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Original Research

Reclassification of *RAS/BRAF* allele mutations predicts the survival benefit of triplet chemotherapy in metastatic colorectal cancer

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Abstract

Background: Different *RAS/BRAF* allele mutations imply distinct biological properties in various solid tumors. Recently, several studies have focused on the predictive and prognostic roles of various *RAS/BRAF* allele mutations in colorectal cancer (CRC) but the results remain controversial.

Methods: Between March 2017 and September 2022, the patients diagnosed as stages I–IV CRC with detailed medical records including next-generation sequencing (NGS) data and clinicopathological follow-up information available at our center were enrolled. Survival data were estimated using the Kaplan–Meier method, and the difference was tested in a log-rank test. Multivariate tests were carried out using Cox models.

Results: A total of 1029 CRC patients were included, and the incidence of *RAS/BRAF* mutation was 58.4%. The hypermutated cohort was defined as patients with microsatellite instability-H or POLE/D mutation. In the non-hypermutational cohort, only KRAS G13D mutation was associated with a higher incidence and inferior disease-free survival in patients with stage I-III CRC. In the cohort of patients with non-hypermutated metastatic colorectal cancer (mCRC), we assessed the risk of various RAS/BRAF allele mutations and subsequently reclassified patients into four groups based on first-line median progression-free survival: wild type (group 1), low-risk *RAS*/BRAF mutation (group 2, RAS/BRAF mutations other than *KRAS* G13D/G12V/G12C or BRAF V600E), high-risk *RAS* mutation (group 3, *KRAS* G13D/G12V/G12C), and *BRAF* V600E mutation (group 4). mCRC patients with high-risk *RAS* mutation could significantly benefit from intensive triplet chemotherapy (hazard ratio, 2.54; 95% confidence interval, 1.36–5.12; $p = 0.0091$).

Conclusion: In the non-hypermutated CRC cohort, the prognostic risk of various *RAS/BRAF* allele mutations varied between local and metastatic CRC. *KRAS* G13D mutation tended to be the only prognostic marker for stages I–III CRC; however, *KRAS* G13D/G12V/G12C mutations collectively defined a high-risk subgroup of mCRC patients with poor prognosis, who would benefit from intensive triplet chemotherapy.

Keywords: colorectal cancer, non-hypermutation, *RAS/BRAF* allele mutation, triplet chemotherapy regime

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Introduction

Predominant oncogene *RAS*/*BRAF* mutations occur in 50–60% of colorectal cancer (CRC) patients[1,2] and are considered negative biological markers due to anti-epidermal growth factor receptor (anti-EGFR) therapy resistance and poor prognosis.[3,4] The frequency of the *RAS*/*BRAF* gene and their allele mutations was highly variable across tumor types.[5] For most cancers, such as CRC and lung cancer, *KRAS* is the dominant mutant but in melanoma and thyroid cancer, *NRAS* mutation is more common.[6,7] In lung cancer, *KRAS* G12C is the most popular mutated codon, but in CRC, the top five prevalent *RAS*/*BRAF* allelic mutations are *KRAS* G12D, *KRAS* G12V, *KRAS* G13D, *BRAF* V600E, and *KRAS* G12C.[5,8] Therefore, with the popularization of NGS in precision medicine, our attention to *RAS*/*BRAF* mutations should not be limited to the gene level but should be refined to the allele level.

Recently, an increasing number of studies have shown that different *RAS*/*BRAF* allele mutations have distinct biological functions and lead to disparate clinical outcomes in cancers. *KRAS* mutations at different codons showed different *RAF* affinities and GTP intrinsic hydrolytic activities. G12A/G12V/G12R/Q61 showed high *RAF* affinity, while the G12R/G12D/G12V mutation showed low affinity. According to the intrinsic hydrolytic activity of GTP, the mutant sites could be divided into a high-activity group (G12C, G12D, and G13D) and a low-activity group (G12A, G12R, G12V, Q61L, and Q61H). Studies have shown that high RAF affinity and low intrinsic Guanosine Triphosphatase (GTPase) activity will exhibit more intense and sustained RAF activation.[9] Meanwhile, the genetic commutation network of oncogenic *KRAS* mutations is allele- and tissue-specific. In CRC, *KRAS* allele-specific derived networks are weaker than those in lung cancer, with only a few genes involved in integral *KRAS* signaling pathways linking the alleles together, including an increased co-mutation interaction between *KRAS* G12A and *MAP2K3*, a reduced co-mutation interaction between *KRAS* G12D and ERBB4, and a very strong increased rate of co-mutation between *KRAS* G12C and *STK11*.[10] There are discrepancies in the main downstream activation pathways and activation intensities at different *KRAS* site mutations.[11,12] In non-small-cell lung cancer, the *KRAS* G12D mutation activated *PI3K*/*AKT*, whereas the *KRAS* G12C mutation decreased growth-factor-dependent Protein Kinase B (AKT) activation.[13] Among various *NRAS* allelic mutations, discrepancies in biochemical and signaling properties were also observed.[14] Approximately 200 *BRAF* mutant alleles have been identified in human tumors. Based on their mechanisms of activation, oncogenic *BRAF* mutants may be divided into three categories.[15] The heterogeneity of the biological function of *RAS*/*BRAF* allele mutations means that site-specific considerations are required for their value in tumor prognosis and treatment decision-making.

Previous research has shown that the *KRAS* codon 12 mutation, but not the codon 13 mutation, is associated with a worse prognosis in CRC[16]; however, other studies have shown opposite results: the *KRAS* G13D mutation exhibited the worst prognosis.[17,18] Some heterogeneous retrospective series have evaluated the prognostic value of *KRAS* G12D with inconsistent results.[19,20] The prognostic value of G12C for CRC is also controversial.[21–23] By contrast, the conclusion that the *KRAS* G12V mutation is associated with greater aggressiveness and poorer outcomes was relatively consistent[24–26] as well as *BRAF*-V600E.[27] In addition to prognosis, different *RAS*/*BRAF* mutation alleles lead to different drug susceptibilities. While *KRAS*/*NRAS* mutations have been considered to be nonresponsive to anti-EGFR therapy, it was shown that patients with *KRAS* G13D mutations may benefit from cetuximab.[28] The classification of *BRAF* mutant alleles determines their sensitivity to inhibitors; class 1 *BRAF* mutations (*BRAF* V600 mutations) are *RAS*-independent, signal as monomers, and are sensitive to current RAF 'monomer' inhibitors; class 2 *BRAF* mutants are *RAS*-independent, signal as constitutive dimers, and are resistant to vemurafenib but may be sensitive to novel *RAF* dimer inhibitors or *MEK* inhibitors; class 3 *BRAF* mutants have impaired kinase activity or are very sensitive to ERK-dependent feedback of *RAS* and are also resistant to vemurafenib and may be effectively treated with combinations that include inhibitors of the RTKs responsible for driving *RAS* activation.[15]

