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Molecular mechanisms underlying the resistance of BRAF V600E-mutant metastatic colorectal cancer to EGFR/BRAF inhibitors

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Abstract

Background: Combinatorial inhibition of epidermal growth factor receptor (EGFR) and BRAF shows remarkable clinical benefits in patients with *BRAF* V600E-mutant metastatic colorectal cancer (mCRC). However, the tumor may inevitably develop resistance to the targeted therapy, thereby limiting the response rate and durability. This study aimed to determine the genetic alterations associated with intrinsic and acquired resistance to EGFR/BRAF inhibitors in *BRAF* V600E-mutant mCRC.

Methods: Targeted sequencing of 520 cancer-related genes was performed in tumor tissues and in plasma samples collected from patients with *BRAF* V600E-mutant mCRC, who were treated with EGFR/BRAF ± MEK inhibitors, before and after the targeted treatment. Clinical benefit was defined as an objective response or a stable disease lasting longer than the median progression-free survival (PFS).

Results: In all, 25 patients with *BRAF* V600E-mutant mCRC were included in this study. Those with *RNF43* mutations ($n = 8$) were more likely to achieve clinical benefit from EGFR/BRAF inhibitors than those with wild-type *RNF43* (87.5% *versus* 37.5%, $p = 0.034$). Genetic alterations in receptor tyrosine kinase genes ($n = 6$) were associated with worse PFS ($p = 0.005$). Among the 23 patients whose disease progressed after the EGFR/BRAF-targeted therapy, at least one acquired resistance-related mutation was detected in 12 patients. Acquired mutations were most frequently observed in the mitogen-activated protein kinase pathway-related genes ($n = 9$), including *KRAS* (G12D and Q61H/R), *NRAS* (Q61L/R/K and amplification), *BRAF* (amplification), and *MEK1* (K57T). *MET* amplification and *PIK3R1* Q579fs mutation emerged in three patients and one patient, respectively, after disease progression.

Conclusion: Multiple genetic alterations are associated with clinical benefits and resistance to EGFR/BRAF inhibitors in *BRAF* V600E-mutant mCRC. Our findings provide novel insights into strategies for overcoming resistance to EGFR/BRAF inhibitors in patients with *BRAF* V600E-mutant mCRC.

Keywords: *BRAF* mutation, colorectal cancer, drug resistance, predictive biomarker, targeted therapy

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Background

Colorectal cancer is one of the most common cancers and the second leading cause of cancer-related deaths worldwide.[1] The constitutive activation of mitogen-activated protein kinase (MAPK) pathway can lead to abnormal cell proliferation and differentiation, thereby contributing greatly to the development and progression of

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colorectal cancer.[2,3] *BRAF*, a gene involved in the MAPK signaling pathway, is mutated in approximately 10–20% of the patients with colorectal cancer.[4] The *BRAF* V600E mutation, which is caused by a c. 1799 T>A missense mutation in the *BRAF* gene, accounts for 90% of all *BRAF* mutations in colorectal cancer.[5] The V600E mutation in BRAF confers remarkably increased activity to the serine/threonine kinase, thereby inducing abnormal hyperactivity of the MAPK pathway in a RAS-independent manner.[5]

The overall survival (OS) of patients with *BRAF* V600E-mutant metastatic colorectal cancer (mCRC) is significantly shorter than that of patients with *BRAF* wild-type mCRC.[6] Efforts have been made to develop novel treatments, such as intensive chemotherapy and targeted therapy, and to improve the outcomes of patients with *BRAF* V600E-mutant mCRC. The selective RAF inhibitor, vemurafenib, had been approved by the United States Food and Drug Administration (U.S. FDA) for the treatment of *BRAF*-mutant metastatic melanoma in 2011. However, *BRAF* V600E-mutant mCRC did not respond to BRAF inhibitors and *BRAF*-mutant melanoma; the response rate was only 5% in *BRAF* V600E-mutant mCRC treated with vemurafenib. This finding was subsequently ascribed to epidermal growth factor receptor (EGFR)-mediated feedback reactivation of MAPK signaling.[7] Thus, combinatorial treatment with BRAF inhibitors and anti-EGFR antibodies, with or without MEK inhibitors, was used to improve the therapeutic efficacy against *BRAF* V600E-mutant mCRC.[7] Specifically, BRAF inhibitors, such as dabrafenib, vemurafenib, and encorafenib, in combination with cetuximab or panitumumab, exhibited better curative effects with respect to CRC, with the objective response rate ranging between 10% and 26.8%, and the progression-free survival (PFS) ranging between 3.5 and 4.5 months.[8–12] A combination of encorafenib and cetuximab was approved by the U.S. FDA – in April 2020 – for the treatment of patients with *BRAF* V600E-mutant mCRC, in whom the disease progression was observed after one or two previous regimens, based on the results of the BEACON trial.[13,14]

