BRAF V600E mutation is a predictive indicator of upfront chemotherapy for stage IV colorectal cancer

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Received March 15, 2016; Accepted February 7, 2017

DOI: 10.3892/ol.2017.7553

Abstract. In stage IV colorectal cancer (CRC), initial resection of the primary tumor is considered to be an important strategy for improving disease outcome. However, there is no consensus on the timing as to when the surgical intervention of the primary tumor should occur. The present study hypothesizes that genetic profiles in CRC may indicate the appropriate treatment strategies for patients with stage IV CRC, and a cohort of 113 patients with stage IV CRC resected primary lesions at various periods were analyzed for the presence of mutations in the *KRAS*, exon 2, and *BRAF* genes, exon 15, and for the microsatellite instability status of the tumor. These data were additionally correlated with various clinicopathological features. Although *BRAF*-mutant was revealed to be an independent negative prognostic factor in stage IV CRC (HR, 8.42; 95% confidence interval, 2.72-26.02), *BRAF*-mutant samples exhibited better prognoses if they were treated with chemotherapy prior to tumor resection. Thus, the presence of *BRAF* mutations provides a compelling rationale for the establishment of intensive upfront chemotherapy to improve survival in stage IV CRC.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortality and the most common type of cancer, with >1 million incident cases diagnosed annually worldwide (1,2). Of patients with synchronous distant metastases, defined as stage IV by Union for International Cancer Control tumor node metastasis (TNM) staging 7th edition (3), at the time of diagnosis, ~25% exhibit poor prognoses, with a 5-year survival rate of ~12% (4,5). Amongst patients diagnosed with a stage IV CRC, liver metastases is the most common type, occurring in 20-30% of patients, whereas peritoneal and lung metastases occur in 10-15% and 10-25% patients, respectively, and other non-rectal or non-colon metastases occur rarely (6). Amongst patients with synchronous distant metastases, ~80% exhibit metastases that cannot be curatively resected and the 10-30% who undergo resection of the primary tumor experience complications such as perforation or hemorrhage (7).

Previously, several prospective studies revealed that initial tumor resection is an important step toward improving the overall survival rates (OS) of patients with stage IV CRC (8,9). However, the optimal timing of primary tumor resection remains controversial. Poultsides *et al* (10) demonstrated that upfront systemic therapy may be safely administered to patients with stage IV CRC, avoiding the need for palliative primary tumor resection in the majority of cases. Additionally, a randomized phase III study [European Organisation for Research and Treatment of Cancer (EORTC) 40983] demonstrated that amongst patients with initially resectable liver metastases, including patients with stage IV disease and those with tumor recurrence, perioperative (pre- and postoperative) chemotherapy with folinic acid, fluorouracil and oxaliplatin (FOLFOX4) significantly improved progression-free survival (PFS) compared with surgery alone, although no differences in OS were observed (11,12). The aforementioned study suggested that perioperative chemotherapy with FOLFOX4 reduced the risk of progression in a subset of patients with initially resectable liver metastases. However, the molecular characteristics of these patients were not examined, and the requirements of initial tumor resection and perioperative chemotherapy remain debatable for patients with stage IV CRC.

CRC progresses through a series of well-defined steps that are associated with characteristic mutations, including genetic and epigenetic alterations in various oncogenes and tumor suppressor genes (13-15). Point mutations in the *KRAS* oncogene are typically observed in codons 12 and 13 and less

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Key words: colorectal cancer, *BRAF* mutation, *KRAS* mutation, microsatellite instability, upfront chemotherapy, stage IV

frequently in codons 59, 61, 117 and 144. Additionally, pathogenetic activating point mutations are primarily observed in codon 600 of the *BRAF* oncogene (16). These mutations in the *KRAS* and *BRAF* oncoproteins activate signaling cascades that mediate cellular responses such as cell proliferation, apoptosis, adhesion, invasion and angiogenesis (17,18). Previously, mutations in the *KRAS* gene, including minor mutations, have been associated with a resistance to anti-EGFR antibodies (16,19,20). Although the *BRAF* gene is located downstream of *KRAS*, the activating V600E *BRAF* mutation is not considered a predictive biomarker for resistance to anti-EGFR antibodies. However, mutations in this gene have been suggested to be strong prognostic indicators of poor prognoses in patients with stage II and III CRC subsequent to curative resection, and in patients with unresectable metastatic CRC (16,21-25).

