Comparison of Mismatch Repair Status Between Primary and Matched Metastatic Sites in Patients With Colorectal Cancer

Wen-Zhuo He, MD^{a,*}; Wan-Ming Hu, MD^{b,c,d,*}; Fang Wang, MD^{e,*}; Yu-Ming Rong, MD^a; Lin Yang, MD^a; Qian-Kun Xie, MD^a; Yuan-Zhong Yang, MD^b; Chang Jiang, MD^a; Hui-Juan Qiu, MD^a; Jia-Bin Lu, MD^b; Bei Zhang, MD^a; Pei-Rong Ding, MD^f; Xiao-Jun Xia, PhD^g; Jian-Yong Shao, MD^e; and Liang-Ping Xia, MD^a

ABSTRACT

Background: Differences between the features of primary cancer and matched metastatic cancer have recently drawn attention in research. This study investigated the concordance in microsatellite instability (MSI) and mismatch repair (MMR) status between primary and corresponding metastatic colorectal cancer (CRC). **Methods:** Consecutive patients with metastatic CRC who had both primary and metastatic tumors diagnosed at our institution in January 2008 through December 2016 were identified. Immunohistochemistry was used to test the MMR status of both primary and matched metastatic tumors, and PCR analysis was performed to test MSI in patients with deficient MMR (dMMR) status. **Results:** A total of 369 patients were included. Of the 46 patients with MSI-high primary tumors, 37 (80.4%) also had MSI-high metastatic tumors, whereas 9 (19.6%) had microsatellite stable (MSS) metastatic tumors. A high concordance was found in patients with liver, lung, or distant lymph node metastases. Interestingly, the discrepancy was more likely to be limited to peritoneal (5/20) or ovarian (4/4) metastasis (chi-square test, $P < .001$). These organ-specific features were also found in the pooled analysis. Along with the change of MSI-high in primary cancer to MSS in metastatic cancer, lymphocyte infiltration decreased significantly ($P = .008$). However, the change did not influence survival; the median overall survival of MSI-high and MSS metastatic tumors was 21.3 and 21.6 months, respectively ($P = .774$). The discrepancy rate was 1.6% for patients with proficient MMR primary tumors. **Conclusions:** For patients with dMMR primary tumors, the concordance of MSI and MMR status in primary CRC and corresponding metastatic cancer is potentially organ-specific. High concordance is found in liver, lung, and distant lymph node metastases, whereas discrepancy is more likely to occur in peritoneal or ovarian metastasis. Rebiopsy to evaluate MSI-high/dMMR status might be needed during the course of anti-PD-1 therapy in cases of peritoneal or ovarian metastasis.

J Natl Compr Canc Netw 2019;17(10):1174–1183
doi: 10.6004/jnccn.2019.7308

Background

Colorectal cancer (CRC) is among the most common cancers worldwide,¹ and distant metastasis is extremely common in CRC.² Recently, different profiles between the primary CRC and corresponding metastatic cancers have been found, particularly genetic discordances between the primary and the metastatic sites.^{3–6} Moreover, oxidative DNA damage and some chemokines were significantly higher in metastases than in primary tumors,^{7,8} and the level of tumor-infiltrating lymphocytes and expression of PD-L1 differed between primary and metastatic tumors,^{9,10} with the discrepancy being site-specific in some cases.¹¹ These differences indicate that metastases may provide a better “predictive window” for targeted therapy than the primary cancer alone.³

The mismatch repair (MMR)/microsatellite instability (MSI) pathway significantly contributes to CRC development^{12,13} and is associated with the immune microenvironment.^{14,15} However, although the distinct features of MSI-high (MSI-H) tumors have been identified,¹⁶ MSI status of the primary CRC versus its metastatic lesions has not been fully explored. Change in MSI status is important because studies have shown that only patients with deficient MMR (dMMR) or MSI-high tumors benefit from anti-PD-1 therapy.^{17,18} Haraldsdottir et al¹⁹ reported a 100% concordance

^aVIP Region and ^bDepartment of Pathology, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangdong, P. R. China; ^cDepartment of Pathology, School of Basic Medical Sciences, and ^dDepartment of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou, P. R. China; and ^eDepartment of Molecular Diagnostics, ^fDepartment of Colorectal Surgery, and ^gDepartment of Experimental Research, Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou, Guangdong, P. R. China.

*These authors contributed equally.

See JNCCN.org for supplemental online content.

See page 1263 for related commentary.

rate in MMR status between the primary tumor and corresponding metastatic tissue in 25 patients with CRC, whereas Fujiyoshi et al²⁰ found a discordance in 3 patients and Jung et al²¹ found a concordance rate of only 77%.

Our study evaluated concordance in MSI and MMR status between primary and corresponding metastatic tumors in CRC. Because prior studies examining this were small, we also performed a pooled analysis.

Methods

Patients and Ethical Concerns

This study evaluated consecutive patients with metastatic CRC diagnosed with both primary and metastatic tumors at Sun Yat-sen University Cancer Center in January 2008 through December 2016. Those with insufficient primary tumor tissue, frozen metastatic tumor tissue, and a history of receiving neoadjuvant chemoradiation before surgery were excluded (Figure 1). This study was approved by the ethics review board at Sun Yat-sen University Cancer Center, and informed consent was obtained from all patients.

Assessment of MMR Status

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue was selected per case, and immunostaining was performed using standard protocols. The 4 most common MMR proteins, namely, MLH1, MSH2, MSH6, and PMS2, were analyzed using the anti-MLH1, anti-MSH2, anti-MSH6, and anti-PMS2 antibodies, respectively (1:50; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd). Methods are described in detail in eAppendix 1, available with this article at JNCCN.org.

Assessment of Lymphocyte Infiltration

Tumor microenvironment parameters were assessed as previously reported.^{22,23} Briefly, absent and weak increases in lymphocytes were considered low-grade infiltration, whereas moderate to severe increases were considered high-grade (see eAppendix 1).

DNA Isolation and Assessment of Tumor MSI

Genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN). PCR analyses were performed to test for MSI when tumors were classified as dMMR. MSI analysis was performed using 5 microsatellite markers (*BAT-25*, *BAT-26*, *D2S123*, *D5S346*, and *D17S250*) recommended by NCI.

Germline MMR Mutational Analysis

Patients with MSI-high tumors underwent germline genetic testing of the MMR genes. The genetic susceptibility panel targeting all exons of *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*, and proximal intronic flanking sequences (± 10 base pairs [bp]) were analyzed for screening of single-nucleotide variation, including insertion and deletions shorter than 20 bp (see eAppendix 1).