Although such combinatorial regimens exhibited improved therapeutic effects in patients with *BRAF* V600E-mutant CRC, not all patients could benefit from EGFR/BRAF inhibitors,[10] and till now, there is no valid clinical biomarker to predict the treatment outcomes. Furthermore, acquired resistance to EGFR/BRAF inhibitors almost inevitably appeared within 4–6 months in individuals who were initially responsive.[14,15] Therefore, elucidation of the mechanisms underlying the development of primary and secondary resistance against EGFR/BRAF inhibitors is essential for selecting potentially responsive patients and developing novel therapeutic strategies. Preclinical studies have indicated that the reactivation of the MAPK pathway is a key mechanism underlying acquired resistance to EGFR/BRAF inhibitors in *BRAF* V600E-mutant CRC.[15] Studies have reported that a variety of genetic alterations, including *EGFR* amplification, *KRAS* mutations or amplification, *MAP2K1* mutations, and *BRAF* amplification, could be acquired under the selective pressure imposed by the targeted therapy, thereby promoting the progression of *BRAF* V600E-mutant CRC.[15,16] However, whether these alterations contribute to resistance toward EGFR/BRAF inhibitors in real-world clinical practice remains to be explored.

In this study, we characterized the genetic profiles of 25 patients with *BRAF* V600E-mutant mCRC before and after receiving EGFR/BRAF inhibitors. By analyzing the genetic alterations associated with therapeutic outcomes and the mutations that were acquired after treatment, the current study supplemented the existing knowledge regarding mechanisms underlying intrinsic and acquired resistance toward EGFR/BRAF inhibitors in *BRAF* V600E-mutant mCRC.

Methods

Patients and sample collection

In total, 25 patients with *BRAF* V600E-mutant mCRC, treated with a combination of cetuximab and BRAF inhibitor (dabrafenib, vemurafenib, or encorafenib) with/without trametinib, at the Peking University Cancer Hospital, between December 2018 and December 2020, were enrolled in this study. The choice of the BRAF inhibitor was based on medical advice and patient preference. The dosage of each drug was adjusted based on treatment tolerance. Computed tomography scans were performed every 6 weeks to evaluate the treatment response in accordance with the Response Evaluation Criteria in Solid Tumors, v1.1.

Blood samples from each patient – collected before and after the targeted therapies – were

stored at −80°C until further analysis. Tumor tissues obtained from the patients – if available – were formalin-fixed and paraffin-embedded (FFPE). Baseline samples were available from 24 patients, whereas the blood sample from the remaining patient was excluded owing to the failure in extracting circulating tumor DNA (ctDNA). Among the participants, 14 patients had paired baseline tumor tissue and plasma samples, seven had only plasma specimens, and three had only FFPE specimens. All baseline tumor tissue samples were collected at the time of diagnosis and the median time from biopsy to MAPK-targeted therapy was 6.03 months [95% confidence interval (CI): 4.24–7.83 months]. In all, 21 patients provided after-progression plasma specimens, and seven of them underwent re-biopsy of the tumor tissue. For one patient, who could not provide after-progression plasma or tumor tissue, we used pleural effusion for detection (Supplemental Figure S1).

This study was approved by the Ethics Committee of Peking University Cancer Hospital (Petition number: 2020KT150). Written informed consent was obtained from each patient. Permission was obtained for the use of treatment information and biospecimens for research purposes.

Next-generation sequencing and analysis of the results

Tumor tissues and plasma samples from the 25 patients were analyzed for a customized panel of 520 cancer-related genes, as described previously.[17] Further details regarding the sequencing and bioinformatic analyses are provided in the Supplemental materials.

Statistical methods

Patients were classified into two groups (clinical benefit group and non-benefit group), based on their response to the targeted therapy. Generally, the clinical benefit rate is defined as the percentage of cases where disease control lasts longer than 6 months. However, considering the high invasiveness of *BRAF*-mutant mCRC, the criterion was not appropriate for this particular population. In this study, objective response and stable disease exceeding median PFS were classified as clinical benefits. Chi-square and *t* tests were performed to identify the genomic differences between the two groups. PFS and OS were analyzed using the Kaplan–Meier method, and the log-rank test was used to compare PFS and OS between different groups. Significance was defined as a two-sided p value <0.05. Statistical analyses were performed using R statistical software (R version 3.5.3; https://www.r-project.org/).

