High concordance rate of *KRAS*/*BRAF* mutations and MSI-H between primary colorectal cancer and corresponding metastases

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Abstract. Genetic testing is needed for the treatment of colorectal cancer (CRC), especially molecular-targeted therapy. The effects of anti-EGFR therapy and prognosis are affected by the presence of *KRAS* mutations. However, whether primary CRC or metastatic tissues are appropriate in the analysis is still unclear. In the present study, we assessed the concordance of *KRAS*/*BRAF* mutation status and microsatellite instability (MSI) in primary CRC and corresponding metastases. This study enrolled 457 patients with surgically resected primary and corresponding metastatic CRC (499 synchronous metastases and 57 metachronous metastases) and seven local recurrences, and *KRAS*/*BRAF* mutation and MSI status were analysed for these tumours. The concordance rates of *KRAS* mutation, *BRAF* mutation, wild-type, MSI-H and MSS between primary CRC and corresponding metastases were 93.9% (214/228), 100% (30/30), 99.3% (304/306), 87.5% (21/24) and 100% (137/137), respectively. These high concordance rates were not different between synchronous and metachronous metastases. In conclusion, a high concordance of *KRAS*/*BRAF* mutation status and MSI status was observed between primary CRC and corresponding metastases in this study. Either primary CRC or metastatic tissues can be used for testing *KRAS*/*BRAF* mutation status and MSI status.

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Abbreviations: CRC, colorectal cancer; MSI, microsatellite instability; MSI-H, MSI-high; MMR, mismatch repair; HRM, high resolution melting; PCR, polymerase chain reaction; MSI-L, MSI-low; MSS, microsatellite stable

Key words: KRAS, *BRAF*, microsatellite instability, colorectal cancer, heterogeneity

Introduction

Colorectal cancer (CRC) is the most common gastrointestinal cancer and one of the leading causes of cancer-related deaths worldwide. Various biomarkers have been identified for chemotherapy in advanced CRC. Particularly, *KRAS*/*BRAF* mutation status and microsatellite instability (MSI) status are known to be effective as predictive biomarkers. One of the important signalling pathways in CRC, activation of the RAS-RAF-MAPK pathway, which consists of *KRAS*/*BRAF*, is known (1). The pathway lies downstream from the epidermal growth factor receptor (EGFR), a transmembrane protein receptor, and contributes to cell proliferation, survival, growth, apoptosis resistance, invasion and migration (2,3). EGFR is overexpressed in most CRCs and antibodies against it inhibit stimulation of several intracellular signalling pathways, such as RAS-RAF-MAPK pathways (4). However, previous studies have shown that *KRAS*-mutant CRC is resistant to EGFR antibodies (5,6). *KRAS* mutation occurs in approximately 40% of CRC cases (6). Therefore, analysis of *KRAS* mutations is important for the selection of anti-EGFR therapy, and it is necessary before treatment in advanced CRC. In addition, CRC with wild-type *KRAS* is not always sensitive to EGFR antibodies and *BRAF*-mutant CRC has a poor prognosis (7). It is suggested that the efficiency of EGFR antibodies is further restricted to CRC, with both *KRAS*/*BRAF* wild-types.

BRAF, a member of the RAF family of serin/threonine kinases, is directly downstream from *KRAS*. *BRAF* mutations lead to constitutive activation of a MAPK pathway. *KRAS*/*BRAF* mutations are considered to be mutually exclusive. *BRAF* mutations are present in approximately 6% advanced CRC cases (5,7-9). Patients with *BRAF*-mutant advanced CRC are more likely to be older, of the female gender, have right-sided primary tumours and show an unusual pattern of metastatic spread, including frequent peritoneal and distant lymph node involvement. *BRAF*-mutant advanced CRC has proven to be a poor prognosis (5,7,9). The *BRAF* inhibitor vemurafenib as well as dabrafenib, have resulted in significantly prolonged progression-free survival and overall survival in patients

with *BRAF*-mutated advanced melanoma (10,11). However, in contrast to *BRAF*-mutant melanoma, *BRAF*-mutant advanced CRC has shown a lack of sensitivity to *BRAF* inhibitor monotherapy in previous clinical trials (12). Nevertheless, FOLFOXIRI + bevacizumab and *BRAF* inhibitor + MEK or EGFR inhibitors, might be a reasonable therapy for *BRAF*-mutant advanced CRC (13-16). *BRAF* is a good biomarker, not only for a poor prognosis but also for the selection of molecular-targeted therapy.

