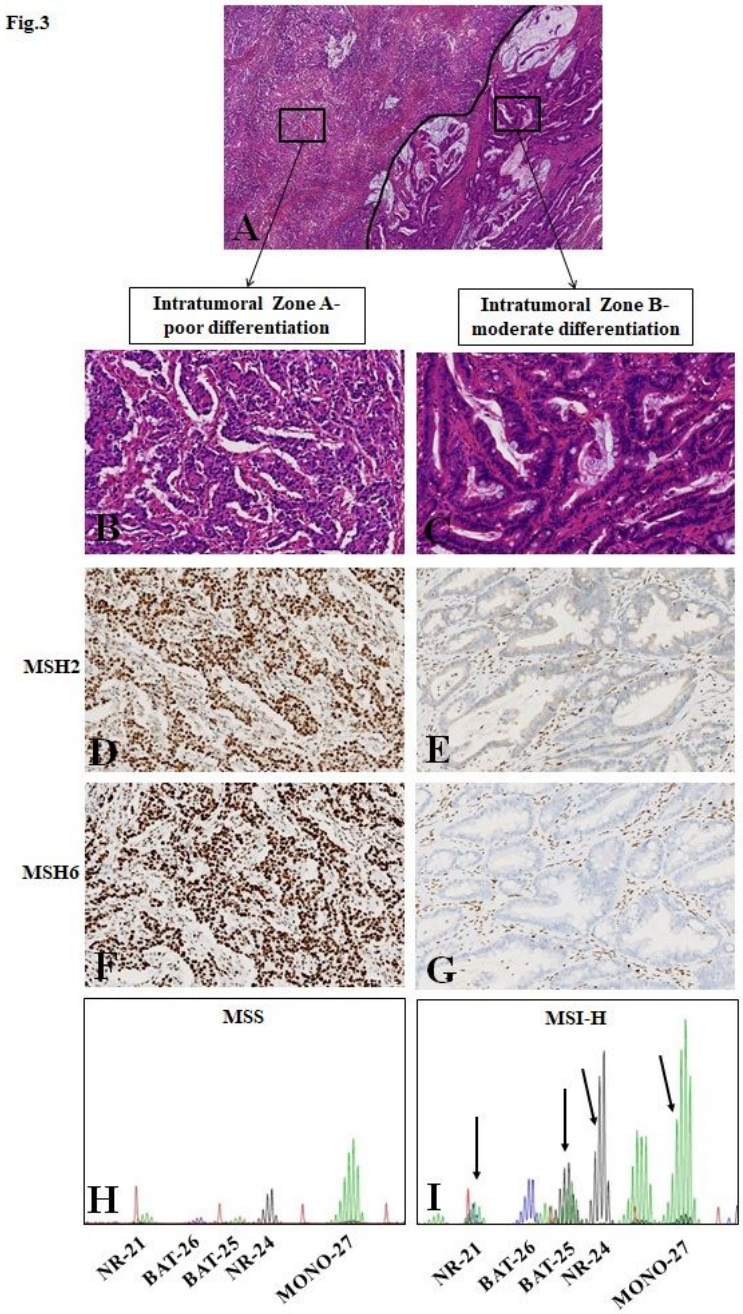

Figure 3

Histopathological morphology, immunohistochemical staining of MMR protein expression (including MSH2 and MSH6) and the status of microsatellite instability (MSI) in different intra-tumoral zones (Zone A and Zone B) with heterogenous MMR protein expression (Case 4). Histopathological morphology of primary CRC at low magnification(A). Intratumoral Zone A (B, D, F, H) with pMMR and MSS was poorly differentiated; Intra-tumoral Zone B (C, E, G, I) with dMMR (MSH2/MSH6 loss) and MSI-H was moderate differentiation.

Supplementary Files

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