BRAF V600E Mutation Analysis

In patients with MSI-high tumors, a *BRAF* V600E mutation analysis was performed with quantitative real-time PCR using the minor groove binder probes (see eAppendix 1).

Tumor Mutational Burden Analysis

Tumor mutational burden (TMB) was measured by counting all nonsynonymous missense mutations that

Figure 1. Flowchart of patient selection.

Abbreviations: dMMR, deficient mismatch repair; pMMR, proficient mismatch repair.

had not been previously reported as germline alterations. The workflow of TMB analysis according to HaploX Biotechnology's laboratory standardization²⁴ is available in eAppendix 1.

MLH1 Promoter Methylation Analysis

Methylation in the region near the start codon of *MLH1* was assessed using bisulfite-treated DNA. The PCR conditions were first set at 98°C for 4 minutes; following this, 20 cycles were completed at 94°C for 45 seconds, 66°C for 45 seconds, and 72°C for 1 minute. Then 20 cycles were completed at 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 1 minute, and a final extension was completed at 72°C for 8 minutes. The PCR reaction was performed with the following primers: M254-3F (5'-GTT TTG GTT TTA GTT TAG GAG TAT TGG-3') and M254-3R (5'-CCR AAT TCA AAA ACT TAC TAT AAA CCT AC-3').

Identification of Studies and Eligibility Criteria

Related studies published before August 31, 2018 were searched in PubMed with the following keywords: ('microsatellite instability' OR 'MSI' OR 'mismatch repair' OR 'MMR') AND ('cancer' OR 'neoplasm' OR 'carcinoma' OR 'adenocarcinoma' OR 'tumor') AND ('colorectal' OR 'colon' OR 'rectal' OR 'rectum') AND ('metastases' OR 'metastasis' OR 'metastatic'). All items were limited in [Title/Abstract]. We included only cohort or case-control studies in which the MMR or MSI status in the primary tumor and the MMR or MSI status in a paired metastatic tumor were reported. Animal studies, review articles, case reports, and duplicate studies were excluded. The study selection flowchart is summarized in supplemental eFigure 1.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics, version 13.0 (SPSS Inc). Frequencies and descriptive statistics were used to report patient characteristics and chi-square test was used to detect differences. Overall survival (OS) was defined as the time from diagnosis of metastasis to the date of death or last follow-up (July 31, 2018). Survival curves were calculated using the Kaplan-Meier method, and the differences were compared using the log-rank test. A *P* value $\leq .05$ was considered significant.

Results

Patient Characteristics

In total, 369 patients with a median age at diagnosis of 59 years were evaluated (Figure 1, Table 1). Of these, 221 (59.9%) were men and 148 (40.1%) were women, and 72 (19.5%) and 297 (80.5%) had metachronous and synchronous metastases, respectively. Among those with metachronous metastases, the median time

interval was 15.6 months (range, 4.0–96.7 months). A total of 112 (30.4%), 147 (39.8%), and 110 patients (29.8%) were diagnosed with right-sided colon cancer, left-sided colon cancer, and rectal cancer, respectively. Patient and tumor characteristics are shown in Table 1.

Patients With dMMR Primary Tumors

A total of 48 patients had dMMR primary tumors, in whom PCR analyses were performed to test for MSI; 2 patients had MSI-low tumors, and their corresponding metastatic tumors were also MSI-low. The remaining 46 patients had MSI-high primary tumors. Among these, 37 (80.4%) had MSI-high and 9 (19.6%) had microsatellite stable (MSS) metastatic tumors. The site of metastasis was the perineum in 20 patients (43.5%), the liver in 10 (21.7%), distant lymph nodes in 9 (19.6%), the ovaries in 4 (8.7%), and the lungs in 3 (6.5%). No discrepancy regarding MSI status was observed in patients with liver, lung, or distant lymph node metastasis. Surprisingly, 5 of 20 patients with peritoneal metastasis and all 4 patients with ovarian metastasis had an MSS metastatic tumor (Figure 2A, B). MSI status was more likely to be different between primary and metastatic sites in patients with MSI-high primary tumors who developed peritoneal (5/20) or ovarian (4/4) metastasis rather than other types of metastasis ($P < .001$; Table 2). Detailed information for these 9 patients is shown in Table 3. No significant association between the MSI-high discrepancy rate and patient age ($P = .135$), sex ($P = .719$), primary tumor location ($P = .580$), and synchronous versus metachronous metastasis ($P = 1.00$) was noted (Table 4).

We also analyzed the TMB in both primary and metastatic tumors, because it has been reported to be associated with MMR status.²⁵ Only 5 patients had acceptable DNA for analysis. Of these patients, 3 had MSI-high primary and MSS metastatic tumors (Patients 2, 5, and 8 in Table 3), and we observed a corresponding decrease in TMB from primary to metastatic tumors (Figure 3A). Two patients had MSI-high primary and metastatic tumors, and TMBs were comparable between their primary and metastatic tumors (Figure 3B). We were unable to perform a statistical analysis because of the limited number of patients.

We then studied whether lymphocyte infiltration reflected a corresponding change in MSI status. For the 9 patients with MSI-high primary and MSS metastatic tumors, the hematoxylin-eosin (HE)-stained tissue sections of 5 patients were reviewed (Patients 1, 2, 3, 6, and 9 in Table 3). These patients had high-grade lymphocyte infiltration in a primary tumor and low-grade lymphocyte infiltration in a metastatic tumor ($P = .008$; Figure 4). For the 37 patients with MSI-high primary and metastatic tumors, 32 had available HE-stained tissue sections;

Table 1. Patient and Tumor Characteristics

Characteristic	All	dMMR	pMMR	P Value
Total, n	369	48	321	
Median age (IQR), y	59 (43–66)	60 (42–68)	55 (44–66)	
Sex, n (%)				.007
Male	221 (59.9)	20 (41.7)	201 (62.6)	
Female	148 (40.1)	28 (58.3)	120 (37.4)	
Primary tumor location, n (%)				.001
Ascending	87 (23.6)	20 (41.7)	67 (20.9)	
Transverse	25 (6.8)	15 (31.3)	10 (3.1)	
Descending	29 (7.9)	7 (14.6)	22 (6.9)	
Sigmoid	118 (32.0)	3 (6.2)	115 (35.8)	
Rectum	110 (29.8)	3 (6.2)	107 (33.3)	
Metastatic site, n (%)				.001
Liver	139 (37.7)	11 (22.9)	128 (39.9)	
Lung	15 (4.1)	3 (6.3)	12 (3.7)	
Peritoneum	97 (26.3)	21 (43.8)	76 (23.7)	
Ovarian	42 (11.4)	4 (8.2)	38 (11.8)	
Distant lymph node	21 (5.6)	9 (18.8)	12 (3.7)	
Others	55 (14.9)	0 (0)	55 (17.2)	
Metastasis, n (%)				.329
Synchronous	297 (80.5)	36 (75)	261 (81.3)	
Metachronous	72 (19.5)	12 (25)	60 (18.7)	
Differentiation, n (%)				.876
Well or moderate	213 (57.7)	27 (56.3)	186 (57.9)	
Poor	156 (42.3)	21 (43.7)	135 (42.1)	
First-line therapy, n (%)				.542
Oxaliplatin-based	277 (75.1)	39 (81.3)	238 (74.1)	
Irinotecan-based	67 (18.2)	7 (14.6)	60 (18.7)	
Fluorouracil	25 (6.8)	2 (4.2)	23 (7.2)	