MSI is a genetic change caused by a deficiency in the mismatch repair (MMR) system. The MMR system detects and repairs the mismatches that occur during DNA replication. It has been reported that approximately 15% of CRC cases show MSI in western countries, and approximately 6% of CRC cases in Asian countries (9,17). Recently, advanced CRC with MSI-H have been shown to have a high response rate to programmed death-1 (PD-1) inhibitor therapy, namely an immune checkpoint inhibitor (18). MSI status may be a helpful biomarker for immune therapy.

Based on the above, evaluating *KRAS/BRAF* mutation status and MSI status may be important to choose the regimen and predict the prognosis for advanced CRC. However, acquiring the various mutations during the CRC progression causes cancer-cell heterogeneity. The prevalence of intratumoural genetic heterogeneity was investigated in the cases of resistance to cancer therapy in previous studies, and the resistance to therapy may be explained by the presence of intratumoural heterogeneity (19). Evaluation of whether *KRAS/BRAF* mutation status and MSI status could change during the progression of metastatic disease might be useful to decide appropriate treatment for advanced CRC. *KRAS* mutation is recognized as an early event in colorectal carcinogenesis (20,21). Therefore, concordance of *KRAS* mutation status between primary CRC and corresponding metastases should be expected, and previous studies demonstrated high concordance rate (22-24). Nevertheless, some other studies reported discordance of *KRAS* mutation status between primary CRC and corresponding metastases. Therefore, there is still conflict about its concordance. Besides, concordance of *BRAF* mutation status and MSI status between primary CRC and corresponding metastases, is still unclear because of the small number of advanced CRC cases with *BRAF* mutation or MSI-H. In the present study, we assessed the concordance of *KRAS/BRAF* mutation status and MSI status in primary CRC and corresponding metastases.

Materials and methods

Patients and tissue samples. A total of 457 patients with surgically resected CRC at the Saitama Cancer Center, from July 1999 to August 2013, were enrolled in this study. Four hundred and fifty-seven primary CRCs, 557 corresponding metastases (499 synchronous metastases and 57 metachronous metastases) and seven local recurrences were analysed. Primary CRCs and corresponding metastatic tissues were paired with normal colorectal tissues and stored at -80˚C. Patients who had a history of preoperative radiotherapy or chemotherapy, inflammatory bowel disease, or a history of familial adenomatous polyposis were excluded. The cases with three or less metastatic lymph nodes were also excluded.

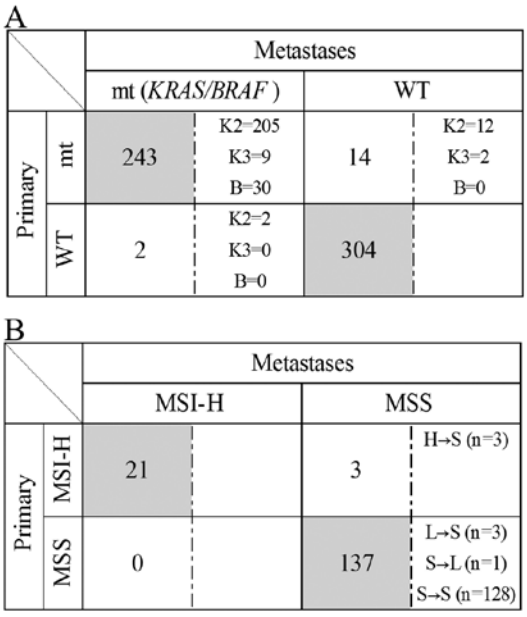

Figure 1. Concordance of *KRAS/BRAF* mutation status and MSI status between primary CRC and corresponding metastases. The grey column shows the concordance between primary CRC and corresponding metastases. The white column is the discordance between primary CRC and corresponding metastases. Local recurrences were included in the metastases. (A) *KRAS/BRAF* mutation status are shown in primary CRC and corresponding metastases. Only one individual had both a *KRAS* exon 3 mutation and a *BRAF* mutation. (B) MSI status is shown in primary CRC and corresponding metastases. K2, *KRAS* exon 2; K3, *KRAS* exon 3; B, *BRAF* V600E; H, MSI-H; L, MSI-L; S, MSS.

