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Intraindividual Tumor Heterogeneity of Mismatch Repair Status in Metastatic Colorectal Cancer

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Abstract: Heterogeneous mismatch repair (MMR) status in metastatic colorectal cancer (mCRC) may associate with refractoriness to immunotherapy. We aimed here to study the concordance in MMR status between primary and paired metastasis in mCRC. Our study included 84 patients diagnosed with mCRC with primary and matched metastatic cancers. Immunohistochemistry was used to determine the MMR status of primary lesions and matched metastases. Pooled analysis of 913 cases was used to evaluate the prevalence and organ specificity of MMR status heterogeneity. The correlations between MMR pattern heterogeneity and clinical outcomes were analyzed. MMR status heterogeneity between primary and corresponding metastatic sites was exhibited by 10 (11.9%) patients. The prevalence of the heterogeneous MMR phenotype was significantly higher in primary tumors with deficient MMR (dMMR) than with proficient MMR (pMMR), which was verified in the pooled analysis ($P < 0.001$). Among patients with a dMMR primary tumor, the discrepancy was detected in liver, lung, ovary, peritoneum, and distant lymph node metastases. However, the discrepancy was confined to liver (26/440) or peritoneum (7/112) ($P = 0.02$) in patients with a pMMR primary tumor. Patients with or without MMR status heterogeneity experienced comparable overall survival ($P = 0.452$). Heterogeneous MMR patterns generally existed in a subset of patients with mCRC, particularly those with dMMR primary tumors. Testing the metastatic site may be valuable because the discordance of MMR status may potentially affect immune surveillance and immunotherapy.

Key Words: DNA mismatch repair, metastatic colorectal cancer, intraindividual tumor heterogeneity, prognosis

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Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death.[1] Among newly diagnosed patients with CRC, 20% have metastatic disease and a 5-year survival rate <20%.[2] The underlying oncogenic mechanism in 15% of patients with CRC is deficient mismatch repair (dMMR), which is typically associated with microsatellite instability (MSI).[3] Testing for tumor DNA mismatch repair (MMR) status has become indispensable in the treatment of metastatic colorectal cancer (mCRC) because of the sensitivity of dMMR mCRC to immune checkpoint inhibitors (ICIs).[4] Unfortunately, ~30% of dMMR CRCs exhibit primary resistance to ICIs, and a substantial fraction of patients acquire resistance after experiencing an initial benefit. Immune evasion is associated with alterations of the WNT and JAK-STAT signaling pathways. Furthermore, refractoriness to ICIs is linked to mutations in the genes encoding components of the antigen presentation machinery.[5]

Another key mechanism affecting the efficacy of ICIs is tumor heterogeneity.[6] High concordance of genomic alterations was revealed by the analysis of primary tumors and the corresponding metastases, particularly for oncogenic drivers.[7] Evidence indicates that genomic alterations associated with invasion and metastasis occur during the early stage of CRC, and analysis of a primary or metastatic lesion is appropriate for clinically significant genomic drivers.[8] Intratumoral heterogeneity MMR status is exhibited by a small subset of CRCs, which may affect immune surveillance.[9,10] However, the mechanism of MMR heterogeneity is unknown, and whether it will affect prognosis requires further analysis.[5,11] The discovery of cancers with MMR status discrepancies between paired primary tumors and metastases may challenge the dogma of proper immunotherapy. Here we therefore investigated the heterogeneity of MMR status between primary and metastatic tumors in mCRC, and a pooled

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analysis was performed to identify the characteristics of MMR heterogeneity.

METHODS

Patients and Ethics Approval

We analyzed the records of all patients diagnosed with mCRC who underwent surgical resection from January 2016 to January 2020 at the Department of General Surgery, Tianjin Medical University General Hospital, Tianjin, Chin. The Ethics Committee of this hospital approved this study (Ethics approval document number: IRB2021-WZ-198). Criteria for excluding patients from the study are as follows: (1) history of neoadjuvant radiotherapy, chemotherapy, or both; (2) inability to obtain matched samples; (3) insufficient tumor tissue or frozen metastatic tumor tissue; (4) patients without informed consent.

Assessment of MMR Status

The MMR status of samples was analyzed using immunohistochemistry (IHC) and polymerase chain reaction (PCR) to test for MSI when samples were classified as dMMR. MSI analysis was performed using the microsatellite markers Bat25, Bat26, NR21, NR24, Mono27, and NR27. The specimens were fixed with 10% neutral formalin, embedded in paraffin, and stained with hematoxylin-eosin. Each formalin-fixed paraffin-embedded tumor sample was serially sectioned. Immunohistochemical analyses of the most common MMR proteins MLH1, MSH2, MSH6, and PMS2 were performed using the standard Envision 2-step procedure with the respective cognate antibodies (1:50; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd). Phosphate buffered saline was used instead of the primary antibody as the negative control. Non-neoplastic colorectal mucosa, stromal cells, and infiltrating lymphocytes, or the centers of lymphoid follicles served as internal positive controls. Known dMMR CRC served as an external negative control. Two pathologists uninformed of patients' clinical data independently evaluated the staining results. Normal expression was defined as nuclear staining within tumor cells, whereas negative protein expression was defined as the complete absence of nuclear staining within tumor cells with concurrent internal positive controls. If internal non-neoplastic tissues showed invalid negative staining, the procedure was repeated. When the opinions of the 2 pathologists differed, an agreement was reached through careful discussion.