FOLFOXIRI plus targeted therapy has become one of the standard treatment options for mCRC recommended by the European Society of Medical Oncology and American Society of Clinical Oncology clinical practice guidelines.[29,30] Patients receiving the intensive triplet regimen have a better objective response rate, progression-free survival (PFS), and overall survival (OS).[31] However, strong chemotherapy-related grades

3–4 toxicities limit its clinical use. Therefore, we need to accurately identify patients who can benefit from a triplet regimen. The *BRAF* V600E mutation accounts for approximately 95% of all *BRAF* mutations in CRC and is responsible for its poor prognosis.[8,32] *BRAF* V600E is a reliable predictor for triplet chemotherapy but studies have been inconsistent on whether mCRCs with *RAS* mutations benefit from this regimen. The TRIBE 2 study showed that the combination of triplet chemotherapy and bevacizumab was a better choice for patients with *RAS* mutation or right-sided colon cancer.[33–35] A subsequent large meta-analysis confirmed the above conclusions.[36] However, in another study, multivariate analysis showed that *RAS* mutation status was not a predictive factor for triplet chemotherapy.[37] Given the poor prognosis of *KRAS* G12C, a recent study suggests that this subgroup of mCRCs may benefit from intensive triplet chemotherapy compared to standard doublet chemotherapy.[38] We speculate that the reason for this inconsistency is that mCRC with RAS mutation is a very heterogeneous population due to various alleles.

Therefore, we conducted a large retrospective study to estimate the prognostic and predictive roles of various *RAS*/*BRAF*-mutated alleles in CRC. Based on the first-line median progression-free survival (mPFS) of metastatic CRC patients, we reclassified *RAS*/*BRAF* allele mutations and explored valid treatment regimens for different groups.

Methods

Patients and study design

From March 2017 to September 2022, patients who were diagnosed with CRC and had available genomic data as well as clinicopathological follow-up information at the First Affiliated Hospital of Zhejiang University were included in our study. All data were retrospectively collected from electronic medical records, and the inclusion criteria were as follows: (a) histologically confirmed CRC, (b) patients with available genomic status based on NGS, using Onco-Screen Plus panel or the Colon Core panel, (c) baseline as well as post-treatment imaging was accessible at our center for efficacy evaluation, and (d) adequate organ function. Exclusion criteria were as follows: (a) multiple primary malignancies, (b) *RAS*/*BRAF* multisite mutations, (c) patients have available genomic status but not based on NGS or not using Onco-Screen Plus panel or the Colon Core panel, (d) less than 3 months of follow-up as of September 2022, and (e) irregular monitoring rhythmicity. The study was approved and supervised by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (No. IIT20210185B), and carried out in accordance with the Declaration of Helsinki. Due to the retrospective nature of our study, written informed consent was exempted. We have followed the relevant Equator guidelines and the reporting of this study conforms to STROBE guidelines (https://www.equator-network.org/)[39] (Supplemental Table S1).

Genomic status

RAS/BRAF mutation and hypermutation status were identified using the Onco-Screen Plus panel or the Colon Core panel that targeted 520/41 cancer-related genes, based on surgically resected or punctured tissue of cancer, provided by Burning Rock, a clinical laboratory based in Guangzhou, China. The hypermutated cohort was defined as those harboring either microsatellite instability (MSI)-H or pathogenic POLE/D mutations. The DNA isolation and targeted sequencing procedures were conducted at Burning Rock Biotech, a commercial clinical laboratory that holds accreditation from the College of American Pathologists and certification from the Clinical Laboratory Improvement Amendments. The process of target capture involved the utilization of a commercially available panel comprising 520/41 genes associated with cancer, which collectively spanned 1.64 megabases of the human genome. The obtained sequence data were aligned to the reference human genome using Burrows-Wheeler Aligner version 0.7.10. Subsequently, local alignment optimization, duplication marking, and variant calling were conducted using Genome Analysis Tool Kit version 3.2 (Manufacturer: Broad Institute) and VarScan version 2.4.3 (Manufacturer: Koboldt Laboratory at Washington University School of Medicine). To identify somatic variants, the tissue samples were compared against their respective white blood cell controls. In addition, an in-house algorithm called markSV was employed for the analysis of structural rearrangements.

Outcomes

The response was assessed according to the Response Evaluation Criteria version 1.1. Disease-free survival (DFS) was defined as the interval between surgery and the emergence of locoregional

failure, distant metastasis, or death from any cause. PFS was defined as the time from first-line chemotherapy initiation to disease progression or death from any cause. Our follow-up protocol is based on National Comprehensive Cancer Network (NCCN) guidelines: for stage I CRC patients, CEA was tested every 6–12 months for a total of 5 years, and chest/abdominal/pelvic computed tomography (CT) was performed when CEA is abnormal or clinical symptoms are present. For stages II–III CRC patients, CEA was examined every 3–6 months for 2 years and every 6 months thereafter for a total of 5 years; chest/abdominal/pelvic CT scans were performed every 6–12 months for a total of 5 years. For stage IV CRC patients, who received first-line therapy are evaluated for efficacy every 2 months based on CEA and chest/abdominal/pelvic CT scans. For all patients, colonoscopy was performed in 1 year after surgery, if advanced adenoma, repeat in 1 year, if no advanced adenoma, repeat in 3 years, then every 5 years.

Statistical analysis

We used the χ^2 test or Fisher exact test to estimate differences between categorical values, and the χ^2 test, *t*-test, and Mann–Whitney to assess differences between wild-type and *RAS*/*BRAF* allele mutations. Survival estimates for the study population were generated using the Kaplan–Meier method. The association of several variables with DFS was assessed using the COX proportional risk regression model, and clinicopathological variables of known prognostic significance, such as age, sex, T-stage, primary tumor location (colon or rectum), regional lymph node status, CEA level, and metastatic organs, were tested for stepwise exclusion, and those that were statistically significant in univariate analysis ($p > 0.05$) were excluded. Variables were retained in the multivariate model. The independence of independent factors was assessed by calculating the previously described β coefficients. All analyses were performed using Statistical Package for the Social Sciences (SPSS) statistical software (version 26; IBM) and R (version 3.5.3). All tests were two-sided, with $p < 0.05$ defined as statistically significant.