Table I. Concordance rate of *KRAS/BRAF* mutation status and MSI status between primary CRCs and corresponding metastases.

	Concordance rate (%)		
Status	Total	Synchronous	Metachronous
KRAS/BRAF	94.6 (243/257)	95.1 (215/226)	90.3 (28/31)
KRAS	93.9 (214/228)	94.6 (194/205)	87.0 (20/23)
BRAF	100 (30/30)	100 (22/22)	100 (8/8)
WT	99.3 (304/306)	99.6 (272/273)	97.0 (32/33)
MSI-H	87.5 (21/24)	94.1 (16/17)	71.4 (5/7)
MSS	100 (137/137)	100 (113/113)	100 (24/24)

Concordance rate, number of concordance cases/number of primary CRCs with each status. WT, the cases without both *KRAS* mutation and *BRAF* mutation. One individual had both *KRAS* mutation and *BRAF* mutation.

Since our preliminary study demonstrated that discordant rate of *KRAS* mutation between primary CRC and macroscopically suspected metastatic lymph node increased in the cases with three or less metastatic lymph nodes comparing to the cases with more.

Informed consent was obtained from all the patients included in this study. Furthermore, the ethics committee of the Saitama Cancer Center approved this study.

			Metastases							
			Synchronous n=499				Metachronous n=64			
			mt (*KRAS/BRAF*)		WT		mt (*KRAS* or *BRAF*)		WT	
Primary	Node	mt	129	K2=110 K3=4 B=16	10[a]	K2=10	5	K2=4 B=1	0	
		WT	0		192		0		7	
	Liver	mt	61	K2=54 K3=4 B=3	1[b]	K2=1	8	K2=6 K3=1 B=1	1[d]	K2=1
		WT	1[c]		64		1[e]		18	
	Peritoneum	mt	24	K2=21 K3=0 B=3	0		10	K2=4 B=6	1[f]	K3=1
		WT	0		13		0		4	
	Spleen	mt	1	K2=1	0		1	K2=1	0	
		WT	0		3		0		0	
	Lung	mt	0		0		1	K2=1	0	
		WT	0		0		0		0	
	Local	mt	-		-		3	K2=3	1[g]	K3=1
		WT	-		-		0		3	

Figure 2. Concordance of *KRAS/BRAF* mutation status between primary CRC and each site of corresponding metastases. The grey column shows the concordance between primary CRC and corresponding metastases. The white column shows the discordance between primary CRC and corresponding metastases. a, b, c, d, e, f and g: corresponding to each case shown in Table IV. Seven local recurrences were included in the metastases.

Analysis of KRAS/BRAF mutations. Genomic DNA was extracted from fresh-frozen tissue samples using the standard phenol-chloroform extraction method. *KRAS* mutations in exon 2 and 3 were detected by denaturing gradient gel electrophoresis or high resolution melting (HRM) analysis, using a Rotor-Gene Q (Qiagen, Hilden, Germany), as previously described (25,26). *BRAF* mutations in exon 15 (codon 600) were detected using either polymerase chain reaction (PCR)-restriction fragment length polymorphism or HRM, as previously described (27).

Analysis of microsatellite status. MSI analysis was performed using fluorescence-based PCR, as previously described (9). MSI status was determined using five Bethesda markers (BAT25, BAT26, D5S346, D2S123 and D17S250). MSI status was graded as MSI-H when there were two or more unstable markers, MSI-low (MSI-L) when only one unstable marker, and microsatellite-stable (MSS) when no unstable markers. MSI-positive markers were re-examined at least twice to confirm the results. MSI-L was included with MSS in this study.

