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Tumor Location Is Associated With the Prevalence of *Braf* And *Pik3ca* Mutations in Patients with Wild-Type *Ras* Colorectal Cancer: A Prospective Multi-Center Cohort Study in Japan

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ABSTRACT

BACKGROUND: Primary tumor location is a critical prognostic factor that also impacts the efficacy of anti-epidermal growth factor receptor (EGFR) therapy in wild-type *RAS* (*KRAS/NRAS*) metastatic colorectal cancer (CRC). However, the association between the incidence of *BRAF* and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations and primary tumor location remains unclear. **METHODS**: We prospectively collected tumor samples and clinical data of patients from 15 hospitals between August 2014 and April 2016 to investigate *RAS*, *BRAF*, and *PIK3CA* mutations using a polymerase chain reaction-based assay. According to the primary tumor location, patients were classified to right-sided (from cecum to splenic flexure) and left-sided (from descending colon to rectum) tumor groups. **RESULTS**: In total, 577 patients with CRC were investigated, 331 patients (57%) had CRC with wild-type *RAS*; of these 331 patients, 10.5%, 4.8%, and 5.9% patients harbored $BRAF^{V600E}$, $BRAF^{non\text{-}V600E}$, and *PIK3CA* mutations, respectively. *BRAF/PIK3CA* mutations were more frequent in females, patients with right-sided tumors, and patients with peritoneal metastasis cases and less frequent in patients with liver metastases. The prevalence rates of $BRAF^{V600E}$ and *PIK3CA* mutations were higher in patients with right-sided tumors than in those with left-sided tumors (32.3% vs. 4.8% and 17.2% vs. 3.6%, respectively). **CONCLUSIONS**: More than half of the patients with right-sided CRC and wild-type *RAS* harbored *BRAF/PIK3CA* mutations, including $BRAF^{non\text{-}V600E}$, which may contribute to the difference in the anti-EGFR efficacy between the right- and left-sided CRC.

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Introduction

Colorectal carcinoma (CRC) is the third most common cancer and the third most common cause of cancer-related deaths worldwide [1,2]; CRC is also the most frequent cancer in Japan with approximately 135,000 cases per year. Approximately 25% of patients have distant metastases at the time of diagnosis, and the most common site of metastasis, which develops in 50% of patients with CRC, is liver. The poor prognosis of metastatic CRC has been the driving force for the ongoing efforts to develop treatment approaches that can improve patient outcomes. Fluoropyrimidines have been the backbone of chemotherapy for CRC for more than 40 years; however, considerable progress has been achieved during the last 10 years, which led to a median overall survival (OS) of 30 months [3,4]. This improvement was due to the development of new cytotoxic agents such as oxaliplatin and irinotecan. Moreover, targeted therapies including the monoclonal antibodies against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) have shown favorable efficacy in metastatic CRC [5].

Cetuximab, a human-mouse chimeric monoclonal IgG1 antibody targeting EGFR, has significantly improved the survival of patients with metastatic CRC refractory to standard therapies with irinotecan, oxaliplatin, and fluoropyrimidines when compared to the best supportive care [6]. Furthermore, treatment with cetuximab plus irinotecan was shown to result in a higher response rate than cetuximab alone in the BOND-1 study [7]. Panitumumab, a fully human monoclonal IgG2 antibody that targets EGFR, also led to a significantly prolonged survival with manageable toxicity compared to the best supportive care in patients with CRC refractory to standard care [8].

KRAS mutations are the first validated negative predictive markers for the outcomes of anti-EGFR therapy in patients with metastatic CRC. Oncogenic *KRAS* mutations are found most frequently in codons 12 and 13 and occur in approximately 30%–45% of the tumors. The CRISTAL study, which assessed the efficacy of cetuximab plus FOLFIRI compared to FOLFIRI alone as first-line treatment revealed that cetuximab prolonged the progression-free survival (PFS) and OS compared to FOLFIRI alone in metastatic CRC; however, the benefit of adding cetuximab was limited to tumors with wild-type *KRAS* exon 2 [9]. This finding was subsequently confirmed by the prospective analysis of other randomized phase III trials which evaluated the efficacy of adding anti-EGFR therapies including cetuximab or panitumumab.