Results

Clinical information

In all, 25 patients with *BRAF* V600E-mutant mCRC were included in this study. The baseline clinical characteristics and treatment regimens are summarized in Table 1. Majority of the patients had undergone at least one line of systemic chemotherapy. Four of the patients were treated with MAPK-targeted therapy as first-line treatment owing to their older age or intolerance to previous adjuvant chemotherapy. Immunohistochemistry and next-generation sequencing (NGS) indicated that all patients had microsatellite stable CRC. In terms of treatment regimens, 18 patients were treated with cetuximab and vemurafenib; five were treated with a combination of cetuximab, vemurafenib, and trametinib; one was treated with a combination of cetuximab, dabrafenib, and trametinib; and one was treated with a combination of cetuximab and encorafenib.

As of April 2021, 23 patients had progressed with the combinatorial therapies. One patient manifested a complete response (CR) after 19 months of treatment and is still undergoing treatment. Six patients reported partial response (PR). The overall objective response rate was 28.0%. One patient received five cycles of cetuximab combined with vemurafenib and achieved partial regression. However, due to the outbreak of COVID-19, the patient stopped the administration of cetuximab, but continued to take vemurafenib. Interestingly, the tumor of the patient continued to shrink and the PFS had exceeded 15.67 months at the time of writing this manuscript. In all, 14 patients had stable disease as their best response. Median PFS of the entire cohort was 4.00 months (95% CI: 2.57–7.77 months) [Supplemental Figure S2(a)] and median OS was 9.57 months (95% CI: 6.47 months–not reached) [Supplemental Figure S2(b)].

Genetic profiles and predictive biomarkers of BRAF V600E-mutant mCRC

The samples obtained from 24 patients, before they received EGFR/BRAF±MEK inhibitors

Table 1. Clinical characteristics of 25 BRAF V600E mutant mCRC in this study.

Characteristics	BRAF mutant mCRC patients ($n=25$)
Age	
Median	61 (range: 35–86)
Gender	
Male	12
Female	13
Primary tumor location	
Right side	14
Left side	11
Microsatellite status	
MSI	0
MSS	25
Prior lines of therapy	
0	4
1	13
2	7
3	1
Treatment regimen	
Cetuximab + vemurafenib	18
Cetuximab + vemurafenib + trametinib	5
Cetuximab + dabrafenib + trametinib	1
Cetuximab + encorafenib	1

mCRC, metastatic colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable

(hereafter defined as pretreatment samples), were subjected to NGS using a panel of 520 cancer-related genes as reference. Among them, *TP53* (91.6%), *RNF43* (33.3%), *LRP1B* (29.1%), *SMAD4* (29.1%), *PIK3CA* (25.0%), and *APC* (25.0%) were found to be most frequently altered in *BRAF* V600E-mutant mCRC (Figure 1). *KRAS* and *NRAS* mutations were not identified in the pretreatment samples – as expected – because *RAS* and *BRAF* hotspot mutations are mutually exclusive. Mutational characteristics of the Wnt signaling pathway in patients with *BRAF* V600E-mutant mCRC were remarkably different from those in patients with *BRAF* wild-type colorectal cancers. Specifically, *APC* was mutated in only 25.0% of the patients with *BRAF* V600E-mutant mCRC (compared to approximately 80% of the entire CRC population), whereas *RNF43* was more frequently mutated in patients with *BRAF* V600E-mutant mCRC (33.3%) [compared to the entire CRC population (6–13%)].[17,18]

We classified the 24 patients into two groups based on their best response and PFS. Patients with a CR/PR or a PFS exceeding 4.00 months (the median PFS of our cohort) were classified into the clinical benefit group ($n=13$), whereas the others were classified into the non-benefit group ($n=11$). The ctDNA tumor mutational burden (TMB) was higher in the non-benefit group than that in the clinical benefit group (median TMB: 3.99 mutations/Mb *versus* 6.98 mutations/Mb, $p=0.047$) [Supplemental Figure S3(a)]. However, a similar trend was not observed in the TMB of tumor tissues ($p=0.771$) [Supplemental Figure S3(b)]. The maximal allelic fractions of ctDNA and tumor tissues in the two groups were similar [Supplemental Figure S3(c) and (d)]. *RNF43* was the only differentially mutated gene between the two groups. Of the eight patients with *RNF43* mutant mCRC, seven (87.5%) achieved clinical benefit from targeted therapy; the benefit rate for *RNF43* wild-type patients was only 37.5% ($p=0.034$) [Figure 2(a)]. Median PFS was significantly better in *RNF43* mutant mCRC (10.18 months *versus* 3.37 months, $p=0.038$) [Figure 2(b)]. The median OS was 18.93 months (95% CI: 18.23 months–not reached) and 6.47 months (95% CI: 5.27 months–not reached) in *RNF43*-mutant and wild-type tumors, respectively ($p=0.055$) [Figure 2(c)]. Interestingly, the clinical benefit rate for *APC*-mutant mCRC was lower than that for *APC* wild-type mCRC in our cohort (33.3% *versus* 61.1%, $p=0.360$), although it was not statistically significant.