Results

Characteristics of primary CRCs and corresponding metastases. Five hundred and fifty-six corresponding metastases (499 synchronous and 57 metachronous metastases) and seven local recurrences that matched primary CRC were included in this study. The metastatic samples included 343 lymph node metastases (331 synchronous and 12 metachronous), 155 liver metastases (127 synchronous and 28 metachronous), 52 peritoneal metastases (37 synchronous and 15 metachronous), five splenic metastases (4 synchronous and 1 metachronous), one pulmonary metastasis (1 metachronous metastasis) and seven local recurrences. *KRAS* exon 2, 3 and *BRAF* exon 15 mutations were analysed in 457 primary CRC cases and 556 corresponding metastases (499 synchronous and 57 metachronous metastases) and seven local recurrences (Figs. 1 and 2). *KRAS* and *BRAF* mutations were detected in 228 and 30 primary CRCs, respectively. MSI status was analysed in 482 primary CRC, 155 corresponding metastases (130 synchronous and 25 metachronous metastases) and six local recurrences. Four hundred and two metastases were not analysed for MSI

Primary			Metastases: Synchronous n=130 MSI-H		Metastases: Synchronous n=130 MSS		Metachronous n=31 MSI-H		Metachronous n=31 MSS	
Node	MSI-H		12		1[h]	H→L (n=1)	0		0	
	MSS		0		70		0		2	
Liver	MSI-H		1		0		1		0	
	MSS		0		29[i]	L→S (n=2)	0		10[j]	S→L (n=1)
Peritoneum	MSI-H		3		0		4		2[k,l]	H→L (n=1) H→S (n=1)
	MSS		0		11		0		5[m]	L→S (n=1)
Spleen	MSI-H		0		0		0		0	
	MSS		0		3		0		1	
Lung	MSI-H		0		0		0		0	
	MSS		0		0		0		0	
Local	MSI-H		-		-		0		0	
	MSS		-		-		0		6	

Figure 3. Concordance of MSI status between primary CRC and each site of corresponding metastases. The grey column shows the concordance between primary CRC and corresponding metastases. The white column shows the discordance between primary CRC and corresponding metastases. h, i, j, k, l and m: corresponding to each case in Table IV. Six local recurrences were included in metastases.

status (Figs. 1 and 3). Eighteen MSI-H CRC cases were identified in this study and consisted of 3 Lynch Syndrome cases, 10 *MLH1* hypermethylated and 5 *MLH1* unmethylated cases without germline mutation (Table II).

Concordance rate of KRAS mutation, BRAF mutation and MSI-H between primary CRCs and corresponding metastases. The concordance rate of *KRAS/BRAF* mutation between primary CRC and corresponding metastases was 94.6% (243/257). The concordance rates of *KRAS* mutation, *BRAF* mutation or wild-type (*KRAS* wild-type and *BRAF* wild-type) between primary CRC and corresponding metastases were 93.9% (214/228), 100% (30/30) and 99.3% (304/306), respectively. High concordance rate was observed in either synchronous or metachronous metastases (Table I).

The concordant rates of MSI-H and MSS (included MSI-L) were 87.5% (21/24) and 100% (137/137), respectively. Discordance of MSI status was found in 3 cases and all of them were *MLH1* unmethylated cases. *KRAS* and *BRAF* mutation status in primary MSI-H CRC was consistent with that in metastases except one case (Table II).

Concordance rate of KRAS/BRAF mutation or MSI status between primary CRCs and each site of corresponding metastases. *BRAF* mutation status of each metastatic tissue was perfectly consistent with primary CRC. In each metastatic tissue, a high concordance rate of *KRAS* mutation was shown as well. Local recurrences (75.0%) had lower concordance rates with each metastatic tissue. Regarding MSI status, a high concordance rate of MSI-H was also observed in each metastatic tissue. Peritoneal metastases (77.8%) had lower concordance rates in each metastatic tissue (Table III).

Discordant cases. Twenty-three cases were discordant between primary CRC and corresponding metastases. Discordant cases were observed in the 16 cases with *KRAS* mutation and 3 cases with MSI-H, but not in *BRAF* mutation cases. Of the 16 discordant cases with *KRAS* mutation, 10 cases were lymph node

Table II. Characteristics of primary CRCs with MSI-H and corresponding metastases.