Identification of Published Studies and Eligibility

Related studies published in the PubMed database were searched up to March 2022 using the following keywords: ("cancer" OR "carcinoma" OR "tumor") AND ("colorectal " OR "colon" OR "rectal" OR "rectum") AND ("metastasis" OR "metastatic") AND ("microsatellite instability" OR "MSI" OR "mismatch repair" OR "MMR"). Only case-control and cohort studies that reported the MMR or MSI status in the primary tumor and paired metastatic tumor were included. We excluded animal studies, review articles, case reports, and duplicates.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics (version 22.0). Overall survival (OS) was defined as the time from diagnosis of mCRC to the date of death or the last follow-up (April 1, 2022). OS rates were calculated using the Kaplan-Meier method, and the differences were compared using the log-rank test. $P \leq 0.05$ indicated a significant difference.

RESULTS

Patients' Characteristics

We enrolled 183 patients diagnosed with mCRC who were treated at the Department of General Surgery of Tianjin Medical University General Hospital from January 2016 to January 2020. We excluded 99 patients with a history of neoadjuvant radiation or chemotherapy or both, and insufficient paired samples. Thus, 84 patients were included. The sites of metastasis included the peritoneum (n = 28, 31.8%), liver (n = 50, 56.8%), ovaries (n = 4, 4.6%), and lungs (n = 6, 6.8%). The study included 1 patient with 3 metastases and 2 patients each with 2 metastases. All primary tumors and metastases exhibited proficient MMR (pMMR).

Heterogeneity of MMR Status

Among the primary tumors, 9/84 (10.7%) were dMMR (dMMR_PT) and 75/84 (89.3%) were pMMR primary tumors (pMMR_PT). Heterogeneity of MMR status between primary and metastatic tumors was exhibited by 10/84 (11.9%) primary tumors. Figures 1A and C shows the heterogeneity of MMR status for Cases 4 and 16. PCR analyses of MSI were performed in dMMR samples and their paired samples. High concordance of MSI and MMR status was verified in dMMR samples and their paired samples. Figures 1B and D shows the MSI status of paired samples from Cases 4 and 16. Among the patients with dMMR_PT, 4 showed proficient expression of MMR in metastatic tumors (pMMR_MT) and 5 showed deficient expression of MMR in metastatic tumors (dMMR_MT). Among patients with pMMR_PT, 6 had dMMR_MT, and the remaining 69 patients had pMMR_MT (Fig. 2A). Heterogeneity of MMR status was exhibited by 5/28 (17.9%) patients with peritoneal metastasis and 5/50 (10%) patients with liver metastasis. There was not a discrepancy of MMR status in patients with ovarian and lung metastasis (Fig. 2B). Of the 2 patients with dMMR in primary tumors and metastases, the expression of MMR proteins in paired samples was interestingly heterogeneous. Patients' clinicopathologic features are shown in Table 1.

Analysis of the Present Study and the Literature

The relatively small number and low incidence of patients with dMMR mCRC required pooled analysis to evaluate the correlation of MMR status between primary