We further explored the genetic characteristics that might predict the treatment efficacy for *BRAF* V600E-mutant mCRC at the pathway level. Alterations in receptor tyrosine kinase (RTK) genes were detected in six patients before treatment (one with *EGFR* amplification, one with *MET* amplification, one with *RET* amplification, one with *ERBB2* amplification, one with an *ERBB2* missense mutation, and two with *NTRK* mutations). Only one of the six patients benefited from EGFR/BRAF inhibitors, with stable disease lasting for 4.51 months (p values for benefit rate,

Figure 1. Mutation landscapes of the 24 BRAF V600E mutant mCRC (pretreatment samples). Each column represents one patient, and each row represents an alternation. The best response, treatment regimen, and sample types of each patient were indicated in the upper bars. For each patient, the left side represents the ctDNA mutational profile, and the right side represents the mutation profile of tumor tissue.
ctDNA, circulating tumor DNA; mCRC, metastatic colorectal cancer.

PFS, and OS were 0.061, 0.005, and 0.053, respectively) [Figure 2(d)–(f)]. Mutations in genes related to the PI3K pathway ($p > 0.999$) and TGF-β pathway ($p = 0.100$) were not associated with the clinical benefits of the targeted therapy. Specifically, mutations in the genes related to the PI3K pathway were detected in 15 patients; nine of these mutations were in patients of the benefit group and six were in patients of the non-benefit group. Mutations in *PIK3CA*, *AKT1*, and *PTEN* could not predict clinical benefit in our cohort, although these genes had previously been reported to be associated with resistance to BRAF-targeted therapy in CRC and melanoma.[19,20]

Mechanisms underlying acquired resistance to EGFR/BRAF inhibitors

We analyzed 29 after-progression samples (7 patients with paired after-progression tumor tissue and plasma, 14 patients with blood samples, and 1 patient with pleural effusion sample) from 22 patients whose tumors had developed resistance to combinatorial therapy with cetuximab and