Results

RAS/BRAF *allele landscape*

Between March 2017 and September 2022, a total of 1029 CRC patients were identified, and 59 patients were excluded due to multiple primary tumors ($n = 54$) and no baseline data ($n = 5$) [Figure 1(a)]. Finally, 970 patients met the criteria and were enrolled in the subsequent analysis. Baseline characteristics of the population are listed in Table 1. The incidence of MSI was 57 (5.8%), and the incidence of *POLD*/*E* was 11 [1.1%, 7 MSI-H, 4 microsatellite stable (MSS)]. *KRAS* G13D (20.3% *versus* 9.6%, $p = 0.008$) and *BRAF* V600E (11.86% *versus* 6.08%, $p = 0.040$) occurred more frequently in the hypermutated cohort than in the non-hypermutated cohort, while *KRAS* G12V (0% *versus* 10.6%, $p = 0.008$) showed the opposite tendency (Supplemental Table S2). Considering the distinct biological characteristics of the hypermutated cohort, we excluded this cohort and further explored the roles of *RAS*/*BRAF* mutations in the non-hypermutated cohort. In the 880 non-hypermutated CRCs, the incidence of *KRAS*, *NRAS*, and *BRAF* mutations was 47.6%, 3.3%, and 7.1%, respectively. The most prevalent *KRAS*-mutated alleles were *KRAS* G12D (14.7%), *KRAS* G12V (10.7%), *KRAS* G13D (9.8%), *KRAS* G12C (3.2%), and *KRAS* A146T (2.0%). The most prevalent N*RAS*-mutated alleles were N*RAS* Q61K (0.8%) and N*RAS* G12C (0.6%). The most prevalent *BRAF*-mutated allele was *BRAF* V600E (6.1%). The remaining mutated *RAS*/*BRAF* alleles were classified separately in the '*KRAS*/N*RAS*/*BRAF*-Other' group [Figure 1(b)].

KRAS *G13D was the only mutated allele related to higher incidence and inferior DFS in stages I–III CRC patients*

There are different frequencies of *RAS*/*BRAF* site mutations between stages I–III and stage IV CRCs. The incidence of *KRAS* G13D was significantly higher in stages I–III CRCs than in stage IV CRCs (13.1% *versus* 6.5%, $p = 0.001$) [Figure 2(a) and (b), Supplemental Table S3]. By September 2022, disease progression had occurred in 184 patients (43.8%) in the stages I–III population. Among the patients with stages I–III disease, *KRAS* G13D variant was significantly associated with inferior DFS [median 18.0 (95% CI, 14.0–22.0) months *versus* 23.0 (95% CI, 19.3–26.7) months *versus* 21.0 (95% CI, 15.0–25.0) months ($p = 0.0034$) compared with wild-type and non-*KRAS* G13D variant [Figure 2(c) and (e)]. However, no statistically significant differences were observed for *BRAF* V600E or other *RAS*/*BRAF* allele mutations [Figure 2(d), Supplemental Figure S1]. In the multivariate

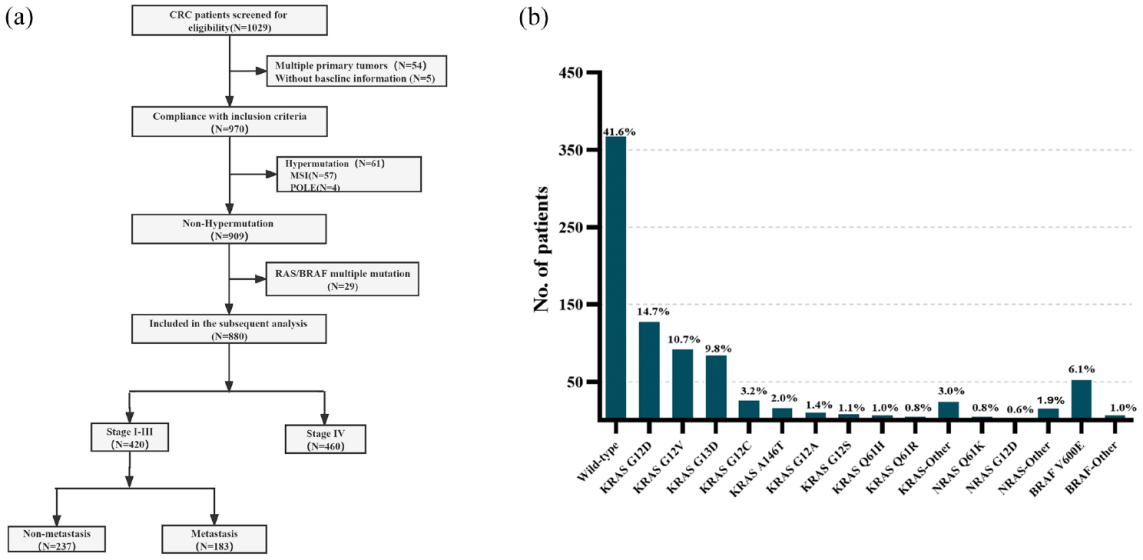

Figure 1. (a) Flowchart of 1029 patients with CRC receiving NGS and (b) frequency of RAS/BRAF site mutation variant in 880 patients with CRC (only mutation sites which were found in more than five patients were listed in the figure).
CRC, colorectal cancer.

Table 1. Baseline clinical characteristics of patients.