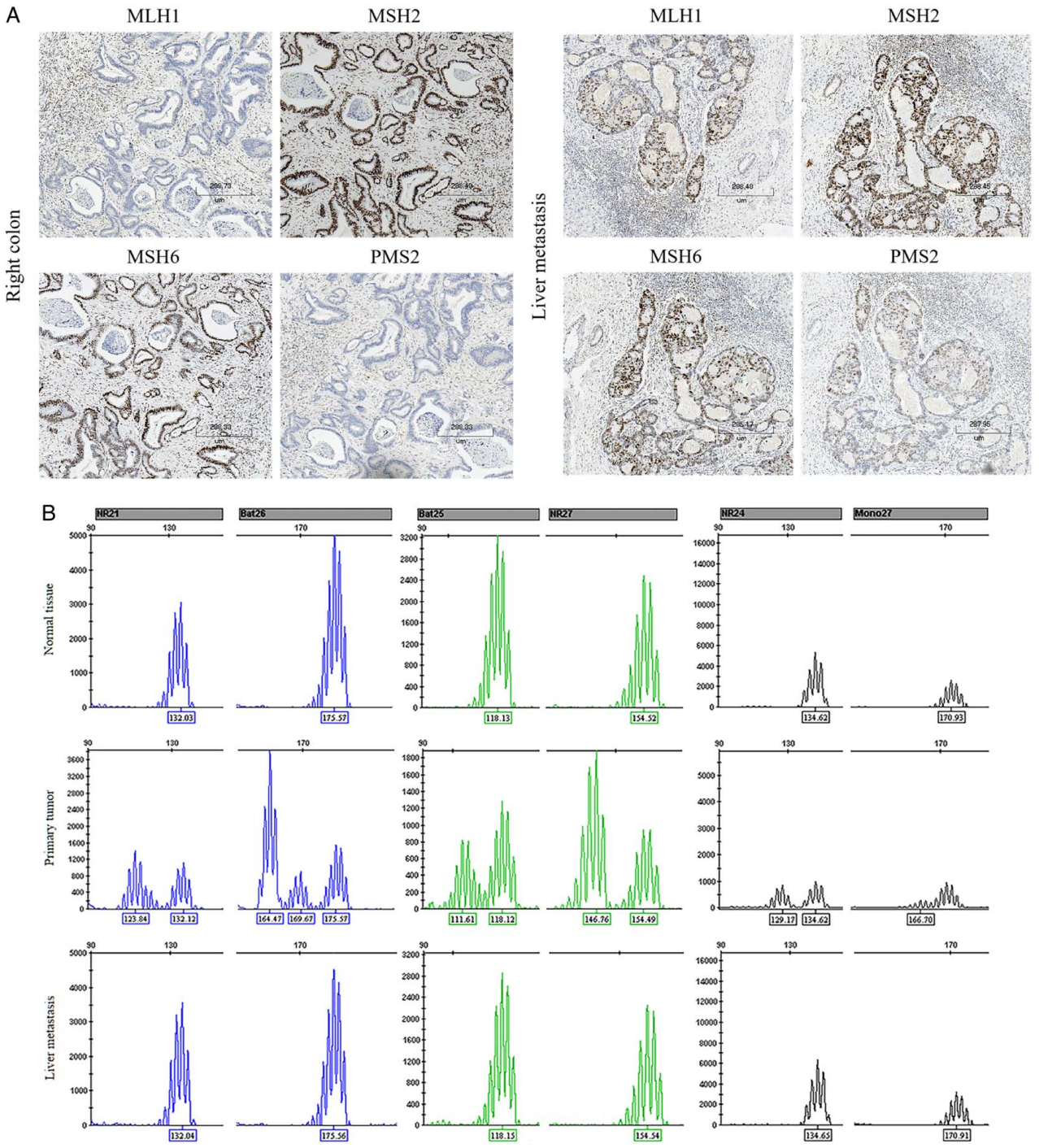

FIGURE 1. A, Mismatch repair immunohistochemistry result of case 4, ×400. B, Microsatellite instability polymerase chain reaction analyses of case 4. C, Mismatch repair immunohistochemistry result of case 16 (×400). D, Microsatellite instability polymerase chain reaction analyses of case 16.

and metastatic lesion in mCRC. This analysis included the present study and 8 other studies (Table 2).[10,12–18] Haraldsdottir's study was excluded because of the absence of data on pMMR primary tumors.[16] We analyzed the incidence of MMR status heterogeneity among 900 patients. Among patients with pMMR in the primary tumor, 761 (95.8%) showed pMMR in metastatic cancers. However, 77 (72.6%) cases showed dMMR in metastatic tumors of patients with dMMR in the primary tumor, and 62 (6.9%) cases exhibited heterogeneity of MMR status between primary and metastatic tumors in patients with a pMMR primary tumor (Fig. 2C). Patients