Case no.	Type	Primary		Type of metastases	Metastases	
		KRAS/BRAF	MSI		*KRAS/BRAF*	MSI
403	LS *(MLH1)*	WT	H	Synchronous node	WT	H
238	LS *(MSH2)*	*KRAS ex2*	H	Synchronous node	*KRAS ex2*	H
238	LS *(MSH2)*	*KRAS ex2*	H	Synchronous Liver	*KRAS ex2*	H
238	LS *(MSH2)*	*KRAS ex2*	H	Synchronous Peritoneum	*KRAS ex2*	H
455	LS *(MSH6)*	WT	H	Synchronous node	WT	H
8	*MLH1* methylated	WT	H	Synchronous node	WT	H
65	*MLH1* methylated	*KRAS ex2*	H	Synchronous node	*KRAS ex2*	H
193	*MLH1* methylated	*BRAF*	H	Synchronous node	*BRAF*	H
396	*MLH1* methylated	*KRAS ex2*	H	Synchronous node	*KRAS ex2*	H
424	*MLH1* methylated	*BRAF*	H	Synchronous node	*BRAF*	H
342	*MLH1* methylated	WT	H	Synchronous node	WT	H
227	*MLH1* methylated	*KRAS ex3/BRAF*	H	Synchronous node	*KRAS ex3/BRAF*	H
149	*MLH1* methylated	WT	H	Synchronous Peritoneum	WT	H
397	*MLH1* methylated	*BRAF*	H	Synchronous Peritoneum	*BRAF*	H
397	*MLH1* methylated	*BRAF*	H	Metachronous Liver	*BRAF*	H
397	*MLH1* methylated	*BRAF*	H	Metachronous Peritoneum	*BRAF*	H
397	*MLH1* methylated	*BRAF*	H	Metachronous Peritoneum	*BRAF*	H
191	*MLH1* methylated	*KRAS ex3*	H	Metachronous Peritoneum	WT	H
4	*MLH1* unmethylated	WT	H	Synchronous node	WT	H
220	*MLH1* unmethylated	WT	H	Synchronous node	WT	L
398	*MLH1* unmethylated	*KRAS ex2*	H	Synchronous node	*KRAS ex2*	H
239	*MLH1* unmethylated	*BRAF*	H	Metachronous Peritoneum	*KRAS ex2*	S
253	*MLH1* unmethylated	*BRAF*	H	Metachronous Peritoneum	*BRAF*	L
253	*MLH1* unmethylated	*BRAF*	H	Metachronous Peritoneum	*BRAF*	H

LS, Lynch syndrome; WT, the cases without *KRAS* and *BRAF* mutations; H, MSI-H; L, MSI-L; S, MSS.

Table III. Concordance rate of *KRAS/BRAF* mutation and MSI status between primary CRCs and each site of corresponding metastases.

Status	Concordance rate (%)					
	Node	Liver	Peritoneum	Spleen	Lung	Local
KRAS/BRAF	93.1 (134/144)	97.2 (69/71)	97.1 (34/35)	100 (2/2)	100 (1/1)	75.0 (3/4)
KRAS	92.2 (118/128)	96.9 (63/65)	96.0 (24/25)	100 (2/2)	100 (1/1)	75.0 (3/4)
BRAF	100 (17/17)	100 (4/4)	100 (9/9)	-	-	-
Wild	100 (197/197)	97.6 (82/84)	100 (17/17)	100 (3/3)	-	100 (3/3)
MSI-H	92.3 (12/13)	100 (2/2)	77.8 (7/9)	100 (2/2)	-	-
MSS	100 (72/72)	100 (39/39)	100 (16/16)	100 (4/4)	-	100 (6/6)

Concordance rate, number of concordance cases/number of primary CRCs with each status. WT, the cases without both *KRAS* mutation and *BRAF* mutation. One individual had both *KRAS* and *BRAF* mutations.

metastases. Most of discordant cases in *KRAS* mutants were lymph node metastases. Of the three cases with MSI-H, one was in lymph node metastases and two cases in the peritoneal metastases (Table IV).

Discussion

High concordance of *KRAS/BRAF* mutation status and MSI status was observed between primary CRC and corresponding

Table IV. The discordant cases.

Case no.	Primary	Metastasis	Location	Time
22[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
230[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
237[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
323[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
361[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
391[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
449[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
474[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
492[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
501[a]	*KRAS* exon2 mutant	WT	Node	Synchronous
466[b]	*KRAS* exon2 mutant	WT	Liver	Synchronous
371[c]	WT	*KRAS* exon2 mutant	Liver	Synchronous
61[d]	*KRAS* exon2 mutant	WT	Liver	Metachronous
270[e]	*KRAS* exon4 mutant	*KRAS* exon2 mutant	Liver	Metachronous
191[f]	*KRAS* exon3 mutant	WT	Peritoneum	Metachronous
380[g]	*KRAS* exon3 mutant	WT	Local	Metachronous
220[h]	MSI-H	MSI-L	Node	Synchronous
126[i]	MSI-L	MSS	Liver	Metachronous
108[i]	MSI-L	MSS	Liver	Metachronous
445[j]	MSS	MSI-L	Liver	Metachronous
253[k]	MSI-H	MSI-L	Peritoneum	Metachronous
239[l]	MSI-H	MSS	Peritoneum	Metachronous
296[m]	MSI-L	MSS	Peritoneum	Metachronous

a, b, c, d, e, f, g, h, o, j, k, l and m, indicating each case in Figs. 2 and 3.