Abbreviations: dMMR: deficient mismatch repair; IQR, interquartile range; pMMR, proficient mismatch repair.

high-grade lymphocyte infiltration was identified in a primary tumor in 27 patients, and in a metastatic tumor in 20 patients. The lymphocyte infiltrations were similar between primary and metastatic tumors in these patients ($P=.088$; Figure 5).

For patients with MSI-high primary tumors, those with MSI-high and MSS metastatic tumors had comparable OS (median, 21.3 vs 21.6 months; $P=.774$; Figure 6).

Among all 46 patients with MSI-high primary tumors, 13 (28.3%) had germline MMR gene mutations, including 10 with *MLH1* mutations, 2 with *MSH2* mutations, and only 1 with an *MSH6* mutation. In these 13 patients, both primary and metastatic tumors were MSI-high. Only 2 patients had a *BRAF* V600E mutation, and both had MSI-high primary and metastatic tumors.

Of the 46 patients with MSI-high primary tumors, 30 had DNA that qualified for *MLH1* promoter methylation analysis. *MLH1* promoter methylation was observed in 15 patients (50%); 14 had MSI-high primary and metastatic tumors and 1 had an MSI-high primary tumor and MSS ovarian metastasis. We were unable to perform a statistical analysis and draw a final conclusion because of the limited number of patients.

Patients With pMMR Primary Tumors

Overall, 321 patients had proficient MMR (pMMR) primary tumors; of them, 316 (98.4%) had pMMR metastatic tumors and 5 (1.6%) had dMMR metastatic tumors. Among the 5 patients with dMMR metastatic tumors, 3 had MSI-low and 2 had MSI-high metastatic tumors (Figure 2A). The primary tumors were confirmed to be MSS in the 2 patients

Figure 2. Circos diagrams of (A) MMR status between primary tumors and metastatic tumors and (B) MSI status of metastatic tumors in patients with MSI-H primary tumors.

Abbreviations: dMMR, deficient mismatch repair; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable; MT, metastatic tumor; pMMR, proficient mismatch repair; PT, primary tumor.

with MSI-high metastatic tumors. Both of these patients were women and had sigmoid colon cancer; one had synchronous liver metastasis and the other had synchronous peritoneum metastasis.

Patients With 2 Sites of Metastases

There were 49 patients with matched samples available from both the primary tumor and metastatic tumors from 2 different organ sites. Of these patients, 47 had both MSS primary and metastatic tumor. The remaining 2 patients had an MSI-high primary tumor; one had lung and liver metastases, with both tumors MSI-high, and the other had peritoneal and liver metastases, with the peritoneal metastasis MSS and the liver metastasis MSI-high.

Pooled Analysis of Published Literature

Given the relatively small number of patients with MSI-high CRC, we searched the literature and performed a pooled analysis to determine the correlation between primary and metastatic CRC with respect to MMR status. A total of 7 studies were included in the final analysis (supplemental eFigure 1)^{19–21,26–29}; 4 studies used immunohistochemistry to detect MMR status (supplemental eTable 1) and 3 used PCR to detect MSI (supplemental eTable 2). Overall, 354 patients had a pMMR/MSS primary tumor; among them, 343 also had pMMR/MSS metastatic cancers, with a concordance rate of 96.9%. Of the 59 patients with a dMMR/MSI-high primary CRC,

52 had a dMMR/MSI-high metastatic tumor (concordance rate, 88.1%). Among patients with a dMMR/MSI-high primary tumor, no difference in MMR/MSI status between the primary and metastatic tumors was observed in those with liver metastasis.

To further study whether the dMMR/MSI-high discrepancy rate was associated with the metastatic site, we performed a pooled analysis that included data from both the searched literature and our study. A total of 94 patients were analyzed, including 34 with peritoneum metastasis, 27 with liver metastasis, 23 with distant lymph node metastasis, 6 with lung metastasis, and 4 with ovarian metastasis. The MMR/MSI discordance was significantly more frequent between primary and metastatic sites in patients with dMMR/MSI-high primary tumors who developed peritoneal metastasis (7/34) or ovarian metastasis (4/4) compared with metastases at other sites (5/56; $P < .001$) (Table 5).

Table 2. MSI Status of Metastatic Tumors in Patients With MSI-High Primary Tumors

Metastatic Site	Total, n	MSI-High, n	MSS, n	P Value
Peritoneum	20	15	5	<.001
Liver	10	10	0	
Distant lymph node	9	9	0	
Ovary	4	0	4	
Lung	3	3	0	

Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable.

Table 3. Characteristics of Patients With MSI-High Primary and MSS Metastatic Tumors (N=9)

Patient	Age, y	Sex	Primary Site	Metastatic Site	Primary Tumor				Metastatic Tumor			
					MLH1	MSH2	MSH6	PMS2	MLH1	MSH2	MSH6	PMS2
1	46	Female	Descending colon	Ovarian	–	+	+	–	+	+	+	+
2	62	Female	Ascending colon	Ovarian	–	+	+	–	+	+	+	+
3 ^a	43	Male	Transverse colon	Peritoneum	–	+	+	–	+	+	+	+
				Liver	–	+	+	–	–	+	+	–
4	52	Female	Sigmoid colon	Ovarian	–	+	+	–	–			+
5	35	Male	Rectal	Peritoneum	+	–	–	+	+	+	+	+
6	37	Male	Descending colon	Peritoneum	+	+	+	–	–	+	+	–
7	43	Male	Transverse colon	Peritoneum	+	+	+	–	+	+	+	+
8	43	Female	Transverse colon	Ovarian	+	–	–	+	+	+	+	+
9	64	Male	Ascending colon	Peritoneum	+	–	–	+	+	+	+	+

Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable.

^aThis patient had peritoneum and liver metastases; the peritoneum metastasis was MSS, whereas the liver metastasis was MSI-high.