Characteristics	*N* = (%)
Patients	*N* = 970
Age	
⩽65	589 (60.7%)
>65	381 (39.3%)
Sex	
Male	624 (64.3%)
Female	346 (35.7%)
Mutate cohort	
MSS	909 (93.7%)
MSI	57 (5.8%)
POLE	4 (0.4%)
Oncogene	
Wild type	386 (39.8%)
KRAS mutation	454 (46.8%)
NRAS mutation	29 (3.0%)
BRAF mutation	75 (7.7%)
Multisite mutation	29 (2.9%)
Right	261 (26.9%)
Left	698 (72.0%)
Missing	11 (1.1%)
Histology	
AC	792 (81.6%)
MC/SRCC	147 (15.2%)
Missing	31 (3.2%)
Tumor staging	
I–III	481 (49.6%)
IV	489 (50.4%)
Metastatic	
Simultaneous	489 (50.4%)
Metachronous	197 (20.3%)

(Continued)

Table 1. (Continued)

(Continued)

Table 1. (Continued)

Characteristics	*N* = (%)
First-line therapy	
5-Fu + OX/IRI + targeted drug	303 (45.4%)
5-Fu + OX + IRI + targeted drug	81 (12.1%)
Metastatic sites	
Liver (%)	423 (63.3%)
Liver limited (%)	249 (37.3%)
Lung (%)	181 (27.1%)
Lung limited (%)	57 (8.5%)
Peritoneum (%)	183 (27.4%)
Peritoneum limited (%)	99 (14.8%)
Distance lymph nodes (%)	117 (17.5%)
Distance lymph nodes-limited (%)	40 (6.0%)
<1 organs involved (%)	453 (67.8%)
⩾2 organs involved (%)	215 (32.2%)

AC, adenocarcinoma; IRI, irinotecan; MC, mucinous adenocarcinoma; MSI, microsatellite instable; MSS, microsatellite stable; OX, oxaliplatin; SRCC, signet-ring cell carcinoma; POLE, polymerase epsilon; KRAS, kirsten rat sarcoma viral oncogene homolog; NRAS, neuroblastoma ras viral oncogene homolog; BRAF, b-raf proto-oncogene.

analysis, *KRAS* G13D variants remained significantly prognostic for DFS [hazard ratio (HR), 3.735, 95% confidence interval (CI), 1.468–9.505, $p = 0.006$] [Table 2(a)].

Reclassification of RAS/BRAF*-mutated alleles based on first-line mPFS in metastatic CRC patients*

Multivariate analysis identified the following factors as significantly associated with mPFS of synchronous mCRC: CEA, peritoneum metastasis, number of distal metastatic organs, *RAS* mutation, and *BRAF* mutation. Further analysis based on mutated alleles found that only *BRAF* V600E, *KRAS* G13D, *KRAS* G12V, and *KRAS* G12C mutants were significantly associated with worse mPFS than the wild-type state [Table 2(b)]. The mPFS ranked *BRAF* V600E at 6 months (95% CI, 4.8–7.1, $p < 0.0001$), *KRAS* G13D at 9.0 months (95% 7.6–10.3, $p = 0.0004$), *KRAS* G12V at 10.0 months (95% CI, 8.2–11.7, $p < 0.001$), and *KRAS* G12C at 11.0 months (95% CI, 5.5–16.5, $p = 0.0094$) [Figure 3(a)–(d)]. Based on the above results and multivariate analysis results of mPFS, we regrouped *RAS*/*BRAF* mutations: *BRAF* V600E was still defined as the group with the worst prognosis, *KRAS* G13D/G12V/G12C were classified as high-risk *RAS* mutations with the second worst prognosis, and *KRAS* G12D/A146T, N*RAS*, and *RAS*/*BRAF*-Other were classified as low-risk *RAS*/BARF mutations [Table 2(b), Supplemental Table S4]. The results were also consistent when all patients with simultaneous or heterogeneous metastases were included (Supplemental Figure S3). Therefore, all patients with mCRC were reclassified into four groups: wild type (group 1), low-risk *RAS*/*BRAF* mutation (group 2), high-risk *RAS* mutation (group 3), and *BRAF* V600E mutation (group 4) (Supplemental Table S4). After regrouping, there was a statistically significant difference in mPFS between the four groups [$p < 0.0001$, Figure 3(e)], as well as between the high-risk *RAS* and low-risk *RAS*/*BRAF* groups [$p < 0.0019$, Figure 3(f)]. Moreover, there was no statistically significant difference in mPFS between alleles in the high-risk *RAS* or low-risk *RAS*/*BRAF* group [Figure 3(g) and (h)]. All of the above results prove that our grouping is reasonable. The results were also consistent when all patients with simultaneous or heterogeneous metastases were included.

Then, we further analyzed whether the reclassification of *RAS*/*BRAF* allele mutations was associated with the clinical and pathological characteristics of CRC patients. In this study, the primary tumor located on the right colon, aggressive histopathology (mucinous adenocarcinoma; signet-ring cell carcinoma), peritoneal metastasis, and distant lymph nodes were the variables different among the four groups (Table 3). However, no difference was found between group 2 and group 3, so we speculate that group 4 (*BRAF* V600E) may be responsible for these differences (Supplemental Table S5).

Intensive triplet regimens significantly improved the mPFS of mCRC patients in group 3

A total of 363 mCRC patients received first-line oxaliplatin- or irinotecan-based chemotherapy combined with targeted therapy. Among them,