The present study hypothesized that mutations in *BRAF* and *KRAS* genes may also indicate the appropriate treatment strategies for patients with stage IV CRC. Thus, the presence of mutations in these genes was determined in a consecutive series of patients with stage IV CRC, including those with resectable and unresectable metastatic lesions at diagnosis, and determined their clinical significance using correlations with clinicopathological characteristics that are associated with patient outcomes and survival.

Materials and methods

Study population. A total of 113 consecutively diagnosed patients with stage IV CRC were treated with colectomy or proctectomy at the Okayama University Hospital, Okayama, Japan, between May 2000 and February 2013. All cases were histologically confirmed as adenocarcinoma, and all familial CRC, such as Lynch syndrome and familial adenomatous polyposis, were excluded.

The present study was approved by the Institutional Review Board of the Okayama University Hospital. All patients gave written informed consent for the use of tissues and clinical data for research purposes. Histological diagnoses of tumors were made according to the World Health Organization International Histological Classification of tumors (26), and tumors were subclassified as differentiated (well and moderately differentiated tubular adenocarcinoma) or undifferentiated types (poorly differentiated adenocarcinoma and mucinous adenocarcinoma) (27). Pathological stage was determined according to the 7th edition Union for International Cancer Control TNM classification of malignant tumors (5).

Analysis of KRAS and BRAF mutations. Direct sequencing was performed to identify mutations in *KRAS* exon 2, including codon 12 and 13, and *BRAF* exon 15, including codon 600, using purified DNA from formalin-fixed and paraffin-embedded tissues or from fresh frozen tissues from each case. Primer sequences for *KRAS* and *BRAF* and polymerase chain reaction (PCR) conditions were described previously (28). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA, USA) and directly sequenced using an ABI PRISM® 310-Avant™ and a 310R Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Analysis of microsatellite status. Multiplex PCRs with the mononucleotide microsatellite markers BAT26, NR27 and NR21 were performed to determine the microsatellite instability (MSI) status of the CRC tissues. Tumors exhibiting genomic instability in ≥1 mononucleotide markers were classified as MSI, and types of cancer with no mutations in these markers were categorized as microsatellite stable (MSS). Previously, we demonstrated that data analyses with the mononucleotide markers BAT26, NR27 and NR21 were comparable or superior compared with those with the five markers recommended by the National Cancer Institute workshop for detecting high MSI, or mismatch deficiencies, in CRC (29).

Statistical analysis. Statistical analyses were performed using SPSS v. 20.0 software (IBM SPSS, Armonk, NY, USA). Categorical variables were compared using Fisher's exact test, and continuous variables were compared using the Kruskal-Wallis test. OS curves were calculated using the Kaplan-Meier method, and differences in the survival times amongst the subgroups were compared using the log-rank test. Univariate and multivariate analyses were performed using Cox proportional hazard regression models. Significant factors from univariate analyses were included in multivariate analysis to determine independent prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. Amongst the 113 patients with stage IV CRC, 57.5% were male and 42.5% were female (Table I), and the median age was 64 years, with a range of 35-88 years. The median serum CEA level was 34.0 ng/ml, with a range of 1.0-9092.0 ng/ml. Tumor locations were categorized as proximal colon, from the cecum to the splenic flexure of the transverse colon, or distal colon, from the splenic flexure of the descending colon to the rectum. A total of 24.8% (28) tumors were in the proximal colon and 75.2% (85) tumors were in the distal colon. The majority of tumors, 85.8% (97/113) were histologically diagnosed as differentiated adenocarcinoma, and 14.2% (16/113) of tumors were categorized as undifferentiated adenocarcinoma. Distant metastatic lesions in a single organ such as the liver or the lung occurred in 64.6% (73/113) of patients and 35.4% (40/113) of patients exhibited metastases in multiple organs (Table I). Chemotherapy including fluoropyrimidine plus oxaliplatin or irinotecan was administrated to 68.1% (77/113) of patients. Of these, 77 were treated with chemotherapy: 23 received chemotherapy prior and subsequent to resection of the primary tumor (upfront chemotherapy) and 54 received chemotherapy subsequent to resection of the primary tumor (postoperative chemotherapy). Amongst all patients with stage IV CRC, 63.7% (72) of patients received curative resection of primary and metastatic sites, defined by the absence of residual disease; the remaining 36.3% (41) of patients received local excisions of primary tumors alone, defined by the presence of residual disease.