More recent analyses indicated that additional activating mutations in exons 3 and 4 of *KRAS* and exons 2, 3, and 4 of *NRAS*, another member of the *RAS* family, were negative predictive markers for the efficacy of anti-EGFR therapies [10]. These mutations have been identified in 10%–15% of tumors with wild-type *KRAS* exon 2. The PRIME study, which compared FOLFOX4 plus panitumumab as the first-line treatment to FOLFOX4 alone showed a significant survival benefit in patients with tumors harboring wild-type *KRAS* and *NRAS* [11]. In addition, the objective response rate and PFS were also favorable in tumors with wild-type *RAS* based on other phase III studies that assessed anti-EGFR therapy as any treatment line and conducted extended *RAS* analyses. The *RAS* testing is widely used in daily practice to guide treatment decisions regarding anti-EGFR therapy.

Moreover, clinical trials report that the primary tumor location plays an important prognostic role in CRC, particularly in patients with wild-type *RAS* who are treated with anti-EGFR antibodies; the studies have showed improved survival outcomes in patients with left-sided tumors [12,13]. Conversely, right sidedness is a negative prognostic factor in the efficacy of anti-EGFR therapy and may predict resistance. Therefore, primary CRC location is also recognized as a key factor in the treatment of metastatic CRC with wild-type *RAS*.

Other current predictive biomarker candidates for anti-EGFR therapy include microsatellite instability and $BRAF^{V600E}$ and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations. The prevalence of these biomarkers was also reported to be different between patients with right-sided and left-sided primary tumors [14,15]. The aim of the study was to present our institutional experience with patients with CRC who underwent clinical mutation profiling in Japan. We also evaluated the differences in the patient characteristics with $BRAF^{V600E}$ and $BRAF^{non\text{-}V600E}$ mutations.

Materials and Methods

Patients

Patients with CRC who received any treatment between August 2014 and April 2016 at one of the 15 study hospitals that participated in the Aichi Cancer Network Project were included in this prospective observational study. Tumor and nodal staging were performed according to the TNM Classification of Malignant Tumors (7th edition). The study was performed according to the ethical principles of the Declaration of Helsinki, and the study protocol was approved by the Institutional Review Boards of the participating institutions (registry number at Aichi Cancer Center Hospital; 2014–3-194). All patients provided written informed consent for participating in the study before enrollment.

PCR-Based Multiplex Assay

Genomic DNA was isolated from formalin-fixed, paraffin-embedded surgical or biopsy specimens obtained from patients with CRC. Extracted DNA samples were diluted to an approximate concentration of 10–20 ng/μL with sterile TE buffer (1 mmol/L Tris–HCL pH 8.0, 0.1 mmol/L ethylenediaminetetraacetic acid). Assays were performed according to the manufacturer's protocol using the Luminex xMAP bead-based multiplex immunoassay system (Luminex). Briefly, a 5-μL sample template was conjugated with 20 μL master mix including Taq DNA polymerase, uracil-DNA-glycosylase, and primers. For PCR, the samples were heated at 40 ˚C for 5 minutes and at 95 ˚C for 2 minutes, followed by 10 cycles of 20 seconds at 94 ˚C and 30 seconds at 62 ˚C; 45 cycles of 90 ˚C for 20 seconds, 60 ˚C for 30 seconds, and 72 ˚C for 30 seconds; 72 ˚C for 1, and 94 ˚C for 10 minutes. Approximately 5 μl of the products and 45 μl of the hybridization solution containing probe-coupled beads were hybridized for 2 minutes at 95 ˚C, followed by incubation for 30 minutes at 55 ˚C. The complexes were washed and incubated with streptavidin-phycoerythrin for 15 minutes at 52 ˚C. The median fluorescence intensities were determined for the color-coded beads and PE using the Luminex 100/200 system (Luminex, Austin, TX) to determine the mutation types and their signal intensities.