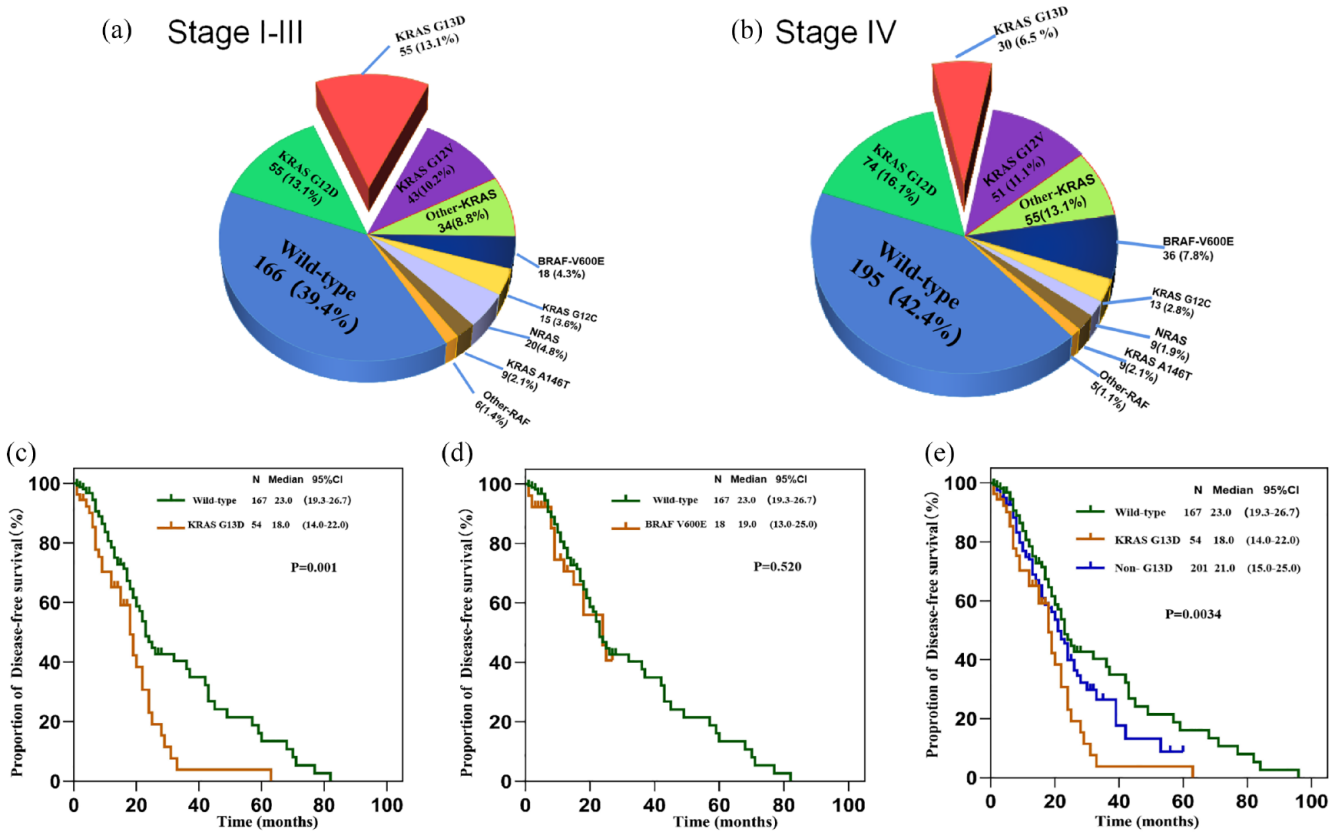

Figure 2. Frequencies of RAS/BRAF mutation subtypes in I–III (a)/IV stage (b) CRC. DFS between KRAS G13D (c)/BRAF V600E (d)/KRAS G13D (e), and wild type in I–III stage CRC.
CRC, colorectal cancer; DFS, disease-free survival.

Table 2. Univariate and multivariate analyses of prognostic factors in non-hypermutated I–III/IV stage CRC.

(a) DFS of non-hypermutated I–III CRC.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Age (⩽65 *versus* >65)	0.939 (0.702–1.257)	0.673		
Sex (male *versus* female)	0.068 (0.562–1.021)	0.068		
CEA (<100 ng/mL *versus* ⩾100 ng/mL)	0.791 (0.412–1.516)	0.479		
Primary site (right *versus* left)	1.050 (0.746–1.478)	0.778		
Histology (AC *versus* MC + SRCC)	1.308 (0.869–1.969)	0.198		
T stage (T1–2 *versus* T3–4)	0.876 (0.548–1.282)	0.496		
N stage (N0 *versus* N+)	0.666 (0.454–0.975)	0.036		
TNM stage(1–2 *versus* 3–4)	0.673 (0.459–0.986)	0.042		
Differentiation (low *versus* high)	0.848 (0.578–1.245)	0.400		
Perineural invasion (yes *versus* no)	3.206 (1.792–5.736)	<0.0001	3.049 (1.583–5.871)	0.001

(Continued)

Table 2. (Continued)

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	***p*-Value**	**HR (95% CI)**	***p*-Value**
Vascular cancer (yes *versus* no)	2.519 (1.459–4.351)	0.001	2.934 (1.374–6.268)	0.005
Tumor germination(G1–2 *versus* G3–4)	0.716 (0.362–1.419)	0.339		
RAS subtype				
RAS *versus* WT	1.510 (1.104–2.065)	0.010	1.271 (0.377–4290)	0.699
KRAS *versus* WT	1.467 (1.067–2.017)	0.018	1.000 (0.276–3.625)	1.000
G12D *versus* WT	1.328 (0.851–2.072)	0.212		
G13D *versus* WT	1.903 (1.244–2.911)	0.003	1.908 (1.208–3.108)	0.006
G12V *versus* WT	1.549 (0.935–2.566)	0.089		
G12C *versus* WT	0.927 (0.370–2.323)	0.871		
A146T *versus* WT	1.830 (0.730–4.589)	0.197		
Other KRAS *versus* WT	0.973 (0.464–2.040)	0.941		
BRAF V600E *versus* WT	1.246 (0.613–2.532)	0.543		
NRAS *versus* WT	2.143 (1.083–4.243)	0.029	2.300 (1.073–4.933)	0.032

(b) PFS of non-hypermutated IV CRC.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	***p* Value**	**HR (95% CI)**	***p* Value**
Age (⩽65 *versus* >65)	0.882 (0.679–1.146)	0.347	–	–
Sex (male *versus* female)	1.154 (0.877–1.519)	0.305		
CEA (⩾200 ng/mL *versus* >200 ng/mL)	1.425 (1.012–2.006)	0.042	1.469 (1.042–2.070)	0.028
Primary site (right *versus* left)	1.219 (0.912–1.628)	0.181		
Histology (AC *versus* MC + SRCC)	0.753 (0.542–1.047)	0.091		
Liver-M (yes *versus* no)	1.075 (0.804–1.436)	0.627		
Only-liver-M (yes *versus* no)	0.759 (0.583–0.988)	0.400		
Lung-M (yes *versus* no)	1.120 (0.838–1.497)	0.445		
Only-lung-M (yes *versus* no)	0.659 (0.338–1.284)	0.221		
Peritoneum-M (yes *versus* no)	1.437 (1.054–1.959)	0.022	1.473 (1.079–2.009)	0.015
Only-peritoneum-M (yes *versus* no)	0.895 (0.559–1.433)	0.643		
DLN-M (yes *versus* no)	1.044 (0.758–1.439)	0.791		
Only-DLN-M (yes *versus* no)	0.643 (0.358–1.156)	0.140		
Metastatic sites (⩽1 *versus* ⩾2)	0.585 (0.450–0.762)	0.000	0.606 (0.457–0.805)	0.001