Frequencies of MSI and mutations in BRAF and KRAS genes in stage IV CRC. In the present cohort of patients with

Table I. Characteristics of 113 patients with stage IV colorectal cancer in relation to the mutational status of *BRAF* and *KRAS* genes.

Characteristic	Total (n=113)	*BRAF*-mutant (n=7)	*KRAS*-mutant (n=31)	Wild-type (n=75)	P-value
Age (years)					0.849[a]
Median (Range)	64 (35-88)	64 (40-79)	61 (35-88)	65 (41-85)	
Gender (%)					0.595[b]
Male	65 (57.5)	3 (42.9)	17 (54.8)	45 (60.0)	
Female	48 (42.5	4 (57.1)	14 (45.2)	30 (40.0)	
Serum carcinoembryonic antigen level (ng/ml)					0.574[a]
Median (Range)	34 (1.0-9092.0)	32 (2.0-1385.0)	31 (1.0-1353.0)	42 (1.0-9092.0)	
Tumor Location[c] (%)					<0.001[b]
Proximal colon	28 (24.8)	6 (85.7)	13 (41.9)	9 (12.0)	
Distal colon	85 (75.2)	1 (14.3)	18 (58.1)	66 (88.0)	
Histology (%)					0.016[b]
Differentiated	97 (85.8)	3 (42.9)	28 (90.3)	66 (88.0)	
Undifferentiated	16 (14.2)	4 (57.1)	3 (9.7)	9 (12.0)	
No. of distant metastatic sites (%)					0.114[b]
Single	73 (64.6)	2 (28.6)	22 (71.0)	49 (65.3)	
Multiple	40 (35.4)	5 (71.4)	9 (29.0)	26 (34.7)	
Chemotherapy[d] (%)					0.431[b]
Upfront[e]	23 (20.3)	2 (28.6)	9 (29.0)	12 (16.0)	
Postoperative	54 (47.8)	2 (28.6)	14 (45.2)	38 (50.7)	
None	36 (31.9)	3 (42.8)	8 (25.8)	25 (33.3)	
Molecularly targeted therapy (%)					0.663[b]
Yes	52 (46.0)	2 (28.6)	14 (45.2)	36 (48.0)	
No	61 (54.0)	5 (71.4)	17 (54.8)	39 (52.0)	
Residual disease (%)					0.045[b]
Present	72 (63.7)	7 (100.0)	16 (51.6)	49 (65.3)	
Absent	41 (36.3)	0 (0.0)	15 (48.4)	26 (34.7)	

P-values were calculated between *BRAF*-mutant vs. *KRAS*-mutant and Wild-type. [a]Kruskal-Wallis test, [b]Fisher's exact test, [c]Proximal colon means from ceacum to splenic flexure and distal colon indicates from splenic flexure to rectum, [d]Regimen including 5-fluorouracil plus oxaliplatin or irinotecan. [e]Upfront includes patients who received with preoperative chemotherapy only and both preoperative and postoperative chemotherapy.