Between August 2014 and June 2015, the mutations in *KRAS* codons 12 and 13 (G12A, G12D, G12C, G12S, G12R, G12V, and G13D) were detected using the MEBGEN *KRAS* mutation detection kit (MBL, Japan) [16,17]. We also used Genosearch™ Mu-Pack™, which detects mutations in *KRAS* codons 61 (Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H) and 146 (A146T, A146S, A146P, A146E, A146V, and A146G); *NRAS* codons 12 (G12A, G12D, G12C, G12S, G12R, and G12V), 13 (G13A, G13D, G13C, G13S, G13R, and G13V), and 61 (Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H); *PIK3CA* codons 542 (E542K), 545 (E545K), 546 (E546K), and 1047 (H1047R and H1047L); and *BRAF* V600 (V600E, V600K, V600D, and V600R) [25]. Starting in July 2015, we used Genosearch™ BRAF kit to detect *BRAF* mutations other than V600, including mutations in exon 11 such as codons 464 (G464E, G464V, and G464R), 466 (G466R, G466V, and G466E), 467 (S467L), 469 (G469A, G469A, G469V, G469R, and G469E), and 485 (L485F). This multiplex kit was also used to detect other *BRAF* mutations in codons 524 (Q524L), 525 (L525R), 581(N581S, N581I, and N581T), 594 (D594N and D594G), 596 (D596R), 597 (L597R, L597S, L597V, L597Q, and L597P), 598 (A598T), 599 (T599_600insT), and 601 (V601E and V601N) [18].

Data Collection

All data were analyzed after reviewing the medical records. The following information was collected: age, sex, primary tumor location, pathology,

and clinical stage. Right-sided primary tumors were defined as those in the splenic flexure, transvers colon, ascending colon, and cecum. Left-sided primary tumors were defined as those in the descending colon, sigmoid colon, and rectum.

Statistical Analysis

The primary endpoint was comparison of the mutation statuses for *RAS*, *BRAF*, and *PIK3CA* between the right- and left-sided tumors. Fisher's exact probability test was used to analyze the differences between the groups. All reported *P* values were based on two-sided tests, and a $P < .05$ was considered to indicate statistical significance. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing).

Results

Mutation Frequencies and Distributions

The current prospective cohort study enrolled 577 patients with colorectal cancer who underwent *RAS* mutation analysis between August 2014 and August 2016. After excluding patients with *RAS*-mutant tumors (n = 235, 41%) and those with unknown *RAS* status (n = 11, 2%), 331 patients with wild-type *RAS* tumors were included in the subsequent analyses (Figure 1). The *BRAF* and *PI3KCA* mutations identified in the study cohort are summarized in Figure 1. Briefly, among the 325 patients who underwent testing for $BRAF^{V600E}$, 34 patients (10.5%) harbored the $BRAF^{V600E}$ mutation. Among the 187 patients who underwent testing for $BRAF^{non\text{-}V600E}$ mutations, 9 patients (4.8%) harbored *BRAF* mutations other than V600E, including seven (3.7%) patients with mutations in exon 15 (K601E, K601N, V600R, T599_V600insT, D594G, and N581T) and two (1.1%) patients with mutations in exon 11 (G466E and G469A) of *BRAF*. Among the 152 patients tested for *PIK3CA* mutations, 9 patients (5.9%) harbored *PIK3CA* mutations, including 5 (3.3%) and 4 (2.6%) patients with mutations in exons 9 (E542K, E545K, and E546K) and 20 (H1047R and H1047L) of *PIK3CA*, respectively. *BRAF* and *PIK3CA* mutations were mutually exclusive in our cohort.