Discussion

This study investigated the concordance in MSI/MMR status between primary and corresponding metastatic CRC. Although the MSI/MMR status was highly concordant, we also observed discrepancies, particularly between MSI-high primary CRC and MSS metastatic lesions, and reduced lymphocyte infiltration. Pooled analysis confirmed that the discrepancy was organ-specific and was stronger in peritoneal and ovarian metastases. To our knowledge, this is the largest study focusing on discordance in MSI/MMR status between primary and matched metastatic CRC tumors.

The exact reasons for the discrepancy in MSI status are unclear. We hypothesized that the primary tumor may be composed of distinct subclones. Bian et al³⁰ found that the primary CRC tumor showed more complex subclonal structures than the metastatic tumor, suggesting that metastasis might be a subclone derived from the primary tumor. Xie et al⁵ compared 2 paired primary CRC and metastatic lesions and found 2 different metastatic patterns. These findings suggest the possibility that only MSS tumor cells develop metastasis, and this is supported by the heterogeneity of MMR defects in primary CRC.^{31–33} A second hypothesis is that the mechanisms underlying MSI may affect tumor concordance. Many of the tumors in this study were Lynch-like; of the 46 evaluated, 13 had germline MMR gene alterations, 2 had *BRAF* V600E mutation, and 28 were without *MLH1* methylation and germline mutation. MSI-high tumors caused by a germline mutation of MMR are unlikely to be inconsistent, because these mutations occur in a very early stage of carcinogenesis. In contrast, MSI-high tumors caused by *MLH1* promoter methylation may be inconsistent. The level

of methylation is targetable by drugs,³⁴ and discrepancies in methylation between primary and metastatic tumors have been reported.³⁵ Consistent with this assumption, we found no discrepancies in MSI-high tumors caused by germline mutations of MMR genes, whereas one patient with a *MLH1* promoter

Table 4. Association Between MSI-High Discrepancy Rate and Characteristics of Patients With MSI-High Primary CRC

Characteristics	Patients, n		P Value
	MSI-High Metastatic Tumor	MSS Metastatic Tumor	
Age, y			.135
>60	2	21	
≤60	7	16	
Sex			.719
Male	5	15	
Female	4	22	
Primary tumor site			.580
Right-sided	5	30	
Left-sided	3	5	
Rectal	1	2	
Metastasis			1.00
Synchronous	7	28	
Metachronous	2	9	
MMR protein expression			.678
MLH1/PMS2 loss	6	28	
MSH2/MSH6 loss	3	9	

Abbreviations: CRC, colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable.

Figure 3. Comparison of tumor mutational burden between primary and matched metastatic tumors in patients with (A) MSI-high primary tumors and MSS metastatic tumors and (B) MSI-high primary tumors and MSI-high metastatic tumors.

Abbreviations: Mb, megabase; MSI, microsatellite instability; MSS, microsatellite stable; Muts, mutations.

methylation and MSI-high primary tumor developed MSS metastatic tumor.

Another notable observation is that only patients with peritoneal or ovarian metastases had MSI-high primary tumors with corresponding MSS metastatic tumors. Peritoneal and ovarian metastases share several other common features, including a lower incidence³⁶ and worse prognosis.^{36,37} Specifically, 2 patients in this study with MSI-high primary tumors

had metastasis to 2 sites, and only the peritoneal metastasis showed MSS, whereas other metastases remained MSI-high. Organ-specific susceptibility of metastasis is common in CRC and is significantly associated with primary tumor location and *BRAF*, *KRAS*, and MSI status.³⁸

Peritoneal metastasis is associated with increased interferon- γ levels and is surrounded by natural killer (NK) cells.³⁷ We previously found a correlation between

Figure 4. Comparison of lymphocyte infiltration between primary and matched metastatic tumors in patients with MSI-high primary tumors and MSS metastatic tumors (hematoxylin-eosin, original magnification $\times 100$). These patient numbers correspond to those in Table 3.

Abbreviations: MSI, microsatellite instability; MSS, microsatellite stable.

Figure 5. Comparison of lymphocyte infiltration between primary and matched metastatic tumors in 3 representative patients with MSI-high primary tumors and MSI-high metastatic tumors (hematoxylin-eosin, original magnification $\times 100$). Abbreviation: MSI, microsatellite instability.

MSI-high CRC and loss of major histocompatibility complex class I (MHC-I) expression,³⁹ which is an activation signal for NK cells. Because MSI heterogeneity exists in primary CRC^{31–33} and MSI-high tumor cells are more susceptible to NK cells because of loss of MHC-I,⁴⁰ it might be more difficult for MSI-high tumor cells to survive peritoneal metastasis in the same patient. In a cohort of 296 female patients with CRC, none with ovarian metastases had an MSI-high tumor, whereas 6 of 96 (6.3%) with extraovarian metastases and 15 of 181 (8.3%) without any recurrence or metastasis had a MSI-high phenotype.³⁶

Our results are valuable in that the discrepancy in MSI/MMR status may be among the mechanisms of resistance to anti-PD-1 therapy. Although peritoneal metastasis is associated with early treatment discontinuation in patients with ovarian cancer receiving anti-PD-1 treatment,⁴¹ it remains unknown whether this correlation exists in patients with CRC. Theoretically, once the MSI status was inconsistent, the immune microenvironment including tumor-infiltrating lymphocytes changed concordantly, as found in our study.

This study has several limitations. First, it was a retrospective study conducted at a single institution. Second, MSI was tested by analyzing 5 microsatellite markers recommended by NCI, and more advanced modalities may have yielded more accurate results. Next-generation sequencing-based tests can scan thousands of loci, allowing for a more thorough assessment.⁴² Third, although we found a stronger discrepancy in MSI-high status in peritoneal and ovarian metastases, only a small number of patients were evaluated.

Conclusions

Although we found a high concordance rate for MSI/MMR status between primary CRCs and their matched metastatic lesions, some discrepancies were noted, particularly a change from MSI-high in primary CRC to MSS in corresponding peritoneal and ovarian metastases. Rebiopsy to evaluate MSI-high/dMMR status and attentive evaluation of treatment response in metastatic lesions at these 2 sites might be needed during the course of anti-PD-1 therapy. Further studies that include a larger population are needed to validate these findings.

Figure 6. Comparison of overall survival in patients with MSI-H primary tumors with MSS versus MSI-H metastatic tumors. Abbreviations: MSI-H, microsatellite instability-high; MSS, microsatellite stable.