stage IV CRC, no tumor displayed MSI, and all 113 tumors were categorized as MSS. *KRAS* and B*RAF* mutation analyses were successful in 113 specimens, and mutations in the two genes occurred in a mutually exclusive manner, with no tumors exhibiting simultaneous mutations in the two genes. Mutated *BRAF* was revealed in 6.2% (7) tumors and encoded the V600E mutation. Mutations in codons 12 or 13 of the *KRAS* gene were revealed in 27.4% (31) of tumors. Amongst the 31 tumors with *KRAS* exon 2 mutations, all exhibited single mutations and the most prevalent types of mutations were GGT to GAT (G12D) in 13.3% (15/113) of tumors, followed by GGT to GTT (G12V) in 6.2% (7/113) of tumors, GGC to GAC (G13D) in 4.4% (5/113) of tumors, GGT to AGT (G12S) in 2.7% (3/113) of tumors, and GGT to TGT (G12C) in 0.9% (1/113) of tumors. Based on the presence or absence of mutations in these two genes, all 113 patients with stage IV CRC were classified as *BRAF*-mutant, *KRAS*-mutant, or wild-type (Table I).

Associations between genetic profiles and clinicopathological characteristics. *BRAF*-mutant tumors were observed significantly more frequently in the proximal colon, 6 in the proximal colon vs. 1 in the distal colon, and *BRAF* mutations were associated with undifferentiated histological phenotypes (P=0.016). Distant metastases in multiple organs were more common in patients with *BRAF*-mutant CRC (71.4%; 5/7) compared with those with *KRAS*-mutant (29.0%; 9/31) and wild-type cancers (34.7%; 26/75; P=0.095).

A total of 74.2% (23/31) patients with *KRAS*-mutant tumors and 57.1% (4/7) patients with *BRAF*-mutant tumors had received one or more course of fluoropyrimidine-based chemotherapy. Upfront chemotherapy was administrated in

Table II. Univariate analysis for survival outcomes in 113 patients with metastatic colorectal cancer.

Variable	Hazard ratio	95% confidence interval	P-value
Age (years): ≥64/≤63	1.16	0.67-2.03	0.594
Gender: Male/Female	0.94	0.53-1.64	0.820
Serum carcinoembryonic antigen level (ng/ml): ≥34/<34	0.98	0.56-1.71	0.945
Tumor location: Distal/Proximal	0.97	0.55-1.71	0.910
Histology: Undifferentiated/Differentiated	5.00	2.48-10.10	<0.001
No. of metastatic sites: Multiple/Single	3.27	1.86-5.75	<0.001
Residual Disease: Present/Absent	4.46	2.29-8.70	<0.001
Chemotherapy: No/Yes	2.70	1.53-4.76	<0.001
Molecularly targeted therapy: No/Yes	2.02	1.11-3.68	0.021
KRAS mutation: Yes/No	0.86	0.45-1.65	0.651
BRAF mutation: Yes/No	11.88	4.55-31.00	<0.001

Table III. Multivariate analysis for survival outcomes in 113 patients with metastatic colorectal cancer.

Variable	Hazard ratio	95% confidence interval	P-value
Histology: Undifferentiated/Differentiated	2.61	1.10-6.21	0.030
No. of metastatic sites: Multiple/Single	1.47	0.74-2.94	0.274
Residual disease: Present/Absent	5.65	2.41-13.16	<0.001
Chemotherapy: No/Yes	3.44	1.60-7.39	0.002
Molecularly targeted therapy: No/Yes	1.66	0.81-3.38	0.167
BRAF mutation: Yes/No	8.42	2.72-26.02	<0.001

28.6% (2/7) patients with *BRAF*-mutant tumors, 29.0% (9/31) of patients with *KRAS*-mutant tumors, and 16.0% (12/75) of patients with wild-type tumors. Postoperative chemotherapy was administrated in 28.6% (2/7) of patients with *BRAF* V600E mutations, 45.2% (14/31) of patients with *KRAS* mutations, and 50.7% (38/75) of patients with wild-type tumors. In contrast, 42.8% (3 of 7) of patients with *BRAF* mutations, 25.8% (8/31) of patients with *KRAS* mutations, and 33.3% (25/75) of patients with wild-type tumors did not receive chemotherapy prior or subsequent to resection of the primary tumor. The molecular targeted agent bevacizumab was administered to 54.8% (17/31) of patients with *KRAS*-mutant tumors, and 28.6% (2/7) of patients with *BRAF*-mutant tumors received bevacizumab and cetuximab. Amongst all patients with stage IV CRC, 63.7% (72 patients) received curative resections that included metastatic sites, as defined by the absence of residual disease, whereas the remaining 36.3% (41 patients) received local excisions of primary tumors alone, as defined by the presence of residual disease. No patients with *BRAF*-mutant tumors received curative resection, whereas 48.4% (15/31) of patients with *KRAS*-mutant tumors and 34.7% (26/75) of patients with wild-type tumors received curative resection (P=0.047).