(Continued)

Table 2. (Continued)

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
KRAS subtype				
KRAS *versus* WT	1.792 (1.315–2.370)	0.000	1.816 (1.373–2.404)	0.001
G12D *versus* WT	1.519 (1.055–2.187)	0.024	1.380 (0.949–2.008)	0.092
G13D *versus* WT	2.216 (1.240–3.961)	0.007	2.079 (1.161–3.722)	0.014
G12V *versus* WT	2.701 (1.732–4.213)	0.000	2.460 (1.567–3.862)	<0.001
G12C *versus* WT	2.640 (1.266–5.507)	0.010	2.640 (1.266–5.507)	0.010
A146T *versus* WT	0.962 (0.351–2.632)	0.962		
Other KRAS *versus* WT	1.368 (0.800–2.338)	0.253		
BRAF(V600E)*versus* WT	3.299 (2.040–5.337)	0.000	3.462 (2.142–5.595)	<0.001
NRAF *versus* WT	1.120 (0.353–3.552)	0.847		

AC, adenocarcinoma; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; TNM, tumor-node-metastasis; CI, confidence interval; DFS, disease-free survival; DLN, distant lymph node metastasis; HR, hazard ratio; M, metastatic; MC, mucinous adenocarcinoma; PFS, progression-free survival; SRCC, signet-ring cell carcinoma; WT, wild type; RAS, rat sarcoma viral oncogene homolog.

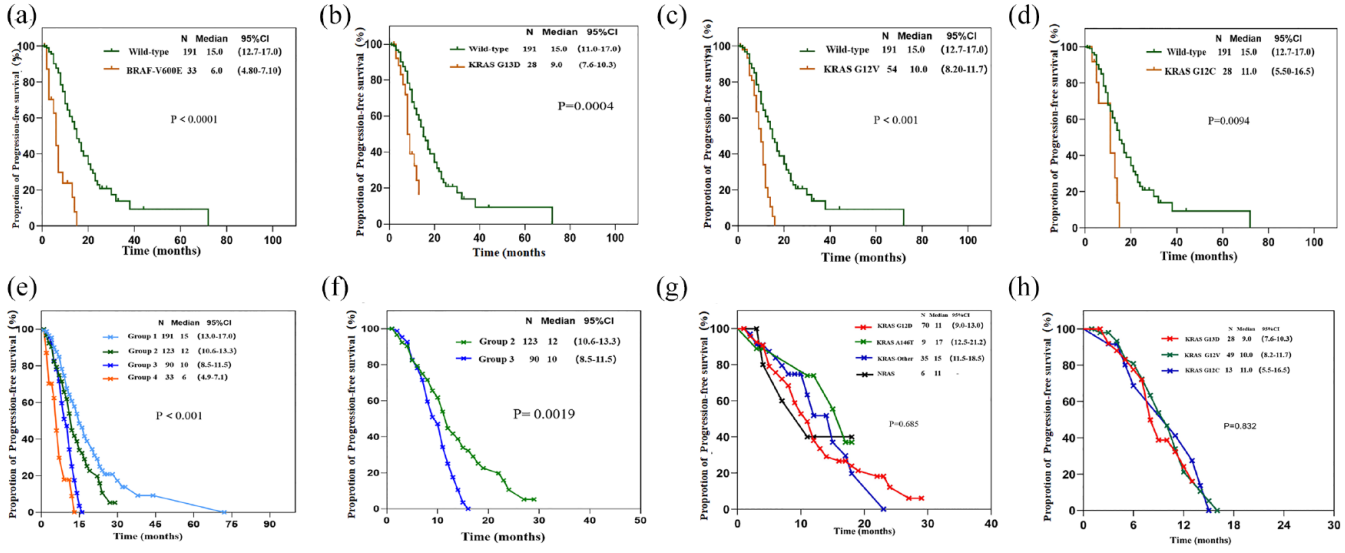

Figure 3. Kaplan–Meier survival curves in RAS/BRAF gene locus mutation in IV stage CRC. (a) PFS according to BRAF V600E/wild type; (b) PFS according to KRAS G13D/wild type; (c) PFS according to KRAS G12C/wild type; (d) PFS according to KRAS G12V/wild type; (e) PFS between group1/2/3/4; (f) PFS between group2/3; (g) PFS in group 2 (KRAS G12D/A146T/other KRAS/NRAS locus mutations); and (h) PFS in group 3 (KRAS G13D/G12V/G12C locus mutations).
CRC, colorectal cancer; PFS, progression-free survival.

Table 3. Baseline clinical characteristics of patients after regroup.

Characteristics	Group 1	Group 2	Group 3	Group 4	*p* Value
Patients, *N* (%)					
874	367 (42.0)	246 (28.1%)	207 (23.7%)	54 (6.2%)	
Age					
⩽65	225 (61.4%)	136 (55.3%)	114 (55.1%)	37 (68.5%)	0.14
>65	142 (38.6%)	110 (44.7%)	93 (44.9%)	17 (31.5%)	
Sex					
Male	255 (69.5%)	156 (63.4%)	123 (59.4%)	38 (70.4%)	0.072
Female	112 (30.5%)	90 (36.6%)	84 (40.6%)	16 (29.6%)	
Location of primary					
Left	300 (81.7%)	169 (68.7%)	155 (74.9%)	31 (57.4%)	0.000
Right	61 (16.6%)	77 (31.3%)	49 (23.7%)	23 (42.6%)	
Missing	6 (1.6%)		3 (1.4%)		
Histology					
AC	319 (86.9%)	203 (82.5%)	179 (86.5%)	33 (61.1%)	0.000
MC/SRCC	38 (10.4%)	34 (13.8%)	24 (11.6%)	16 (29.6%)	
Missing	10 (2.7%)	9 (3.7%)	4 (1.9%)	5 (9.3%)	
Tumor staging					
I–III	165 (45.0%)	116 (47.2%)	113 (54.6%)	18 (33.3%)	0.024
IV	202 (55.0%)	130 (52.8%)	94 (45.4%)	36 (66.7%)	
Metastatic					
Simultaneous	202 (55.0%)	130 (52.8%)	94 (45.4%)	36 (66.7%)	0.257
Metachronous	66 (18.0%)	49 (19.9%)	61 (29.5%)	5 (9.3%)	
First-line therapy					
5-Fu + OX + targeting	121 (33.0%)	89 (36.2%)	65 (31.4%)	12 (22.2%)	0.241
5-Fu + OX + IRI + targeting	28 (7.6%)	13 (5.3%)	16 (7.7%)	19 (35.2%)	
5-Fu + IRI + targeting	4 (1.1%)	7 (2.8%)	3 (1.4%)	0 (0%)	–
Metastatic sites					
Liver (%)	186 (50.7%)	103 (41.9%)	92 (44.4%)	22 (40.7%)	0.136
Liver limited (%)	129 (35.1%)	54 (22.0%)	48 (23.2%)	8 (14.8%)	0.000
Lung (%)	45 (12.3%)	64 (26.0%)	51 (24.6%)	11 (20.4%)	0.000
Lung limited (%)	9 (2.5%)	21 (8.5%)	22 (10.6%)	1 (1.9%)	0.000