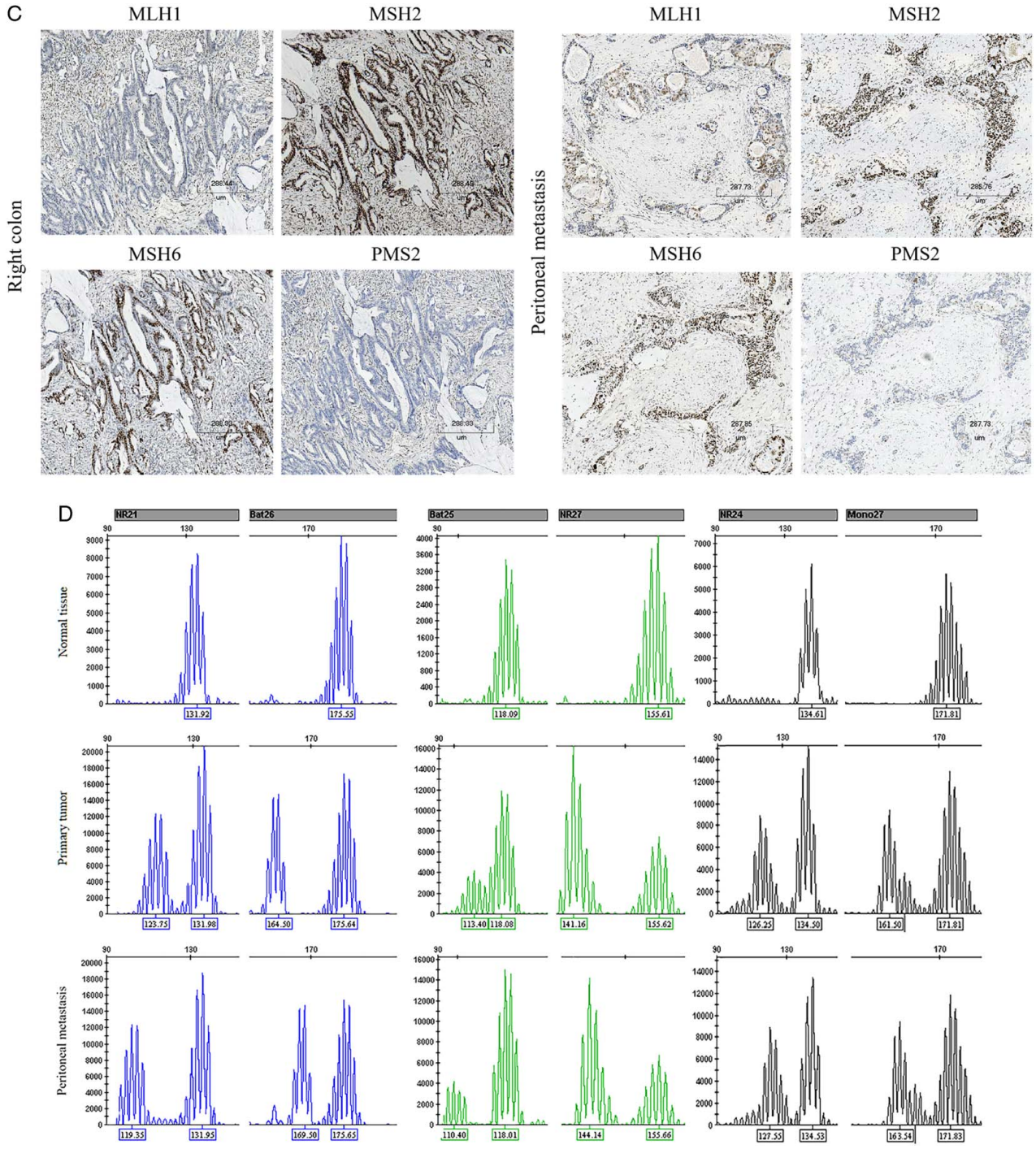

FIGURE 1. (Continued).

with dMMR primary tumors were associated with a significantly greater prevalence of heterogeneous MMR status compared with patients with pMMR primary tumors ($P < 0.001$) (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A368). We next analyzed the tissues of 913 patients to determine whether the heterogeneity of MMR status was associated with the locations of the metastatic sites. Among patients with dMMR primary tumors, discrepancies associated with MMR status were observed in liver, lungs, ovaries, peritoneum, and distant lymph node metastases (Fig. 2D). The discrepancy was more likely to be identified in ovarian metastases (4/4) ($P < 0.001$) (Supplemental Table 2, Supplemental Digital Content 2, http://links.lww.com/AIMM/A369). In contrast, the discordance in patients with pMMR primary tumors was more likely