Survival analyses in stage IV CRC patients with BRAF mutations. The OS in patients with stage IV CRC with mutations in *KRAS* and *BRAF* genes is illustrated in Fig. 1. The median follow-up duration was 17.3 months and patients with *BRAF* mutations exhibited significantly poorer prognoses compared with those with *KRAS* mutations or wild-type tumors, with median survival times (MSTs) of 2.5, 41.2 and 40.3 months, respectively (P<0.001). Univariate analysis revealed several factors associated with poor prognosis, including tumors with undifferentiated histology, multiple metastatic sites, residual disease, no chemotherapy, therapy with molecular targeted drugs and the presence of *BRAF* mutations (Table II). Similarly, multivariate analysis revealed that undifferentiated tumor histology, residual disease, no chemotherapy and mutations in the *BRAF* gene were statistically significant predictors of survival and independent prognostic factors for poor outcomes of stage IV CRC (Hazard ratio; 8.42, P<0.0001; Table III).

Subsequent to correcting for the administration of upfront chemotherapy, clinical outcomes did not differ between patients with stage IV CRC with and without upfront chemotherapy (Fig. 2). However, amongst 7 patients with *BRAF* V600E mutations in primary tumors, 2 received upfront chemotherapy and demonstrated improved survival compared with the 5 patients who did not receive upfront chemotherapy (Fig. 3), highlighting the prognostic value of *BRAF* mutations in patients with stage IV CRC.

Discussion

The present study identified the presence of the *BRAF* V600E mutation in primary tumor tissues and produced data that

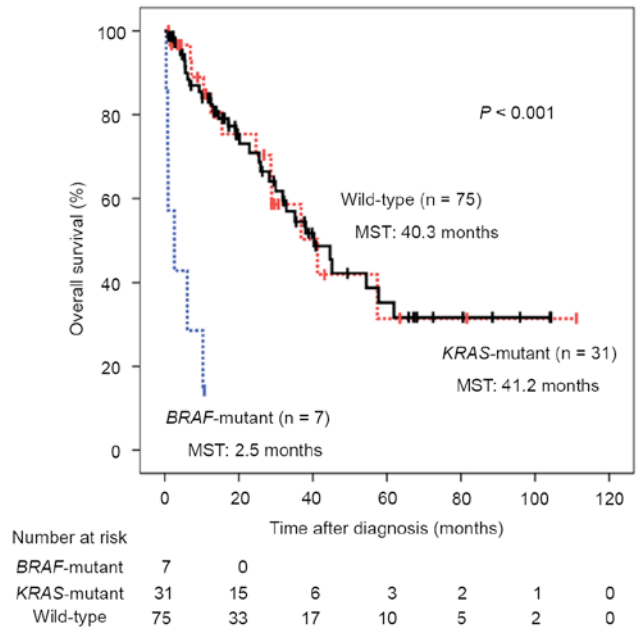

Figure 1. Kaplan-Meier estimates of overall survival in patients with stage IV colorectal cancer with and without *BRAF* and *KRAS* mutations. Patients with *BRAF* mutations exhibited significantly poorer prognoses compared with those with *KRAS* mutations or neither mutation (P<0.001). MST, median survival time.