(Continued)

Table 3. (Continued)

Characteristics	Group 1	Group 2	Group 3	Group 4	*p* Value
Peritoneum (%)	58 (15.8%)	47 (19.1%)	43 (20.8%)	18 (33.3%)	0.017
Peritoneum limited (%)	27 (7.4%)	24 (9.8%)	24 (11.6%)	12 (22.2%)	0.006
DLN (%)	51 (13.9%)	27 (11.0%)	19 (9.2%)	12 (22.2%)	0.048
DLN-limited (%)	18 (4.9%)	12 (4.9%)	5 (2.4%)	3 (5.6%)	0.479
<1 organs involved(%)	188 (51.2%)	113 (45.9%)	99 (47.8%)	24 (44.4%)	0.545
⩾2 organs involved(%)	80 (21.8%)	66 (26.8%)	56 (27.1%)	17 (31.5%)	0.307

AC, adenocarcinoma; DLN, distance lymph nodes; IRI, irinotecan; MC, mucinous adenocarcinoma; OX, oxaliplatin; SRCC, signet-ring cell carcinoma.

289 were treated with a first-line doublet regimen (FOLFOX/FOLFIRI + Cet/Bev), and 74 were treated with a first-line triplet regimen (mFOLFIRINOX + Bev). Disease progression occurred in 191 (52%) of 363 patients (44 in the triple-regimen group and 147 in the double-regimen group). Based on the new reclassification of *RAS*/*BRAF* allele mutations, we found that only patients in group 3 (with high-risk *RAS* mutation) benefited from an intense triplet regimen (HR 2.54, 95% CI, 1.36–5.12; $p = 0.014$) [Figure 4(a)–(c), Supplemental Figure S4]. For mCRC patients with *KRAS* G13D/G12V/G12C mutations, the mFOLFIRINOX group reported a median PFS of 12 months compared with 9 months in the doublet chemotherapy group ($p = 0.0091$) [Figure 4(b)].

Discussion

We retrospectively analyzed *RAS*/*BRAF* allele variants and their association with clinicopathologic characteristics and prognosis in CRC patients. The frequencies of *KRAS*, N*RAS*, and *BRAF* mutations in our cohort were 47.6, 3.3, and 7.1%, respectively. The top five most prevalent *RAS* allelic mutations were *KRAS* G12D (14.7%), *KRAS* G12V (10.7%), *KRAS* G13D (9.8%), *KRAS* G12C (3.2%), and *KRAS* A146T (2.0%), which is in accordance with previous studies.[20,40] Furthermore, *BRAF* V600E and *KRAS* G13D were significantly more frequent in hypermutated CRCs, while G12V appeared more frequently in non-hypermutated CRCs but never in hypermutated populations, which is also in concordance with other reports.[41,42]

In stages I–III CRC patients, the prognostic value of *RAS*/*BRAF* is controversial. Roth *et al.*[43] showed that *KRAS* mutation status does not have prognostic value based on a study of 1404 stages II–III CRC patients. In addition, a study concluded that *KRAS* mutation was not associated with DFS in stage III colon cancer patients.[44] However, some studies,[45,46] as well as a large retrospective analysis of stage III MSS CRC by Taieb *et al.*,[47] have yielded opposite results. The prognostic value of *BRAF* is also controversial.[48,49] We suspect that the reason for this controversy is that most previous studies have included hypermutated cohorts with MSI and POLE/POLD1 mutations. However, the prognosis of the hypermutated population is excellent, and previous studies and our study have clearly shown that many *RAS*/*BRAF* sites differ significantly between populations with and without hypermutation. Considering the distinct biological characteristics of the hypermutated cohort, we further explored the roles of *RAS*/*BRAF* allele mutations only in the CRCs with non-hypermutation. In our univariate analysis, prognosis was worse in CRCs with *RAS*/*KRAS*/*NRAS* mutations than in those with wild type but similar trends were not observed in the multivariate analysis. Further multivariate analysis found that patients with *KRAS* G13D mutation showed a worse DFS than those without *KRAS* G13D mutation, and the *KRAS* G13D mutation was the most significant independent prognostic factor associated with DFS. Based on our study, for patients with stages I–III CRC, we recommend that not only MMR status but also *RAS*/*BRAF* allele mutations be determined as early as