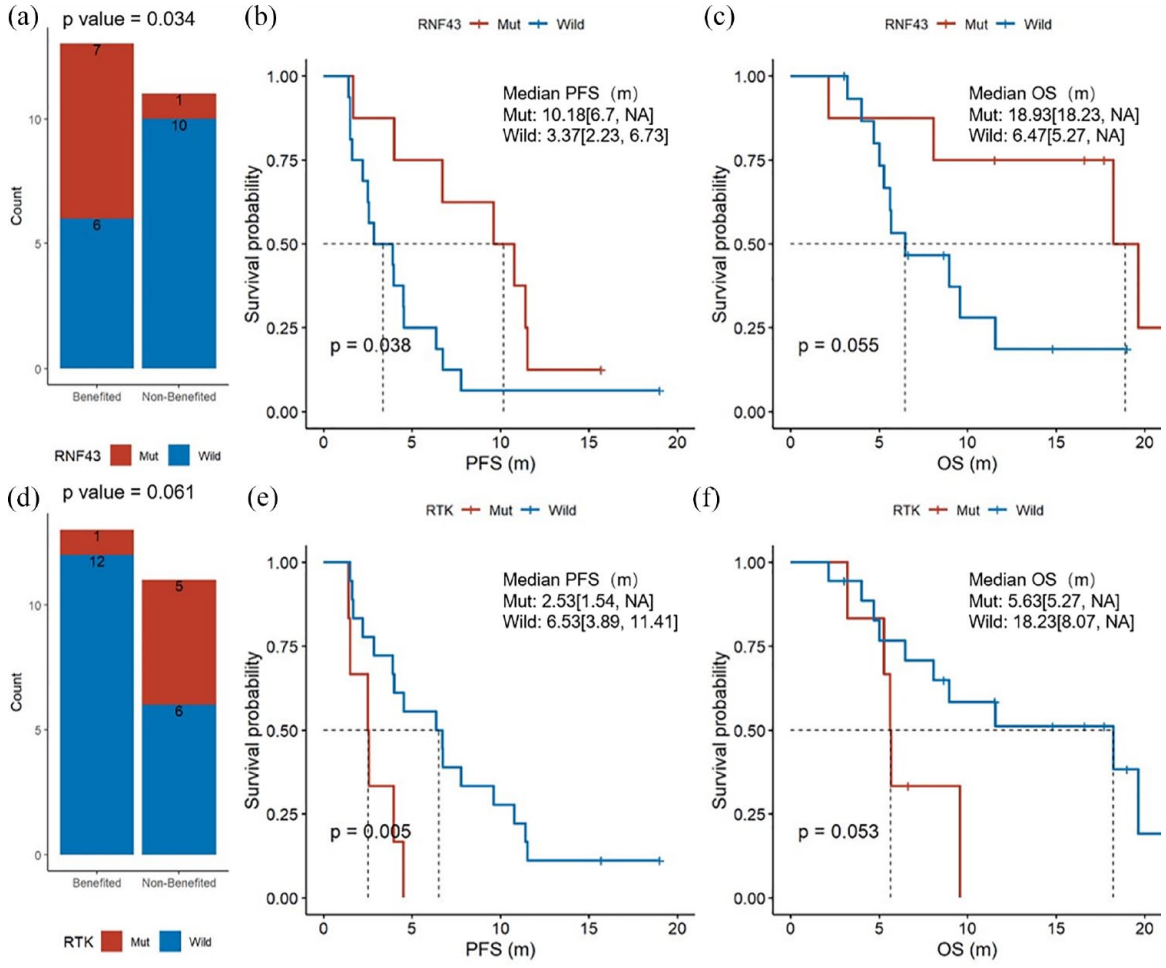

Figure 2. Genetic characteristics associated with clinical benefits. (a) RNF43 mutations in benefit group and non-benefit group. (b) PFS of RNF43 mutant and RNF43 wild-type BRAF V600E mutant mCRC. (c) Overall survival of RNF43 mutant and RNF43 wild-type BRAF V600E mutant mCRC. (d) RTK gene mutations in benefit group and non-benefit group. (e) PFS of BRAF V600E mutant mCRC with or without RTK mutations. (f) OS of BRAF V600E mutant mCRC with or without RTK mutations.
mCRC, metastatic colorectal cancer; OS, overall survival; PFS, progression-free survival; RTK, receptor tyrosine kinase.

BRAF inhibitor with and without MEK inhibitor (Figure 3). The genetic profiles of these samples were compared to those of patient-matched pre-treatment samples to identify the acquired genetic alterations in tumor cells resistant to the combinatorial therapy. ctDNA analyses of three patients yielded negative results due to insufficient ctDNA shedding and low tumor burden. Therefore, these patients were excluded from subsequent analyses.

BRAF V600E was identified in all samples obtained from the remaining 19 patients. Newly acquired mutations were detected in 12 patients (63.2%). The potential resistance-related genetic alterations are listed in Table 2. Hotspot mutations in *RAS* are the most commonly detected acquired mutations after disease progression. *NRAS* mutations (Q61L, Q61R, and Q61K) were detected in four (21.1%) patients, and *KRAS* mutations (G12D and Q61H) were observed in three (15.8%) patients. In addition, *NRAS* amplification was detected in one patient (5.3%). Focal amplification of *BRAF* was detected in three patients, with copy numbers ranging from 3.0 to 4.3. In one patient, *BRAF* amplification was identified as the only potential mechanism underlying acquired resistance. Furthermore, an acquired oncogenic *MEK* mutation (K57T) was detected in two patients, who were treated with cetuximab and vemurafenib. One of them was subsequently treated with a combination of cetuximab, dabrafenib, and