Patient Characteristics

We compared the characteristics of the patients who were wild-type for $RAS/BRAF^{V600E}$ (wild-type group) and those harboring mutations in either *BRAF* or *PIK3CA* (mutation group). As summarized in Table 1, the frequencies of occurrence of $RAS/BRAF^{V600E}$ over either *BRAF* or *PIK3CA* were higher for female sex (62% vs. 36%), right-sided primary tumors (57% vs. 11%), differentiated histology (20% vs. 9%), and peritoneal metastasis (35% vs. 15%) in the mutation group compared with the wild-type group, whereas the frequency of liver metastases was lower in the mutation group compared with the wild-type group (37% vs. 66%).

Mutation Prevalence According to Tumor Sidedness

The study patients were categorized into the right-sided and left-sided tumor groups based on the primary tumor site. After excluding five patients with unknown primary tumor sites, the remaining cohort included 68 and 258 patients with right- and left-sided tumors, respectively. As shown in Table 2, the $BRAF^{V600E}$ and *PIK3CA* mutations were found in 32.3% and 17.2% of the patients with right-sided CRC, respectively. These rates were significantly higher than those in the patients with left-sided CRC. Conversely, the $BRAF^{non\text{-}V600E}$ mutation rates were not different between the right- and left-sided primary tumor groups (8.3% and 4.8%, respectively). The detailed mutational status of the patients with the $BRAF^{non\text{-}V600E}$ mutations is presented in Table 3.

Discussion

In the current prospective cohort study, we compared the *BRAF*/*PIK3CA* mutations and the clinical characteristics between the left- and right-sided tumors in patients with CRC harboring wild-type *RAS*. Our analyses revealed different genetic alterations associated with neoplastic transformation in CRC based on the primary tumor sidedness. Specifically, we found that the *BRAF* and *PIK3CA* mutations were more frequent in the right-sided tumors compared with the left-sided tumors, implicating that these genetic pathways might have potential roles in the mechanisms underlying differences in the clinical characteristics, survival outcomes, and anti-EGFR efficacy between the patients with right- and left-sided CRC.

$BRAF^{V600E}$, which comprises more than 90% of all *BRAF* mutations, is a well-established marker indicating extremely poor prognosis in CRC; this mutation is associated with right-sided and poorly differentiated tumors. Studies previously demonstrated that the prevalence of *BRAF* mutations was relatively lower in East Asian patients (around 5%) compared to Caucasian patients with CRC (approximately 10%) [19,20]. In the present study, we also found that the *BRAF* mutation frequencies in the entire cohort and in those with wild-type *RAS* were 5.9% and 10.5%, respectively. Notably, we detected *BRAF* mutations in more than 30% of the patients with right-sided CRC harboring wild-type *RAS*, which was quite high. The clinical practice guidelines recommend the *RAS* and $BRAF^{V600E}$ testing before the initiation of first-line chemotherapy for metastatic CRC [21,22]. Anti-EGFR therapy for patients with the $BRAF^{V600E}$ mutation remains uncertain, because meta-analyses demonstrated that evidence was insufficient

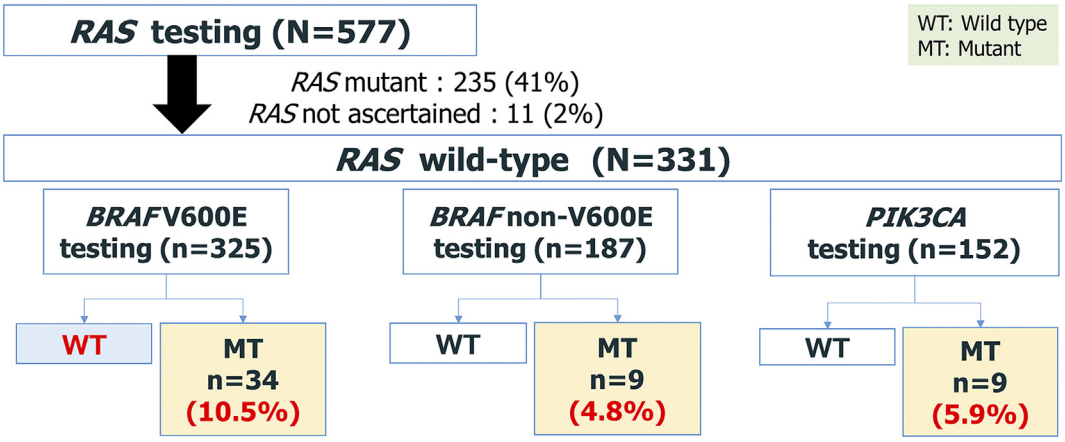

Figure 1. Study population.