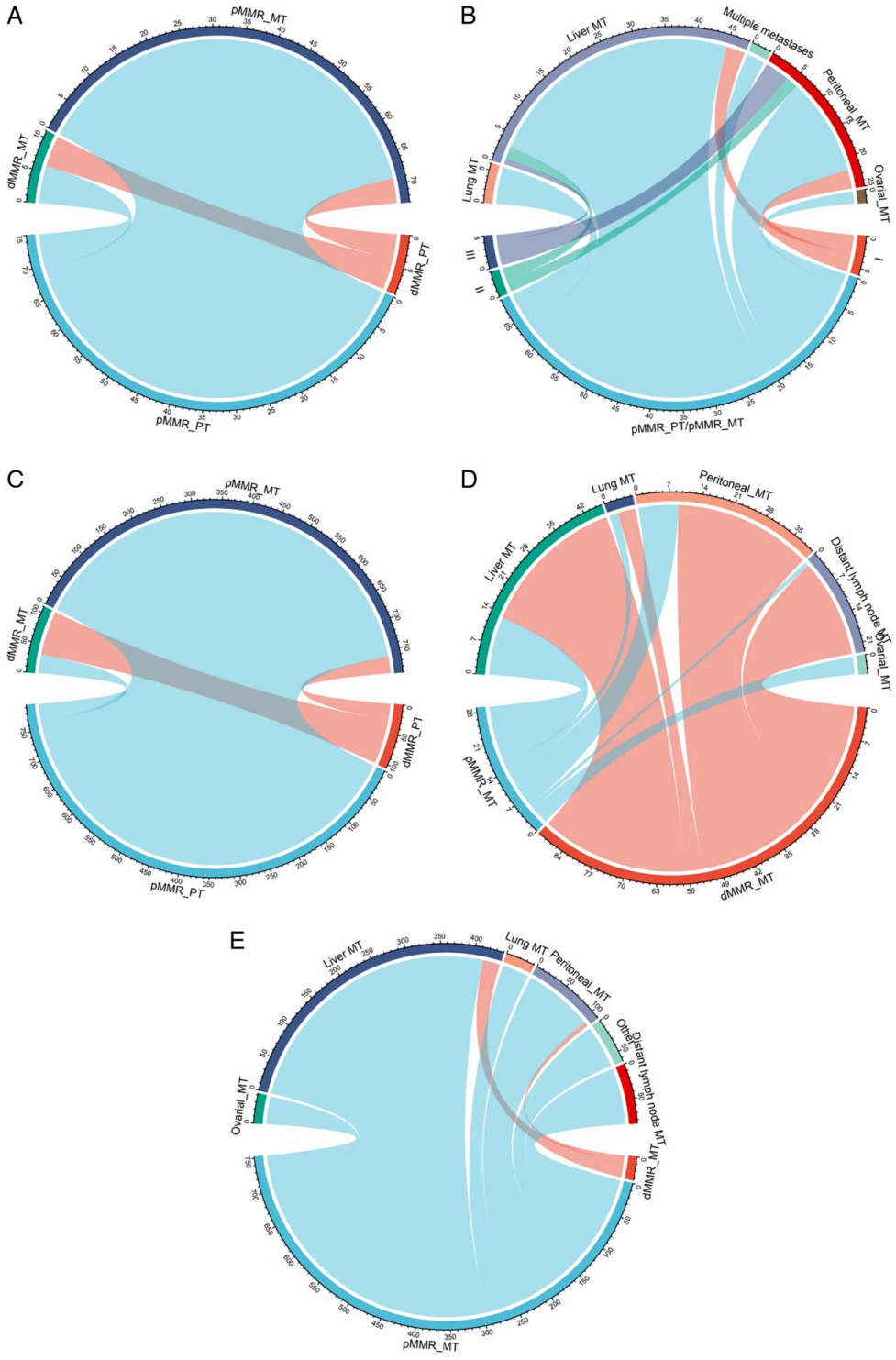

FIGURE 2. A, Circus diagrams of mismatch repair (MMR) status between primary tumors and metastatic tumors. B, Circus diagram of correlation between metastatic site and discrepancy regarding MMR status. C, Circus diagram of MMR status between primary tumors and metastatic tumors in pooled analysis. D, Organ-specific Circus diagram of MMR status between primary and matched metastatic tumors in patients with deficient MMR (dMMR)_primary tumors (PT). E, Organ-specific Circus diagram of MMR status between primary and matched metastatic tumors in patients with proficient MMR (pMMR)_PT. I indicates pMMR_PT/dMMR_MT; II, dMMR_PT/pMMR_MT; III, dMMR_PT/dMMR_MT; MT, metastatic tumors. full color online

TABLE 1. Clinicopathological Features of Patients With Colorectal Cancer and MMR Protein Expression Status in Primary and Metastatic Tumors

Case	Sex	Age (y)	Primary Site	Size (cm)	Histologic Type	Lymph Node	Differentiation	Metastatic Site	Follow-up Time (mo)	Dead (Y/N)	MLH1 (P/M)	MSH2 (P/M)	MSH6 (P/M)	PMS2 (P/M)	MMR (P/M)
1	Female	51	Left colon	2.5	Adenocarcinoma	1/13	Medium-low	Liver	20	Y	I/I	I/I	I/I	I/L	Pr/De
2	Female	59	Right colon	8	Adenocarcinoma, partial MAC	0/8	Medium	Peritoneal	27	Y	I/L	I/L	I/L	I/L	Pr/De
3	Female	21	Right colon	6.5	Mucinous adenocarcinoma	5/15	Low	Peritoneal	32	Y	I/I	I/I	I/L	I/L	Pr/De
4	Female	70	Right colon	10	Adenocarcinoma, partial SRCC	0/21	Low	Liver	15	Y	I/I	I/I	L/I	L/I	De/Pr
5	Male	51	Right colon	4.5	Adenocarcinoma, partial MAC	14/15	Medium-low	Liver	11	Y	I/I	I/I	L/I	L/I	De/Pr
6	Female	59	Right colon	7	MAC, partial SRCC	13/13	Low	Peritoneal	17	Y	I/I	I/I	L/I	I/I	De/Pr
7	Male	74	Rectum	5	Adenocarcinoma	0/14	High-medium	Liver	18	N	I/I	I/I	I/I	I/L	Pr/De
8	Male	68	Rectum	8	Adenocarcinoma, partial MAC	5/14	High-medium	Peritoneal	21	N	I/I	I/I	I/I	L/I	De/Pr
9	Female	61	Rectum	5	Adenocarcinoma	0/15	Medium	Liver	48	N	I/I	I/I	I/I	I/L	Pr/De
10	Male	68	Rectum	5	Mucinous adenocarcinoma	8/14	Low	Peritoneal	12	Y	I/I	I/I	I/I	I/L	Pr/De
11	Male	50	Right colon	7.5	Adenocarcinoma	0/24	Medium	Liver	30	N	I/I	L/L	L/L	I/L	De/De
12	Male	67	Right colon	3	Adenocarcinoma	3/9	Medium-low	Peritoneal	20	Y	I/I	I/I	L/I	L/L	De/De
13	Male	44	Right colon	8	Mucinous adenocarcinoma	0/3	Low	Peritoneal	8	Y	L/L	I/I	I/I	L/L	De/De
14	Male	74	Right colon	6	Adenocarcinoma, partial SRCC	3/13	Medium-low	Peritoneal	28	Y	L/L	I/I	I/I	L/L	De/De
15	Male	62	Right colon	4	Adenocarcinoma	0/18	Medium	Peritoneal	15	Y	I/I	I/I	L/L	L/I	De/De
16	Male	67	Right colon	3	Adenocarcinoma	3/9	Medium-low	Peritoneal	20	Y	I/I	I/I	L/I	L/L	De/De
17	Female	62	Right colon	4	Adenocarcinoma	0/18	Medium	Peritoneal	15	Y	I/I	I/I	L/L	L/I	De/De

De indicates deficient; I, intact; L, low; M, metastasis; MAC, mucinous adenocarcinoma; MMR, mismatch repair; P, primary; Pr, proficient; SRCC, signed-ring cell carcinoma.