Table 5. Concordance Rates of dMMR or MSI-High Status Between Primary Tumor and Corresponding Metastatic Tumors

Studies	Concordance Rate (%)				
	Liver	Lung	Peritoneum	Distant Lymph Node	Ovary
Ishimaru et al ^{26,a}	—	—	—	—	—
Melloni et al ^{28,a}	—	—	—	—	—
Larsen et al ²⁷	1/1 (100%)	—	—	—	—
Murata et al ²⁵	3/3 (100%)	—	—	—	—
Fujiyoshi et al ²⁰	2/2 (100%)	—	7/9 (77.8%)	12/13 (92.3%)	—
Haraldsdottir et al ¹⁹	6/6 (100%)	1/1 (100%)	5/5 (100%)	1/1 (100%)	—
Jung et al ²¹	3/5 (60%)	0/2 (0%)	—	—	—
Present study	10/10 (100%)	3/3 (100%)	15/20 (75%)	9/9 (100%)	0/4 (0%)

Abbreviations: dMMR, deficient mismatch repair; MSI, microsatellite instability.

^aPatients all had proficient mismatch repair or microsatellite stable primary tumor.

Acknowledgments

The authors have engaged the services of Editage, which provides English editing services, to improve language in this manuscript.

Submitted September 25, 2018; accepted for publication April 5, 2019.

Author contributions: Study concept and design: He, Hu, Shao, L.P. Xia. Data acquisition, analysis, or interpretation: He, Hu, Wang, Jiang, Lu, Zhang, Ding, Shao, L.P. Xia. Statistical analysis: He, Hu, Wang, L.P. Xia. Acquisition of funding: X.J. Xia, Shao, L.P. Xia. Administrative, technical, or material support: Rong, L. Yang, Xie, Y.Z. Yang, Qiu, Lu. Supervision: X.J. Xia, Shao, L.P. Xia. Drafting of manuscript: He, X.J. Xia, L.P. Xia. Critical revisions: all authors.

Disclosures: The authors have disclosed that they have not received any financial considerations from any person or organization to support the preparation, analysis, results, or discussion of this article. The data in the study were deposited in the Research Data Deposit system of Sun Yat-sen University Cancer Center [RDDA2019001007].

Funding: This work was supported by grants from the Natural Science Foundation of Guangdong, China (2015A030313010), Science and Technology Program of Guangzhou, China (1563000305), the Guangdong Innovative and Entrepreneurial Research Team Program (2016ZT06S638), the Science and Technology project of Guangdong Province (2017A020215031), and National Natural Science Foundation of China (81773051, 81272641, and 81572409).

Correspondence: Liang-Ping Xia, MD, and Jian-Yong Shao, MD, Sun Yat-sen University Cancer Center, 651 Dongfeng East Road, Guangzhou, Guangdong 510060, P. R. China. Email: xialiangping@163.com; shaojy@sysucc.org.cn

References

- Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017;67:177–193.
- Hugen N, van de Velde CJ, de Wilt JH, et al. Metastatic pattern in colorectal cancer is strongly influenced by histological subtype. *Ann Oncol* 2014;25:651–657.
- Vermaat JS, Nijman IJ, Koudijs MJ, et al. Primary colorectal cancers and their subsequent hepatic metastases are genetically different: implications for selection of patients for targeted treatment. *Clin Cancer Res* 2012;18:688–699.
- Lee SY, Haq F, Kim D, et al. Comparative genomic analysis of primary and synchronous metastatic colorectal cancers. *PLoS One* 2014;9:e90459.
- Xie T, Cho YB, Wang K, et al. Patterns of somatic alterations between matched primary and metastatic colorectal tumors characterized by whole-genome sequencing. *Genomics* 2014;104:234–241.
- Brannon AR, Vakiani E, Sylvester BE, et al. Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. *Genome Biol* 2014;15:454.
- van der Waals LM, Jongen MJM, Elias SG, et al. Increased levels of oxidative damage in liver metastases compared with corresponding primary colorectal tumors: association with molecular subtype and prior treatment. *Am J Pathol* 2018;188:2369–2377.
- Hu D, Du C, Xue W, et al. The expression of chemokine receptors CCR6, CXCR2 and CXCR4 is not organ-specific for distant metastasis in colorectal cancer: a comparative study. *Histopathology* 2013;63:167–173.
- Schweiger T, Berghoff AS, Glogner C, et al. Tumor-infiltrating lymphocyte subsets and tertiary lymphoid structures in pulmonary metastases from colorectal cancer. *Clin Exp Metastasis* 2016;33:727–739.
- Wang HB, Yao H, Li CS, et al. Rise of PD-L1 expression during metastasis of colorectal cancer: Implications for immunotherapy. *J Dig Dis* 2017;18:574–581.
- Kim MJ, Lee HS, Kim JH, et al. Different metastatic pattern according to the KRAS mutational status and site-specific discordance of KRAS status in patients with colorectal cancer. *BMC Cancer* 2012;12:347.
- Germano G, Lamba S, Rospo G, et al. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. *Nature* 2017;552:116–120.
- Yim KL. Microsatellite instability in metastatic colorectal cancer: a review of pathology, response to chemotherapy and clinical outcome. *Med Oncol* 2012;29:1796–1801.
- Hamada T, Soong TR, Masugi Y, et al. TIME (Tumor Immunity in the MicroEnvironment) classification based on tumor CD274 (PD-L1) expression status and tumor-infiltrating lymphocytes in colorectal carcinomas. *Oncol Immunology* 2018;7:e1442999.
- Liu S, Kong P, Wang X, et al. Tumor microenvironment classification based on T-cell infiltration and PD-L1 in patients with mismatch repair-proficient and -deficient colorectal cancer. *Oncol Lett* 2019;17:2335–2343.
- Venderbosch S, Nagtegaal ID, Maughan TS, et al. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: a pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. *Clin Cancer Res* 2014;20:5322–5330.
- Dudley JC, Lin MT, Le DT, et al. Microsatellite instability as a biomarker for PD-1 blockade. *Clin Cancer Res* 2016;22:813–820.
- Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 2015;372:2509–2520.
- Haraldsdottir S, Roth R, Pearlman R, et al. Mismatch repair deficiency concordance between primary colorectal cancer and corresponding metastasis. *Fam Cancer* 2016;15:253–260.
- Fujiyoshi K, Yamamoto G, Takahashi A, et al. High concordance rate of KRAS/BRAF mutations and MSI-H between primary colorectal cancer and corresponding metastases. *Oncol Rep* 2017;37:785–792.