Table 1
Patient characteristics

		RAS WT *BRAF* V600E WT (n = 291)	*RAS* WT *BRAF* or *PIK3CA* MT (n = 52)	*P***
Median (range) age, years		66 (30–90)	65 (40–88)	.66
Mean ± SD		64 ± 11	63 ± 13	
Sex	Male	64%	38%	<.001
	Female	36%	62%	
Primary tumor site	Right-colon	11%	57%	<.001
	Left-colon	34%	12%	
	Rectum	55%	31%	
Stage	Localized	19%	12%	.24
	Metastatic	81%	88%	
Histology*	Tub	91%	80%	.043
	Por/Muc/Sig	9%	20%	
Metastatic tumor site	Liver	66%	37%	<.001
	Lung	32%	17%	.051
	Lymph node	25%	39%	.069
	Peritoneum	15%	35%	.003

Abbreviations: WT, wild-type; MT, mutant; SD, standard deviation; Tub, tubular adenocarcinoma; Por, poorly differentiated adenocarcinoma; Muc, mucinous adenocarcinoma; Sig, signet ring cell carcinoma. * Japanese Classification of Colorectal Carcinoma-Second English Edition. **Fisher's exact test.

to definitively confirm that individuals with the *BRAF*^{V600E} mutation attained improved treatment benefits with anti-EGFR therapy [23,24]. Conversely, in patients with right-sided tumors harboring wild-type *RAS/BRAF*, anti-EGFR therapy as first-line therapy is a treatment option if tumor shrinkage is needed. Considering the high *BRAF*^{V600E} mutation prevalence in the current study, testing for *RAS* as well as *BRAF* mutations is essential for patients with metastatic CRC to determine appropriate chemotherapeutic agents, especially for those with right-sided tumors. Recently, the BEACON CRC trial and SWOG1406 trial demonstrated novel regimens including Braf inhibitor and anti-EGFR therapy resulted in significantly longer OS and a higher response rate than the standard therapy [25,26], and recommended as standard of care in pretreated metastatic colorectal cancer with *BRAF*^{V600E} mutation. Therefore, the *BRAF*^{V600E} analysis informs treatment choice beyond anti-EGFR therapy.

In the current study, we detected *BRAF*$^{non\text{-}V600E}$ mutations in 4.8% of the patients with wild-type *RAS*, which was consistent with previous reports. Cremolini et al. reported the clinical, pathological, and molecular features of metastatic CRC with *BRAF* mutations in codons 594 and 596 vs. those with the *BRAF*^{V600E} mutation, demonstrating that the *BRAF* mutations other than V600E were more frequent in the rectum, exhibited differentiated histology, and were associated with better prognosis [27]. The present study results also demonstrated that tubular adenocarcinoma and rectum were more frequent as the primary tumor location in the patients with *BRAF*$^{non\text{-}V600E}$ mutations, suggesting that CRCs with *BRAF*$^{non\text{-}V600E}$ mutations might be a distinct entity from CRCs with *BRAF*^{V600E}. Some studies reported that anti-EGFR therapy was effective for some patients with *BRAF*$^{non\text{-}V600E}$ mutations [28], whereas others reported contradictory results [29]. Given that CRC with *BRAF*$^{non\text{-}V600E}$ is a small fraction of all CRCs, more studies with larger cohorts are needed for confirmation.

Table 2
Tumor sidedness according to mutations

	Right (n = 68)	Left (n = 258)	*P**
BRAF^{V600E} mutation	32.3% (22/68)	4.8% (12/252)	<.001
BRAF$^{non\text{-}V600E}$ mutation	8.3% (3/39)	4.8% (6/148)	.40
PIK3CA mutation	17.2% (5/29)	3.6% (4/118)	.015

* Fisher's exact test.