metastases in the present study. These high concordance rates were not different between synchronous and metachronous metastases. These results are in agreement with the notion that *KRAS/BRAF* mutations occur early in CRC carcinogenesis (20,28). Lymph node metastases showed a slightly lower concordance rate than other metastatic sites. Mao *et al* demonstrated that lymph node metastases indicated a lower concordance rate with *KRAS* mutation status (29) and this support our results. However, concordance rate of *KRAS/BRAF* mutation and MSI-H was >90% in lymph node suspected metastases macroscopically, metastatic lymph node will be useful for mutation analysis after confirmation of enough tumour cells and content microscopically. With regard to the other metastatic sites, a high concordance was observed between primary CRC and corresponding liver metastases. Knijin *et al* demonstrated a high concordance, i.e. 96.4%, in 305 liver metastases (30). In addition, this study showed high concordance of *KRAS/BRAF* mutation between primary tumour and peritoneal metastases. No other studies have systematically compared the concordance of *KRAS/BRAF* mutation status in primary CRC with corresponding peritoneal metastases.

Regarding MSI status, a high concordance rate was also shown between primary CRC and corresponding metastases. This result suggested that cancer cells do not change their MSI status during progression. MSI-H CRC consists of three types, which harbours a germline mutation in the MMR gene (e.g. Lynch syndrome), acquires epigenetic change in the MMR gene (e.g. *MLH1* promoter hypermethylation) and uncertified germline mutation without *MLH1* promoter hypermethylation (e.g. Lynch-like syndrome). Our results indicated perfect concordance of MSI status was observed in two types, i.e. Lynch syndrome and *MLH1* promotor hypermethylation (3 Lynch syndrome cases and 10 *MLH1* promoter hypermethylation cases) between primary CRC and corresponding metastases (Table II). This is the first study of concordance rate of MSI status between primary and metastatic CRC using Bethesda markers. Recently, Haraldsdottir *et al* reported perfect concordance of MMR deficiency evaluated by immunohistochemistry (IHC) between primary CRC and corresponding metastases (31).

In this study, 23 cases showed discordance of mutation status between primary CRC and corresponding metastases. Several reasons are conceivable. First, it could be speculated that discrepancies may depend on the molecular heterogeneity in primary CRCs. For instance, intratumoural heterogeneity for a *KRAS* point mutation was observed within 20-60% CRC cases in previous studies (32,33). In contrast to *KRAS* mutation status, *BRAF* mutation status did not show heterogeneity in previous studies (34,35). Mao *et al* have reported a higher

concordance rate of *BRAF* mutation status (93.6%) between primary and lymph node metastases (29). Our results, which showed a perfect concordance rate in *BRAF*-mutant cases, is in agreement with these studies.

Second, discordant results could be explained by the acquisition of the mutation during the disease progression. However, *KRAS* mutation occur in early stage of carcinogenesis (20,28), it may be rare that CRC acquired *KRAS* mutation after metastasis (21,36).

Third, selecting improper samples containing a high number of normal or necrotic cells, could create discordance between primary CRC and corresponding metastases. In this study, samples that were suspected to contain enough cancer cells macroscopically by surgeons were used for mutation testing. Consequently, these samples might not include enough cancer cells especially in the lymph nodes. High concordance rate might be shown in previous studies that used laser microdissection-collected cancer cells from lymph node metastases (37,38).

In conclusion, although attention should be paid to selecting and sampling tissue, high concordance rate of *KRAS*/*BRAF* mutation status and MSI status was observed between primary CRC and corresponding metastases, regardless of metastatic sites and synchronous/metachronous types. Therefore, to choose the appropriate regimen for therapy, either primary or metastatic CRC can be used for testing *KRAS*/*BRAF* mutation status and MSI status.

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