TABLE 2. Literature Review of Mismatch Repair Status of Primary and Matched Metastatic Tumor Tissues

					Metastatic Site (patients, n) Histologic Type N Stage					
References	Method	Pairs	Primary Tumor MMR Status	Number of Cases	Ovary (n)	Peritoneal (n)	Liver (n)	Distant LN (n)	Lung (n)	Other (n)
He et al[10]	PCR/IHC	369	dMMR	46	4	20	10	9	3	
			pMMR	323	38	76	128	12	12	
Fujiyoshi et al[14]	PCR/IHC	161	dMMR	24		9	2	13		0
			pMMR	137		16	39	72		10
Jung et al[15]	IHC	61	dMMR	7			5		5	
			pMMR	54			31		23	
Larsen et al[12]	IHC	90	dMMR	1			1			
			pMMR	89			89			
Murata et al[18]	PCR/IHC	26	dMMR	3			3	0		
			pMMR	23			22	1		
Ágoston et al[17]	IHC	69	dMMR	12			12			
			pMMR	57			57			
Wang et al[13]	PCR/IHC	40	dMMR	4			4			
			pMMR	36			36			
Haraldsdottir et al[16]	IHC	13	dMMR	13		5	6	1	1	
This study	IHC	84	dMMR	9	0	4	4		0	
			pMMR	75	4	22	47		6	

dMMR indicates deficient mismatch repair; IHC, immunochemistry; PCR, polymerase chain reaction; pMMR, proficient mismatch repair.

limited to liver (26/440) and peritoneum (7/112) ($P = 0.02$) (Fig. 2E) (Supplemental Table 3, Supplemental Digital Content 3, http://links.lww.com/AIMM/A370). We excluded 65 cases with pMMR primary tumors, because the metastatic sites were not available.[10,14]

When we next analyzed the organ specificity of MMR status heterogeneity, we found that peritoneal metastases had the highest rate of MMR status heterogeneity (10.67%), while distant lymph node metastases had the lowest rate (0.93%) (Fig. 3A). Distant lymph nodes with metastases had the lowest incidence of MMR heterogeneity ($P = 0.016$) (Supplemental Table 4, Supplemental Digital Content 4, http://links.lww.com/AIMM/A371). Among all metastatic sites, except distant lymph nodes, the prevalence of MMR status heterogeneity was higher in dMMR primary tumors than in pMMR primary tumors (Fig. 3B) (Supplemental Table 5, Supplemental Digital Content 5, http://links.lww.com/AIMM/A372).

To identify the mechanism of MMR status heterogeneity, we classified the proportion of heterogeneously expressed MMR proteins and the proportion of patients with heterogeneous MMR status. Among the heterogeneously expressed MMR proteins, MSH6, and PMS2 were expressed at higher levels (Fig. 3C). The proportion of patients with isolated heterogeneous expression of MSH6 and PMS2, as well as paired heterogeneous expression of MSH2/MSH6 and MLH1/PMS2, was relatively higher (Fig. 3D).

Survival Analysis

Survival analysis assessed the relationship between MMR status heterogeneity and prognosis. Patients with pMMR experienced OS comparable with patients with dMMR [hazard ratio (HR): 0.86; 95% CI: 0.36-1.93, $P = 0.719$) (Fig. 4A). Patients with pMMR metastatic tumors with pMMR primary tumors experienced similar OS to patients with dMMR metastatic tumors (HR: 2.02; 95% CI: 0.715.79, $P = 0.189$) (Fig. 4B). Patients with or without heterogenous expression of MMR proteins between primary and metastatic tumors still had a similar prognosis (HR: 0.73; 95% CI: 0.33-1.56, $P = 0.452$) (Fig. 4C).

DISCUSSION

ICIs extend the survival of patients with mCRC with dMMR/MSI-H.[19] The PD-1 blockade was approved as a first-line therapy in the United States, Switzerland, and Japan for treating MSI-H mCRC.[20] Subsequent to the landmark KEYNOTE-177 study results, the US Food and Drug Administration approved pembrolizumab as first-line therapy for patients with unresectable dMMR/MSI-H mCRC.[21] However, ~30% of dMMR CRC tumors exhibit primary resistance, and a significant portion of tumors acquire resistance after initial benefit. Thus, resistance to ICIs is a significant barrier to improving therapy for dMMR CRC.[22]