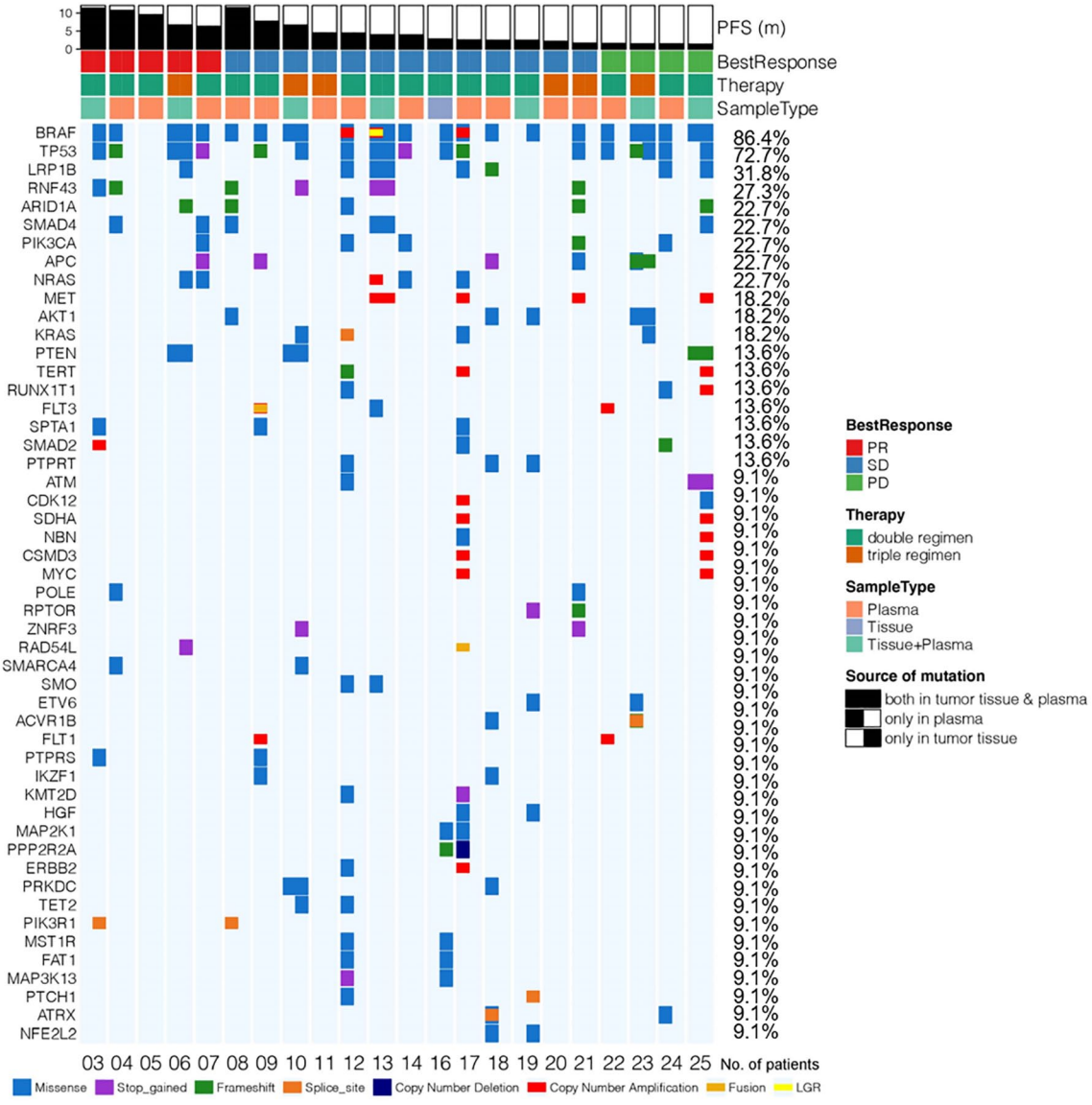

Figure 3. The landscape of molecular alternations detected in after-progression samples.

trametinib. Unfortunately, the patient experienced rapid disease progression thereafter, indicating that *MEK* mutations can also induce resistance toward triplet-targeted regimens. Taken together, 9 of the 19 patients developed resistance toward EGFR/BRAF inhibitors *via* mechanisms involving MAPK reactivation. Furthermore, amplification of *MET* was identified in four patients, with copy numbers ranging from 3.0 to 6.3. A mutation (Q579fs) in *PIK3R1* was acquired in one patient after treatment with cetuximab and vemurafenib. Among the 12 patients who acquired secondary alterations after progression, multiple resistance mechanisms were identified by ctDNA sequencing in two patients. Paired after-progression tumor tissues and ctDNA samples were obtained from one of these patients. Except for the consistently identified *MET* amplification, acquired *NRAS* and *BRAF* amplifications were only detected in ctDNA samples, but not in tumor tissues, thereby indicating that various drug-resistant alterations might occur simultaneously at different metastatic sites.

Discussion

The combination of anti-EGFR antibodies, BRAF inhibitors, and MEK inhibitors has ushered in significant benefits with respect to

Table 2. Patient, treatment regimen, outcome, and acquired alternations.

Patient no.	Age/sex	Treatment	Best of response	PFS (m)	OS (m)	Acquired mutations	
						Tumor tissue	ctDNA
21	64/F	C + V + T	SD	1.7	2.1	NA	MET amp
23	65/M	C + V + T	PD	1.5	5.7	KRAS G12D	No acquired mutation
10	56/M	C + V + T	SD	6.7	18.2	KRAS Q61H	No acquired mutation
6	61/M	C + V + T	PR	6.7	11.6	No acquired mutation	NRAS Q61L
25	86/F	C + V	PD	1.4	9.6	MET amp	No acquired mutation
13	73/M	C + V	SD	4.0	8.1	MET amp	MET amp
							NRAS amp
							BRAF amp
17	35/M	C + V	SD	2.6	5.6	NA	KRAS Q61H
							NRAS Q61R
							BRAF amp
							MET amp
							MEK1 K57T
16	40/M	C + V	SD	2.8	9.0	NA	MEK1 K57T (pleural effusion)
12	61/M	C + V	SD	4.5	Not reached	NA	BRAF amp
7	78/F	C + E	PR	6.4	Not reached	NA	NRAS Q61K
14	50/F	C + V	SD	4.0	5.3	NA	NRAS Q61K
3	53/F	C + V	PR	11.4	Not reached	PIK3R1 Q579fs	Negative*