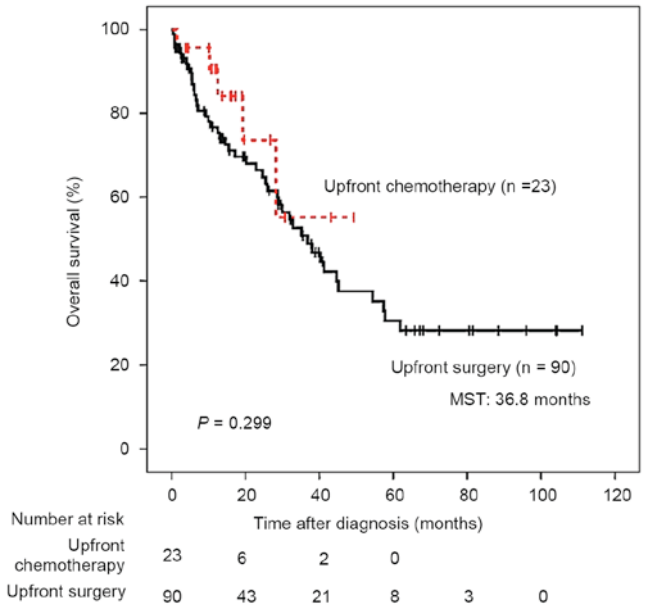

Figure 2. Kaplan-Meier estimates of overall survival based on initial therapy in 113 patients with stage IV colorectal cancer. MST, median survival time.

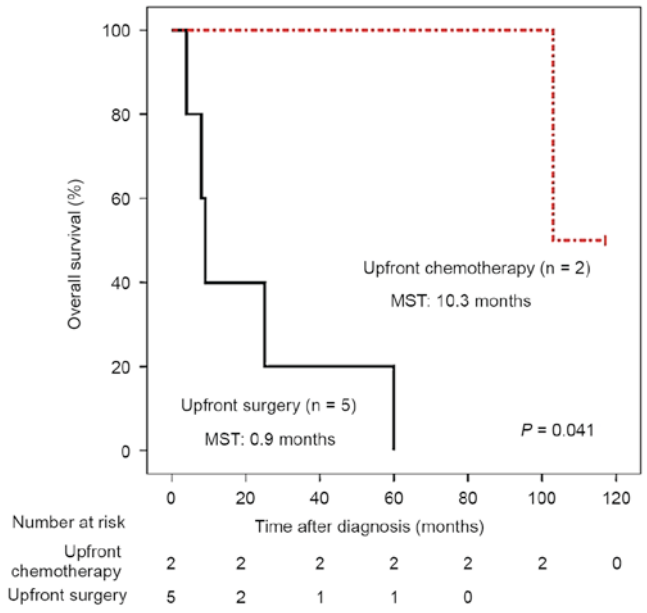

Figure 3. Kaplan-Meier estimates of overall survival based on initial therapy in *BRAF*-mutant CRC. Upfront chemotherapy improved prognoses for patients with *BRAF*-mutant CRC (P=0.041, log-rank test). MST, median survival time; CRC, colorectal cancer.

supported the hypothesis of the potential for individualized treatment strategies for patients with stage IV CRC.

Several studies investigating stage IV CRC have demonstrated that initial tumor resection improves survival (6,8,9,30). However, in certain cases, surgical removal of the primary tumor is accompanied by exceptionally rapid outgrowth of distant metastases (31,32), suggesting that primary tumors inhibit the growth of metastatic lesions in a limited number of cases (33-35). The data of the present study indicate that this subset of patients with stage IV CRC may include those with the *BRAF* V600E mutation, with rapid outgrowth of distant metastases subsequent to the surgical removal of primary tumors.

The importance of upfront systemic chemotherapy was reported in a previous randomized phase III study (EORTC 40983) (11,12), although perioperative combination chemotherapy with FOLFOX4 increased PFS compared with surgery alone in a subset of patients, no differences in overall survival were observed (11,12). Therefore, perioperative chemotherapy may reduce the risk of PFS events in certain patients with stage IV CRC with initially resectable liver metastases. In agreement with this, the data of the present study demonstrates that patients with V600E *BRAF*-mutant advanced CRC are the most likely type of patient to benefit from perioperative chemotherapy.