21. Jung J, Kang Y, Lee YJ, et al. Comparison of the mismatch repair system between primary and metastatic colorectal cancers using immunohistochemistry. *J Pathol Transl Med* 2017;51:129–136.
22. Klintrup K, Mäkinen JM, Kauppila S, et al. Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 2005;41:2645–2654.
23. Park JH, Powell AG, Roxburgh CS, et al. Mismatch repair status in patients with primary operable colorectal cancer: associations with the local and systemic tumour environment. *Br J Cancer* 2016;114:562–570.
24. GeneFuse: detection and visualization of target gene fusions from DNA sequencing data. *Int J Biol Sci* 2018;14:843–848.
25. Zhuang W, Ma J, Chen X, et al. The tumor mutational burden of Chinese advanced cancer patients estimated by a 381-cancer-gene panel. *J Cancer* 2018;9:2302–2307.
26. Murata A, Baba Y, Watanabe M, et al. Methylation levels of LINE-1 in primary lesion and matched metastatic lesions of colorectal cancer. *Br J Cancer* 2013;109:408–415.
27. Ishimaru G, Adachi J, Shiseki M, et al. Microsatellite instability in primary and metastatic colorectal cancers. *Int J Cancer* 1995;64:153–157.
28. Larsen NB, Heiberg Engel PJ, Rasmussen M, Rasmussen LJ. Differential expression of hMLH1 in sporadic human colorectal cancer tumors and distant metastases. *APMIS* 2009;117:839–848.
29. Melloni G, Doglioni C, Bandiera A, et al. Prognostic factors and analysis of microsatellite instability in resected pulmonary metastases from colorectal carcinoma. *Ann Thorac Surg* 2006;81:2008–2013.
30. Bian S, Hou Y, Zhou X, et al. Single-cell multiomics sequencing and analyses of human colorectal cancer. *Science* 2018;362:1060–1063.
31. Tachon G, Frouin E, Karayan-Tapon L, et al. Heterogeneity of mismatch repair defect in colorectal cancer and its implications in clinical practice. *Eur J Cancer* 2018;95:112–116.
32. Chapusot C, Martin L, Bouvier AM, et al. Microsatellite instability and intratumoural heterogeneity in 100 right-sided sporadic colon carcinomas. *Br J Cancer* 2002;87:400–404.
33. Hühns M, Krohn S, Murua Escobar H, et al. Genomic heterogeneity in primary colorectal carcinomas and their metastases: born bad or brought up a villain? *Hum Pathol* 2018;74:54–63.
34. Yu G, Wu Y, Wang W, et al. Low-dose decitabine enhances the effect of PD-1 blockade in colorectal cancer with microsatellite stability by re-modulating the tumor microenvironment. *Cell Mol Immunol* 2018;16:401–409.
35. Moarii M, Pinheiro A, Sigal-Zafrani B, et al. Epigenomic alterations in breast carcinoma from primary tumor to locoregional recurrences. *PLoS One* 2014;9:e103986.
36. Mori Y, Nyuya A, Yasui K, et al. Clinical outcomes of women with ovarian metastases of colorectal cancer treated with oophorectomy with respect to their somatic mutation profiles. *Oncotarget* 2018;9:16477–16488.
37. Seebauer CT, Brunner S, Glockzin G, et al. Peritoneal carcinomatosis of colorectal cancer is characterized by structural and functional reorganization of the tumor microenvironment inducing senescence and proliferation arrest in cancer cells. *OncoImmunology* 2016;5:e1242543.
38. Prasanna T, Karapetis CS, Roder D, et al. The survival outcome of patients with metastatic colorectal cancer based on the site of metastases and the impact of molecular markers and site of primary cancer on metastatic pattern. *Acta Oncol* 2018;57:1438–1444.
39. Liu SS, Yang YZ, Jiang C, et al. Comparison of immunological characteristics between paired mismatch repair-proficient and -deficient colorectal cancer patients. *J Transl Med* 2018;16:195.
40. Konjević G, Vuletić A, Mirjačić Martinović K. Natural killer cell receptors: alterations and therapeutic targeting in malignancies. *Immunol Res* 2016;64:25–35.
41. Boland JL, Zhou Q, Martin M, et al. Early disease progression and treatment discontinuation in patients with advanced ovarian cancer receiving immune checkpoint blockade. *Gynecol Oncol* 2019;152:251–258.
42. Middha S, Zhang L, Nafa K, et al. Reliable pan-cancer microsatellite instability assessment by using targeted next-generation sequencing data. *JCO Precis Oncol* 2017:2017.

See JNCCN.org for supplemental online content.

REGISTER NOW!

NCCN

ANNUAL
CONFERENCE

• Celebrating 25 years of NCCN •

March 20 – 22, 2020 • Rosen Shingle Creek • Orlando, FL

Final call for Abstracts! • Submit by October 23, 2019
NCCN.org/conference

Supplemental online content for:

Comparison of Mismatch Repair Status Between Primary and Matched Metastatic Sites in Patients With Colorectal Cancer

Wen-Zhuo He, MD; Wan-Ming Hu, MD; Fang Wang, MD; Yu-Ming Rong, MD; Lin Yang, MD; Qian-Kun Xie, MD; Yuan-Zhong Yang, MD; Chang Jiang, MD; Hui-Juan Qiu, MD; Jia-Bin Lu, MD; Bei Zhang, MD; Pei-Rong Ding, MD; Xiao-Jun Xia, PhD; Jian-Yong Shao, MD; and Liang-Ping Xia, MD

J Natl Compr Canc Netw 2019;17(10):1174–1183

eFigure 1: Study Selection Flowchart

eTable 1: Mismatch Repair Status Between Primary and Matched Metastatic Tumor Tissues

eTable 2: MSI Status Between Primary and Matched Metastatic Tumor Tissues

eAppendix 1: Supplemental Methods

eFigure 1. Study selection flowchart.

eTable 1. Mismatch Repair Status Between Primary and Matched Metastatic Tumor Tissues

Studies	Year	Pairs	Primary→Matched Metastatic Tumor (Patients, n)			
			pMMR→pMMR	pMMR→dMMR	dMMR→dMMR	dMMR→pMMR
Melloni et al ¹	2006	41	41	0	0	0
Larsen et al ²	2009	90	89	0	1	0
Haraldsdottir et al ³	2016	24	0	0	24	0
Jung et al ⁴	2017	61	44	10	3	4

Abbreviations: dMMR, deficient mismatch repair; pMMR, proficient mismatch repair.