Tumor heterogeneity was proposed as the key mechanism underlying the incomplete response of CRCs to therapy. Intratumoral and intertumoral heterogeneity of KRAS mutations in mCRC are associated with resistance or reduced efficacy of anti-EGFR therapies.[23] However, insufficient data are available regarding the temporal and spatial heterogeneity of MSI/MMR status. To address this gap in our knowledge, here we conducted IHC and PCR analyses to assess MMR status in paired primary and metastatic tumors to reveal intraindividual MMR heterogeneity. We found that 11.9% of patients with mCRC exhibited MMR status heterogeneity between primary and metastatic tumors and that patients with

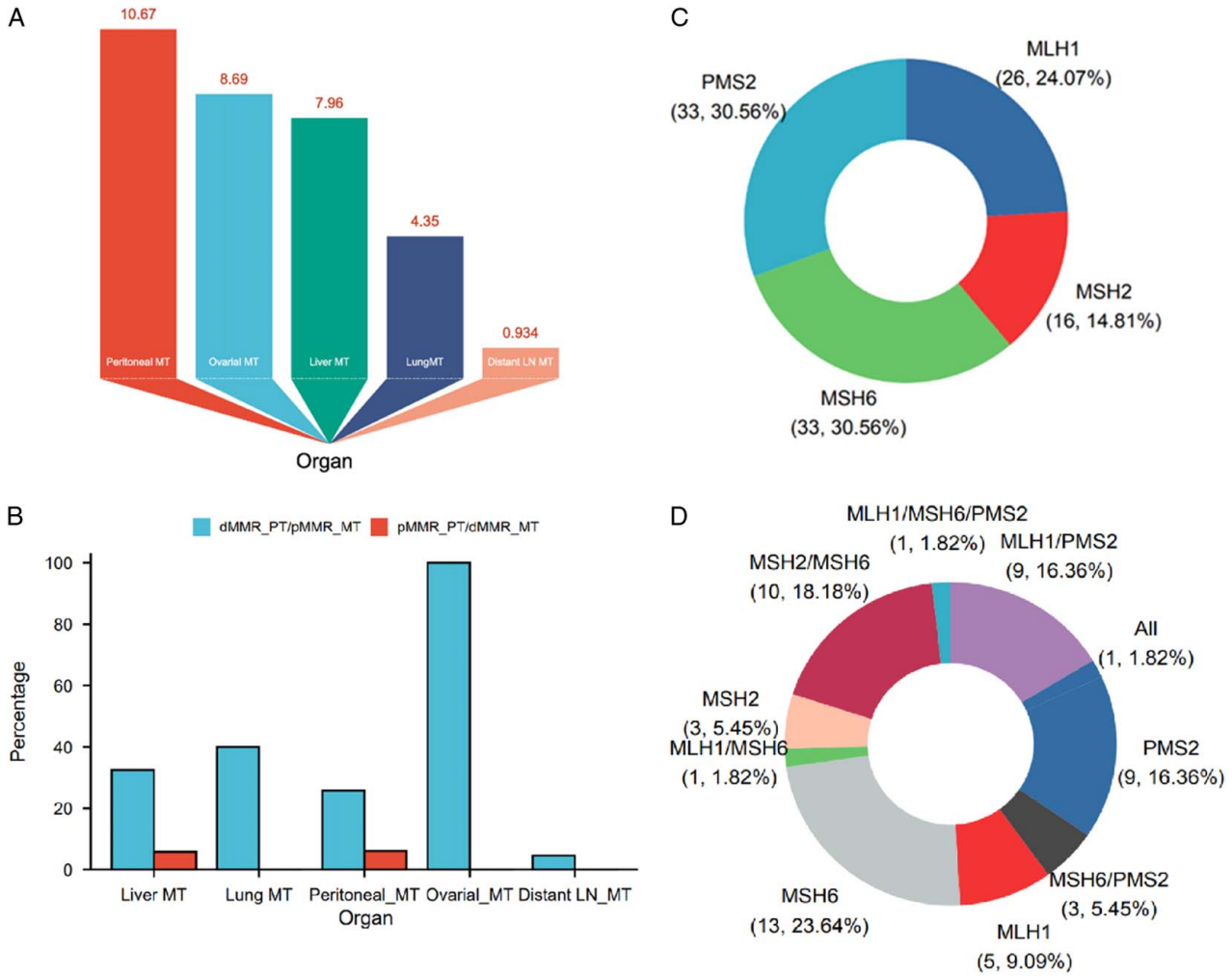

FIGURE 3. A, Incidence of mismatch repair (MMR) status heterogeneity between primary tumors and metastatic tumors in different organs. B, The incidence of MMR status heterogeneity between metastatic tumors and primary tumors with deficient MMR (dMMR) (dMMR_PT) or proficient MMR (pMMR) (pMMR_PT) in different organs. C, Frequency and proportion of mismatch repair proteins with heterogeneous expression. D, Frequency and proportion of patients with different types of mismatch repair protein heterogeneity. The mismatch repair proteins were heterogeneously expressed between primary and metastatic tumors. MT indicates metastatic tumors; PT, primary tumors. full color online

peritoneal and liver metastases were more likely to have MMR status heterogeneity.