PIK3CA is another relevant mutation in CRC, with an approximate prevalence of 15%. In the current study, we detected *PIK3CA* mutations in 5.9% of the patients with wild-type *RAS*, which was lower than the previously reported rates. There are two potential explanations for this discrepancy. First, the polymerase chain reaction (PCR)-based assay used in the current study covers only five of the mutations. Kawazoe et al. utilized the same assay to report that the prevalence of *PIK3CA* mutations was 6.4% in metastatic CRC, a rate comparable to that in the present study [30]. Second, in the current study, we focused on the patients with wild-type *RAS* who were previously reported to have lower incidence of *PIK3CA* mutations compared to patients with mutant *RAS*. In fact, we found that the patients with *PIK3CA*- as well as *RAS*-mutant CRC comprised 8.5% of the current cohort (data not shown). Several studies provide evidence that *PIK3CA* mutations, especially those in exon 20, can confer resistance to anti-EGFR therapy [31,32]. Considering the high prevalence of *PIK3CA* mutations in right-sided tumors with wild-type *RAS*, which was more than 15% in the current study, *PIK3CA* testing may also be recommended for patients with metastatic CRC, especially for those with right-sided primary tumors.

There are several limitations in the current study. First, the cohort size was small; therefore, the incidence of gene mutations, especially that of *BRAF*$^{non\text{-}V600E}$ mutations, was not confirmative. Second, we used PCR-based and not sequencing-based assays, whereas next-generation sequencing allow for more frequent detection of *BRAF*$^{non\text{-}V600E}$ and *PIK3CA* mutations. However, PCR-based assays used in the current study are relatively cheap, simple, and quality-guaranteed methods. Third, the present study did not evaluate the microsatellite instability status, a known key genetic factor in CRC. Deficient mismatch repair (MMR) status is relatively high incidence in right-sided tumors and an independent prognostic factor for favorable prognosis especially for resected colon cancer. *BRAF*$^{non\text{-}V600E}$ mutation is also known to be observed more in deficient MMR CRC, and since it is not a prognostic in the deficient MMR CRC. During study period, MMR status was not routinely tested in many clinical institutions and we cannot discuss the status in this study. Finally, information regarding the efficacy of anti-EGFR therapy in the study cohort was not available.

Conclusions

The current prospective observational study demonstrated that more than half of the patients with right-sided CRC harboring wild-type *RAS*

Table 3
Characteristics of the patients with *BRAF*$^{non\text{-}V600E}$ mutations

Age	Sex	Primary site	Stage	Metastasis site	Histology	*RAS* status	*BRAF* status
67	Female	Rectum	Localized	-	Tub	WT	G466E
74	Male	Rectum	Localized	-	Tub	WT	G469A
55	Female	Rectum	Metastatic	Lymph node	Tub	WT	N581T
39	Female	Rectum	Metastatic	Liver, pleura	Tub	WT	D594G
74	Male	Rectum	Metastatic	Liver, peritoneum	Tub	WT	D594G
68	Female	Ascending	Metastatic	Liver	Tub	WT	T599_V600insT
68	Female	Ascending	Metastatic	Lymph node	Tub	WT	V600R
79	Female	Ascending	Metastatic	Peritoneum	Tub	WT	K601N
44	Male	Rectum	Metastatic	Lung	Tub	WT	K601E

Abbreviations: WT, wild-type; Tub, tubular adenocarcinoma.

had *BRAF* or *PIK3CA* mutations, which might partially contribute to the previously reported resistance to anti-EGFR therapy observed in right-sided CRC. Therefore, testing for *RAS* as well as *BRAF* and *PIK3CA* mutations should be recommended for patients with metastatic CRC before the initiation of first-line chemotherapy for improved treatment approaches.

Declaration of Competing Interests

The authors have declared that no competing interest exists.

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