References

1. Melloni G, Doglioni C, Bandiera A, et al. Prognostic factors and analysis of microsatellite instability in resected pulmonary metastases from colorectal carcinoma. *Ann Thorac Surg* 2006;81:2008–2013.
2. Larsen NB, Heiberg Engel PJ, Rasmussen M, et al. Differential expression of hMLH1 in sporadic human colorectal cancer tumors and distant metastases. *APMIS* 2009;117:839–848.
3. Haraldsdottir S, Roth R, Pearlman R, et al. Mismatch repair deficiency concordance between primary colorectal cancer and corresponding metastasis. *Fam Cancer* 2016;15:253–260.
4. Jung J, Kang Y, Lee YJ, et al. Comparison of the mismatch repair system between primary and metastatic colorectal cancers using immunohistochemistry. *J Pathol Transl Med* 2017;51:129–136.

eTable 2. MSI Status Between Primary and Matched Metastatic Tumor Tissues

Studies	Year	Pairs	Primary→Matched Metastatic Tumor (Patients, n)			
			MSS→MSS	MSS→MSI-H	MSI-H→MSI-H	MSI-H→MSS/MSI-L
Ishimaru et al ¹	1995	14	10	0	—	0
Murata et al ²	2013	26	22	1	3	0
Fujiyoshi et al ³	2016	161	137	0	21	3

Abbreviations: MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable.

References

1. Ishimaru G, Adachi J, Shiseki M, et al. Microsatellite instability in primary and metastatic colorectal cancers. *Int J Cancer* 1995;64:153–157.
2. Murata A, Baba Y, Watanabe M, et al. Methylation levels of LINE-1 in primary lesion and matched metastatic lesions of colorectal cancer. *Br J Cancer* 2013;109:408–415.
3. Fujiyoshi K, Yamamoto G, Takahashi A, et al. High concordance rate of KRAS/BRAF mutations and MSI-H between primary colorectal cancer and corresponding metastases. *Oncol Rep* 2017;37:785–792.

eAppendix 1. Supplemental Methods

Assessment of Mismatch Repair Status

Immunohistochemistry analysis of the 4 most-common mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, and PMS2) was performed with the standard Envision 2-step procedure. Slides were dried overnight at 37°C, dewaxed in xylene, rehydrated with graded alcohol, and immersed in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. These proteins were then pretreated in antigen-retrieval buffer (EDTA buffer, pH 8.0, 100°C, 2 minutes in a pressure cooker) and incubated with 10% normal goat serum at room temperature for 10 minutes to reduce nonspecific binding. Subsequently, the slides were incubated overnight at 4°C using the following antibodies: anti-MLH1, anti-MSH2, anti-MSH6, and anti-PMS2 (1:50; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd). After rinsing 5 times with 0.01 mol/L of phosphate-buffered saline (PBS; pH=7.4) for 10 minutes, detection of the primary antibody was performed using a secondary antibody (Envision; Dako) for 1 hour at room temperature and staining with 3,3-diaminobenzidine after washing in PBS again. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. PBS was used instead of the primary antibody in the negative control. Nonneoplastic colonic mucosa, stromal cells, and infiltrating lymphocytes or the centers of lymphoid follicles were used as internal positive controls. Known MMR-deficient (dMMR) colon carcinomas served as external negative controls. Two experienced pathologists evaluated the staining results independently and without any prior knowledge of the patients' clinical data. 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. Tumors with a loss of the MLH1, MSH2, MSH6, and PMS2 proteins, as visualized using light microscopy, were classified as MLH1/MSH2/MSH6/PMS2-negative. If internal nonneoplastic tissues showed invalid negative staining, the procedure was repeated. When the opinions of the 2 pathologists were different, agreement was reached through careful discussion.

Assessment of Lymphocyte Infiltration

Lymphocyte infiltration was evaluated through histopathologic analysis of hematoxylin-eosin (HE)-stained tissue sections by 2 pathologists who were blinded to the clinical data. We evaluated all HE sections for each patient. In general, lymphocyte infiltration in different sections of a tumor was similar. If there were differences between sections, the most-representative and invasive part of the primary tumor was chosen. We used a 4-degree scale to assess lymphocyte infiltration. A score of 0 was given when there was no increase of lymphocytic cells, whereas a score of 1 denoted mild and patchy increase of lymphocytic cells at the invasive margin, but no destruction of invading cancer cell islets by the lymphocytic cells. A score of 2 was given when lymphocytic cells formed a band-like infiltrate at the invasive margin with some destruction of cancer cell islets by lymphocytic cells. A score of 3 denoted a very prominent lymphocytic reaction, forming a cup-like zone at the invasive margin, and destruction of cancer cell islets was frequent and invariably present. Absent and weak increases in lymphocytes (0–1) were considered low-grade infiltration, whereas moderate to severe increases (2–3) were considered high-grade.

DNA Extraction and MSI Analysis

PCR analyses were performed to test for MSI when tumors were classified as dMMR. For each primary and metastatic tumor, formalin-fixed, paraffin-embedded (FFPE) tissue blocks were reviewed for quality control, and regions rich in tumor cells (>50% of cancer cells available) were selected for macrodissection. Genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN). Microsatellite instability (MSI) analysis was performed using 5 microsatellite markers (BAT-25, BAT-26, D2S123, D5S346, and D17S250) recommended by NCI. DNA was amplified using an Applied Biosystems 2720 Thermal Cycler. Separation and detection of amplified fragments was performed on an Applied Biosystems 3500XL Genetic Analyzer, and data were analyzed with GeneScan Analysis Software (Applied Biosystems). Tumors were classified as MSI-high (≥ 2 markers), MSI-low (1 marker), or MSS (none), according to the number of instability markers. Allelic patterns or genotypes for normal and tumor pairs were compared and scored as MSI-positive if ≥ 1 different alleles were present in the tumor DNA samples that were not found in normal samples from the same individual.

Germline MMR Mutational Analysis

The genetic susceptibility panel targeting all exons of 53 genes covering *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*, and proximal intronic flanking sequences (± 10 base pairs [bp]) were analyzed for screening of single-nucleotide variation,