The low prevalence of MMR status heterogeneity required a pooled analysis, which revealed that the total concordance rate of MMR status was 93.1%. The concordance rates of MMR status of patients with dMMR primary tumors compared with pMMR primary tumors were 72.6% versus 95.8%, respectively. Thus, our discoveries indicate that pMMR primary tumors are exceedingly unlikely to be associated with dMMR metastases. Analysis of the metastatic site may be valuable if the primary tumor is dMMR because metastases with discordant MMR status may be associated with primary or acquired ICI resistance.

So-called seed factors are required for tumor progression and metastasis, and metastases generated by dMMR primary tumors exhibit a degree of MMR status discordance.[24] For example, BRAF mutations, which are present in 34% of MSI CRC versus 6% of MSS CRC,[25] are strongly associated with peritoneal metastases.[26] The microenvironment of an organ as well as the metastatic milieu are required for colonization and metastatic outgrowth.[27] The mode of metastasis may be associated with heterogeneous MMR status. Thus, metastasis diverges before the development of MSI,[28] because the progenitors of malignant cells do not harbor all mutations present in the primary tumor before metastatic seeding, and the tumor mutation burden of these discordant metastases is reduced.[6] However, the reason underlying MMR status heterogeneity is unknown.

The dMMR/MSI phenotype can be acquired through sporadic CRC or Lynch syndrome.[29] The lack of MLH1 expression associated with hypermethylation

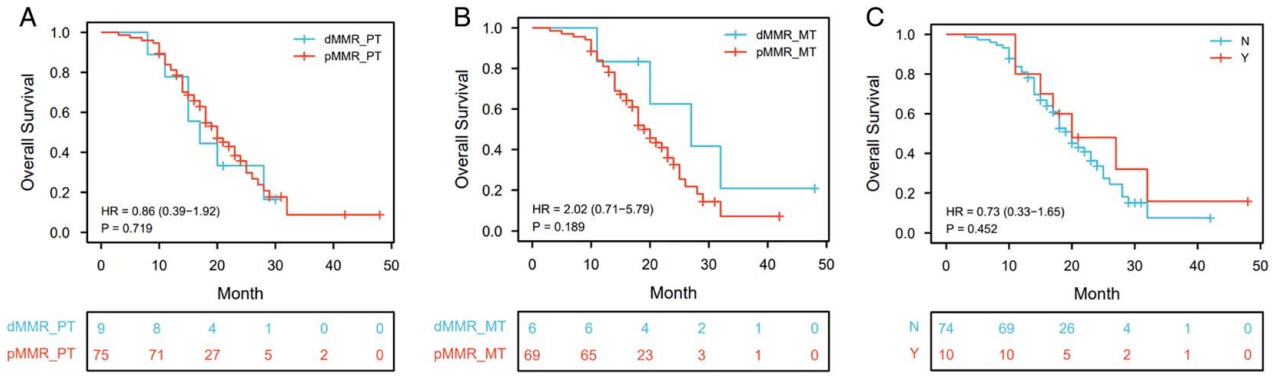

FIGURE 4. A, Comparison of overall survival of patients with deficient mismatch repair (dMMR) versus proficient mismatch repair (pMMR) in primary tumors. B, Comparison of overall survival of patients with pMMR primary tumors with pMMR versus dMMR metastatic tumors. C, Comparison of overall survival of patients with or without heterogeneous expression of mismatch repair (MMR) proteins between primary and metastatic tumors. (Cohort Y, patients with heterogeneously expressed MMR proteins between primary and metastatic tumors; Cohort N, patients without heterogeneously expressed MMR proteins between primary and metastatic tumors). full color online

of the MLH1 gene promoter is the most common cause of sporadic cases. Most Lynch syndrome germline mutations (90%) occur in MLH1 or MSH2. In contrast, ~10% of cases harbor MSH6 and PMS2 mutations.[3] We show here, however, that MSH6 and PMS2 represent a large proportion of proteins that are heterogeneously expressed. Moreover, patients with isolated heterogeneous expression of MSH6 and PMS2, as well as paired heterogeneous expression of MSH2/MSH6 and MLH1/PMS2, experienced higher prevalence. To our knowledge, this is the largest study focusing on discordance among MMR proteins with heterogeneous expression between primary and matched metastatic tumors.

Prospective trials are therefore required to determine the influence of MMR heterogeneity on prognosis and treatment efficacy. We show here that patients with or without heterogeneity of MMR status had comparable OS rates. Unfortunately, the patients included in this study did not undergo immunotherapy, and the number of patients was limited. The efficacy of ICIs administered to patients with mCRC with dMMR heterogeneity status requires further research that must include more samples. Furthermore, data that contribute insights into the dynamic evolution of MMR status during tumor progression, particularly under immunotherapy selection pressure, are not available.

Although this study systematically assessed the intraindividual heterogeneity of MMR status in patients with mCRC, there were limitations. For example, although IHC and PCR analyses are less time-consuming and less expensive, more advanced techniques may yield more accurate results. Thus, tests based on next-generation sequencing scan hundreds of loci, achieving more thorough analysis.[30] Furthermore, the results of the present study may be somewhat biased because of the low incidence of MMR status heterogeneity in mCRC and the absence of relevant research.

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