In the present cohort of patients with stage IV disease, no primary tumors exhibited MSI, which typically indicates defective DNA mismatch repair systems (36). Clinically, CRC with MSI include patients with Lynch syndrome and sporadic MSI cancer (36,37). Lynch syndrome is hereditary and reflects germline mutations in the DNA mismatch repair genes *MLH1*, *PMS2*, *MSH2* or *MSH6* (36,37). In contrast, sporadic MSI usually reflects hypermethylation of the *MLH1* promoter, which causes transcriptional silencing of this proofreading gene (15,28). In the present study, patients with Lynch syndrome were excluded and the entire cohort of stage IV patients exhibited sporadic CRC. Typical features of sporadic MSI CRC include older age, female sex, proximal tumor location, undifferentiated histology, lower clinical stage, slow growth and better overall prognosis (38-40). Therefore, it is unlikely that the tumors in the present study displayed MSI, which is usually uncharacteristic of advanced/metastatic CRC.

Mutations in the *BRAF* oncogene were first identified in 2002 and were demonstrated to be initially associated with

MSI CRC, particularly sporadic MSI tumors (15,41,42). Subsequently, *BRAF*-mutant CRC were recognized as either sporadic MSI tumors or MSS tumors, and the *BRAF* V600E mutation was exhibited in >50% of sporadic MSI tumors and in certain MSS tumors (28). In a retrospective study of several clinical trials, the presence of the *BRAF* V600E mutation was a strong negative prognostic factor for OS in patients with stage II/III CRC, particularly in patients with colorectal tumors with low or stable microsatellite instability, MSI-L MSS or no MSI (25). Several studies demonstrated that patients with CRC with MSI tumors carrying *BRAF* mutations exhibited significantly better prognoses compared with those with *BRAF*-mutant MSS tumors (43,44). Similarly, amongst patients with metastatic CRC treated with combination chemotherapy using molecular targeted agents, *BRAF* mutations were associated with significantly poorer prognoses (16,21-23).

In the prospective FFCD 9601 trial, which examined the benefit of primary tumor resection on survival of patients with CRC with synchronous metastases, stage IV, treated by chemotherapy, survival outcomes were better in patients with distal primary lesions (9). Although the mechanisms behind these observations remain unclear, the authors emphasized that the primary tumor locations and resections were critical clinical factors in the therapeutic management of these patients with CRC. In the present study, *BRAF*-mutant CRC were significantly associated with proximally located primary tumors and poor prognosis, with a median overall survival time of 2.5 months. Therefore, the absence of the *BRAF* V600E mutation may explain the improved survival outcomes in patients with stage IV CRC with distal cancers. Additionally, the present data demonstrate that patients with V600E *BRAF*-mutant stage IV tumors tend to exhibit distant metastases in multiple organs, inhibiting the success of curative resection. Accordingly, none of the present patients with *BRAF* mutations received curative resection, as indicated by the significantly worse prognoses for *BRAF*-mutant CRC.

Although there were only seven CRC exhibiting the *BRAF* V600E mutation, 6.2%, of the 113 stage IV patients with CRC, they demonstrated a trend of improved responses to upfront systematic chemotherapy, which improved prognoses. In contrast, patients with *BRAF*-mutant tumors who received upfront surgical resection of primary tumors instead of chemotherapy exhibited limited MSTs of 0.9 months. These data indicate that intensive upfront chemotherapy improves prognoses of patients with stage IV CRC with *BRAF* mutations.

In conclusion, although the present study was limited to 113 patients, the presence of *BRAF* mutations in primary tumors from patients with stage IV CRC was a significant negative prognostic factor. The present data suggest that intensive upfront chemotherapy enhances survival rates in patients with advanced CRC exhibiting *BRAF* mutations.

Acknowledgements

The authors would like to thank Mr. Toru Nakai, Ms. Tae Yamanishi and Mr. Akihiro Nyuya for technical assistance. The authors would also like to thank Dr. Margaret Hinshelwood, of the Office of Scientific Publications at the Baylor Charles A. Sammons Cancer Center at Dallas for her helpful editorial comments. The present study was supported by KAKENHI (grant nos. 20590572, 25860409, 26462016 and 15H03034).

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