Microsatellite Marker	Primer Sequences
D2S123 (VIC)	Forward: 5'-AAACAGGATGCCTGCCTTTA-3' Reverse: 5'-GGACTTTCCACCTATGGGAC-3'
D17S250 (FAM)	Forward: 5'-GGAAGAATCAAATAGACAAT-3' Reverse: 5'-GCTGGCCATATATATTTAAACC-3'
D5S346 (FAM)	Forward: 5'-ACTCACTCTAGTGATAAATCGGG-3' Reverse: 5'-AGCAGATAAGACAGTATTACTAGTT-3'
BAT-25 (NED)	Forward: 5'-TCGCCTCCAAGAATGTAAGT-3' Reverse: 5'-TCTGCATTTTAACTATGGCTC-3'
BAT-26 (VIC)	Forward: 5'-TGACTACTTTTGACTTCAGCC-3' Reverse: 5'-AACCATTCAACATTTTAAACCC-3'

including insertion and deletion shorter than 20 bp. FFPE tissue blocks of negative surgical margin confirmed pathologically were collected from 46 patients for germline DNA sequencing and variant classification. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN), according to the manufacturer's instructions. DNA concentration was measured using the Qubit double-stranded DNA assay (Thermo Fisher Scientific). Qualified DNA samples were used for the library construction. The insert size and quantity of the library were assessed using the Agilent 2100 Bioanalyzer system and the library was sequenced using the NextSeq500 Sequencing System (Illumina, Inc) with paired-end reads. After filtering the low-quality reads, clean sequencing data were aligned to reference (hg19; UCSC Genome Browser) using the Burrow-Wheeler Aligner program BWA0.7.10, marked for duplication using Picard v1.138, and realigned with GATK v3.2-2 (Broad Institute). After that, the final mapping file (BAM format) was used to detect the SNP/InDel using GATK software. SNP/InDel mutations with <10% mutational frequency were filtered. Mutations were annotated based on RefSeq GTF file, DbSNP 141, ExAC, 1000 Genomes Project, and PFAM databases, and so on. The pathogenicity of mutations was classified according to American College of Medical Genetics and Genomics recommendations. Missense mutations of uncertain significance were reevaluated in silico prediction results, phenotype, and family history, and also checked against the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) database hosted by Leiden Open Variation Database (LOVD) at <https://www.insight-group.org/variants/databases/> to further assess pathogenicity. We performed deep sequencing with the multigene panel to achieve median 200× coverage of the targeted regions. All samples have >99% of all regions with coverage depth >20×.

BRAF V600E Mutation Analysis

Mutations in *BRAF* exon 15 V600E were identified by quantitative real-time PCR (qPCR) using the minor groove binder (MGB) probes. Primers and probes for the V600E assay were as follows:

- Forward: 5'-ATGAAGACCTCACAGTAAAAATAGG-3'
- Reverse: 5'-AGACAACTGTTCAAAGTATGGG-3'
- Mutation anchor: FAM-TCTAGCTACAGAGAAA-MGB
- Wild anchor: HEX-TCTAGCTACAGTGAAA-MGB

Detection was performed using the Applied Biosystems 7500 Real-Time PCR System.

Sequencing and Tumor Mutational Burden Analysis

The workflow of sample preparation for exome sequencing according to HaploX Biotechnology's laboratory standardization¹ is as follows:

1. Quality control of nucleic acid

- Perform quality control of the DNA provided by the customer or entrusted HaploX Biotechnology extracted DNA.
- Use the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) to determine the concentration of the DNA samples.
- Use the NanoDrop One (Thermo Fisher Scientific) to determine the purity of the DNA samples.
- Use a Bioanalyzer DNA 1000 chip and reagent kit for analysis of the DNA samples using the Agilent 4200 Bioanalyzer.

2. Library preparation workflow

2.1 Shear DNA

- High-throughput library is started using sheared, double-stranded DNA. Use the Covaris instruments (Covaris) or endonuclease to shear the DNA and for recovery of target DNA fragments.

2.2 Library preparation and quality control

- 2.2.1 End repair, which produces blunt-ended, 5'-phosphorylated fragments.
- 2.2.2 A-tailing, during which dAMP is added to the 3'-ends of blunt-ended dsDNA library fragments.
- 2.2.3 Adapter ligation, during which dsDNA adapters with 3'-dTTP overhangs are ligated to 3'-A-tailed library fragments.
- 2.2.4 Size selection; requirements vary widely according to specific applications.
- 2.2.5 Library amplification, which uses PCR to amplify library fragments carrying appropriate adapter sequences on both ends.
 - The size distribution of the precapture library should be confirmed with an electrophoretic method.

2.3 Hybridization and capture

- The prepared DNA libraries are hybridized to a targeted-specific capture library using the Roche NimbleGen SeqCap EZ Exome v3 (Roche).

2.4 Enrich capture library

- Run the PCR amplification program to enrich the captured libraries.

2.5 Quality control of capture library

- Use a Bioanalyzer DNA 1000 chip and reagent kit for analysis of the library using the Agilent 4200 Bioanalyzer (Agilent Technologies Inc).
- Library quantitation is validated using the Quant Studio 5 Real-Time PCR System (Thermo Fisher Scientific).

3. Sequencing

- Pool the captured libraries and perform the pair-end sequencing (PE150) on Illumina HiSeq X technology (Illumina) for sequencing.
- WES pipeline: 1. raw data quality control; 2. low quality filter (use After quality control or fastp); 3. sequence alignment (use BWA); 4. duplication (use picardtools); 5. indelrealign (use GATK); 6. call variants (use GATK); 7. call fusion (use factera); 8. call sv (use contra) ; 9. annotate SNP/indel variants (use Annovar)
- Parameters:
 - After quality control use default parameters;
 - Fastp parameters - t 1 -T 1;
 - bwa mem -k 32;
 - duplicate picardtools use default parameters;
 - Indelrealign: GATK RealignerTargetCreator + IndelRealigner + BaseRecalibrator + PrintReads default parameters;
 - Call Variants: GATK HaplotypeCaller default parameters;
 - Extract SNPs & Indels: GATK SelectVariants default parameters;
 - Filter SNPs and Indels: GATK VariantFiltration (SNP parameters: "QD < 2.0 || FS > 60.0 || MQ < 40.0 || SOR > 4.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0"; INDEL parameters: "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0 || SOR > 10.0");
 - call fusion:factera use default parameters;
 - call cnv:contra parameters: -l-small Segment=3-large Segment=30;
 - Annotate SNP/INDEL: Annovar use default parameters;

Tumor mutational burden was defined as the number of somatic, nonsynonymous, coding, base substitution, and indel mutations per megabase (Mb) of genome examined. We filtered detected variants with tumor coverage depth ≥ 20 , normal coverage depth ≥ 10 , tumor variant allele frequency $\geq 5\%$, tumor variation depth ≥ 3 , normal variation depth = 0. Known germline alterations found in the dbSNP database were not counted. Common single-nucleotide polymorphisms were eliminated through comparison with 1000 Genomes Project data (frequency of SNP $\leq .001$). We used 38 Mb as the estimated exome size.

The Cancer Genome Atlas (TCGA) data were obtained from public repositories (<https://github.com/OpenGene/fastp>). For this analysis, we used the somatic mutations as determined by TCGA to count tumor mutation burden.

References

1. Detection of urothelial bladder carcinoma via microfluidic immunoassay and single-cell DNA copy-number alteration analysis of captured urinary-exfoliated tumor Cells. *Cancer Res* 2018;78:4073–4085.