C, cetuximab; ctDNA, circulating tumor DNA; D, dabrafenib; E, encorafenib; NA, no sample was available; PD, progression disease; PR, partial response; SD, stable disease; T, trametinib; V, vemurafenib.
*ctDNA analysis of this patient was negative.

treatment of *BRAF* V600E-mutant mCRC. However, the limited response rate and the rapid development of resistance remain to be addressed. In this study, predictive genetic biomarkers and acquired resistance mechanisms were explored, based on a real-world clinical cohort, to facilitate the treatment of *BRAF* V600E-mutant mCRC.

Many molecular characteristics, such as loss of PTEN expression, *MEK1* mutation, and cyclin D1 amplification, have been reported to be associated with the efficacy of BRAF/MEK inhibitors in melanoma.[21] A BM subtype of *BRAF* V600E-mutated CRC – defined by the transcriptional context of tumors – was reported to be able to predict the outcome of targeted therapy. The BM1 subtype tumors – characterized by KRAS/AKT pathway activation and high levels of epithelial–mesenchymal transition – showed a higher sensitivity to EGFR/BRAF/MEK inhibitors than the cell cycle checkpoint deregulated BM2 subtype tumors.[22] In our study, genetic alterations associated with the response to EGFR/BRAF inhibitors were explored in case of mCRC. We found *RNF43* mutations to be enriched in patients who had achieved favorable outcomes with EGFR/BRAF-targeted treatment. RNF43 is an E3-ubiquitin ligase that negatively regulates

the canonical Wnt/β-catenin pathway and noncanonical WNT5A signaling.[23] *RNF43* and *BRAF* mutations were the molecular events involved in the serrated neoplasia pathway during CRC development.[24] Therefore, *BRAF*-mutated CRC harbored more *RNF43* mutations and fewer *APC* alterations compared to *BRAF* wild-type CRC.[25] In addition, the mutual exclusivity between *RNF43* and *APC* mutations in *BRAF* V600E-mutant CRC was consistently observed in our and others' studies.[25] Fennell *et al.* have shown that *APC* mutations could induce aggressive biological behaviors and poor prognosis in *BRAF* V600E-mutant CRC, whereas *RNF43* mutations were associated with prolonged OS. In summary, *RNF43* mutations were associated with a better response to EGFR/BRAF inhibitors and better prognosis in *BRAF* V600E-mutant CRC, although the underlying mechanisms are yet to be clarified. One possible explanation is the different consensus molecular subtypes (CMSs) of CRC with different Wnt pathway mutations; patients with *RNF43* mutations were enriched in CMS1 (immune) and CMS4 (mesenchymal) subgroups. whereas *APC* mutations were more common in patients with CMS2 subtype (canonical) CRC.[23] Recently, Kopetz *et al.*[26] explored the molecular correlates of clinical benefit in patients with *BRAF* V600E-mutant mCRC from the BEACON study and found that CMS1 and CMS4 subtypes were associated with better response rates than others. However, they did not report the mutational characteristics of those patients. It would be very interesting to determine the CMSs subtypes of the patients in our cohort in the future, to confirm the relationship between *RNF43* mutation and CMS classification in *BRAF* V600E-mutant mCRC.

Our study identified multiple acquired genetic alterations in MAPK pathway-related genes after treatment with EGFR/BRAF inhibitors; these included *RAS* mutations or amplifications, *BRAF* amplification, and *MEK* mutations, which highlighted the significance of MAPK signaling pathway reactivation in the development of drug resistance in *BRAF* V600E-mutant mCRC. Synchronous mutations in *RAS* and *BRAF* are speculated to activate cell-cycle inhibitory proteins and increase oncogene-induced senescence.[27] Thus, RAS and BRAF mutations are mutually exclusive. The occurrence of *RAS* alterations indicates tumor heterogeneity in response to the selective pressure imposed by targeted therapies. *KRAS* and *NRAS* mutations have been detected in 19–48% of the patients with *BRAF* V600E-mutant mCRC after treatment with EGFR/BRAF ± MEK/PI3K inhibitors.[28–30] *RAS* mutations can induce RAF dimerization, resulting in sustained phosphorylation of ERK. The high incidence of acquired alterations in MAPK pathway-related genes emphasizes the significance of the profound blockade of MAPK signaling in the treatment of *BRAF* V600E-mutant mCRC. Unfortunately, the mechanisms underlying acquired resistance are mostly shared between doublet-targeted and triplet-targeted regimens. Incorporation of MEK inhibitors into the treatment regimen could not overcome the resistance conferred by upstream alterations, such as *RAS* mutations. Studies have demonstrated that ERK inhibitors can maintain a robust suppression of the MAPK pathway and inhibit the outgrowth of drug-resistant subclonal *BRAF*-mutated CRC cell lines with genetic alterations in the MAPK pathway, thus constituting a promising treatment strategy.[29] Several ongoing clinical trials have been designed to evaluate the efficacy of different combinations of ERK inhibitors (NCT04294160 and NCT02867270) in *BRAF* V600E-mutant CRC. However, the safety of these combinations still remains a major concern.

The acquisition of *MET* amplification was observed in four patients after disease progression in our cohort. *MET* amplification is known to occur in approximately 2% of the patients with CRC and can induce resistance toward EGFR-targeted therapies in patients with CRC and non-small-cell lung cancer.[31–33] Activation of the HGF/MET signaling pathway could prevent cell death and trigger resistance to BRAF inhibitors by restoring the activation of PI3K and MAPK pathways in melanoma and thyroid carcinoma.[34] Pietrantonio *et al.*[35] reported increased *MET* copy number in a patient with *BRAF*-mutated mCRC after combinatorial treatment with panitumumab and vemurafenib, and the patient achieved remarkable therapeutic effectiveness from a combination of MET inhibitor and BRAF inhibitor.[36] A preclinical study has demonstrated that ectopic overexpression of MET can result in activation of ERK signaling and confer resistance to panitumumab and vemurafenib treatment in *BRAF*-mutated CRC cell lines.[36] The safety and efficacy of combinatorial therapy with MET and BRAF is currently being evaluated in a phase I clinical trial (NCT01531361). Nevertheless, two of the four patients who acquired *MET* amplification in our study also acquired other alterations in *RAS* and *BRAF*. Therefore, the efficacy of

co-inhibition of MET and BRAF in patients who had acquired multiple resistance-related alterations would require further investigation.

In contrast to the mechanisms of resistance against EGFR tyrosine kinase inhibitors in non-small-cell lung cancer, which are dominated by a specific acquired mutation, those for BRAF inhibitors are far more complicated. Corcoran *et al.*[10] showed that in a cohort of patients with *BRAF*-mutated CRC, 6 of the 14 patients acquired more than one resistance mechanism after the failure of EGFR/BRAF-targeted therapies. Interpatient variability and heterogeneity remarkably increased the difficulty associated with overcoming treatment resistance. A combination of multiple mutation-targeted inhibitors might provide limited benefits while causing cumulative toxicity. Combinations of traditional chemotherapies or immunotherapies with targeted therapy are also promising strategies for increasing the response durability. A randomized phase III study of first-line encorafenib plus cetuximab with or without chemotherapy *versus* standard therapy in *BRAF* V600E-mutant CRC is currently underway. Other strategies, such as the use of heat shock protein 90 inhibitors and anti-apoptotic proteins (such as BCL-2), have also been reported to delay the resistance to BRAF inhibitors in melanoma.[37]

The present study had several limitations. First, the sample size was relatively small, as the *BRAF* V600E-mutant mCRC is a relatively rare malignancy. Our results would need to be further verified using large-sample studies. Second, only genetic alterations were analyzed, whereas the mechanisms underlying the development of resistance against BRAF inhibitors involve not only genetic alterations, but also epigenetic, transcriptomic, and immunological transitions, as well as changes in the tumor microenvironment.[37] Evolution of gene expression signatures, before and after the targeted therapy, might provide a more comprehensive profile of intrinsic resistance and acquired resistance mechanisms.

Despite the limitations of this study, we identified *RNF43* mutations as potential biomarkers, predicting favorable response to EGFR/BRAF inhibitors in *BRAF* V600E-mutant mCRC. Our study also suggested that acquired mutations in the MAPK pathway-related genes, RTK genes, and *PIK3R1* are important molecular hallmarks of resistance toward EGFR/BRAF inhibitors in mCRC. Therefore, genetic alterations should be monitored before and during targeted therapy in CRC, and personalized therapies should be established for patients with different resistance mechanisms.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Peking University Cancer Hospital. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

Author contribution(s)

Ting Xu: Data curation; Investigation; Project administration; Software; Writing – original draft.

Xicheng Wang: Conceptualization; Funding acquisition; Investigation; Methodology; Resources; Visualization.

Zhenghang Wang: Project administration; Resources.

Ting Deng: Resources.

Changsong Qi: Project administration; Resources.

Dan Liu: Project administration; Resources.

Yanyan Li: Data curation; Methodology.

Congcong Ji: Investigation; Methodology.

Jian Li: Supervision; Validation; Writing – review & editing.

Lin Shen: Conceptualization; Resources; Validation; Writing – review & editing.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Supplemental material

Supplemental material for this article is available online.

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