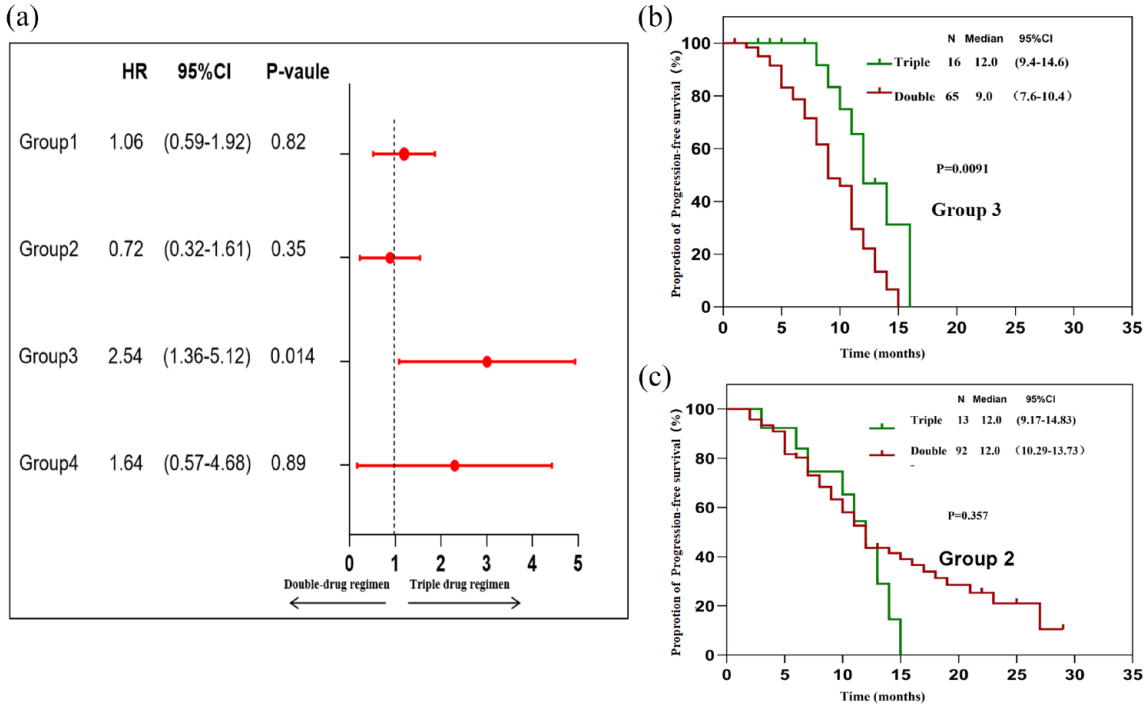

Figure 4. Intensive triplet regimens significantly improved the mPFS of mCRC patients in regroup (a): Forest plots of double drug regime *versus* triple-drug regime within four groups; (b): PFS in KRAS group 3 compared double drug regime with the triple-drug regime; and (c): PFS in KRAS group 2 compared double drug regime with the triple-drug regime.
mCRC, metastatic colorectal cancer; mPFS, median progression-free survival; PFS, progression-free survival.

possible. Patients with *KRAS* G13D but not *BRAF* V600E or other allele mutations in the non-hypermutated cohort may need more intensive adjuvant therapy and a more rigorous follow-up schedule.

It is controversial whether mCRC patients with *RAS* mutations have a poor prognosis, both at the gene and the exon levels.[50–53] For example, one study showed a similar prognosis for *KRAS* exons 2 and 3 mutation,[54] while another study showed a better prognosis for *KRAS* exon 2.[55] Some studies focusing on *RAS*/*BRAF* codon mutations have been more controversial and even come to the opposite conclusions.[16,18,56,57] Therefore, the classification of *RAS*/*BRAF* mutations in terms of genes, exons, and alleles is not accurate, and we need to overcome the limitations of these traditional classifications. In our study, the heterogeneity of mPFS of the first-line treatment in mCRCs between *RAS*/*BRAF* variants was well demonstrated at the allele level. The mPFS for each allele mutation was generally consistent with that previously reported,[58] indicating that our cohort could be representative of the entire mCRC population. We performed an innovative reclassification of *RAS*/*BRAF* mutations based on first-line mPFS and identified a high-risk *RAS*-mutated population with *KRAS* G13D/G12V/G12C, whose survival was much worse than that of the low-risk *RAS*/*BRAF*-mutated population, close to *BRAF* V600E.

Reclassification of *RAS*/*BRAF* mutations is to better stratify treatment. Except for a few patients with targetable mutations, most mCRC patients need to receive doublet or triplet chemotherapy plus targeted drugs (anti-EGFR or anti-VEGF). Previous studies have shown that patients with poorer biological behavior are more likely to benefit from aggressive triplet chemotherapy. For mCRCs with *BRAF* V600E, a first-line intensive triplet regimen is necessary[32] but for *RAS*/*BRAF* wild type, doublet chemotherapy combined with anti-EGFR is sufficient because of the benign biological behavior.[59] However, there has been controversy over patients with *RAS* mutations. More than 100 *RAS* mutant alleles have been identified in CRC, which has different biological functions and leads to different clinical outcomes or drug

responses. As a result, gene-level analysis is no longer enough to accurately stratify treatments. At the same time, it should be taken into account that the mutation frequency of some *RAS*/*BRAF* alleles is very low, and it is difficult to obtain exact conclusions from allelic analysis alone. Regrouping *RAS*/*BRAF* allele mutations with similar prognoses is an effective strategy for stratified therapy. To verify the significance of regrouping for treatment decision-making, we analyzed which strategy of doublet or triplet chemotherapy was better in groups 1–4 and found that only group 3 (the high-risk *RAS* mutation) benefited from an intense triplet regimen. This indicates that for patients with high-risk *RAS* mutations (*KRAS* G13D/G12V/G12C), more intensive triplet chemotherapy plus targeted drug is needed, while for patients with low-risk *RAS* mutations in group 2, doublet regimen is sufficient. The reason for the negative result of group 4 was that the well-documented benefit of first-line triplet chemotherapy for *BRAF* V600E mCRCs influenced real-world treatment decisions at our center, resulting in a control sample size that was too small to analyze. To our knowledge, this is the first study to define a population with high-risk *RAS* mutations (*KRAS* G13D/G12V/G12C) who can benefit from an intensive first-line triplet regimen, and its implication for real-world treatment decision warrants further prospective randomized controlled clinical studies.

This study has some limitations. The main limitation is the insufficient follow-up time. Unable to provide accurate OS Kaplan–Meier curves, more than 60% of the patients survived at the end of the follow-up, and the shortest follow-up time was only approximately 10 months. Thus, the outcomes seem less rigorous. In addition, due to the retrospective nature of the study, a degree of selection bias was unavoidable.

Conclusion

The frequencies of various *RAS*/*BRAF* allele mutations varied between hypermutated and non-hypermutated cohorts or locally advanced and metastatic CRC. In the non-hypermutated cohort, the *KRAS* G13D mutation tended to be more common and aggressive for patients with stages I–III CRC. Our study revealed a significant association between high-risk *RAS* allele mutations and shorter PFS in non-hypermutated mCRC patients undergoing standard first-line therapy. Meanwhile, we found that intensive first-line therapy with triplet chemotherapy in combination with targeted therapy may be considered a viable and effective treatment option for high-risk *RAS* allele mutations.

Declarations

Ethics approval and consent to participate

This study was approved (No. IIT20210185B) and supervised by the Research Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

Consent for publication

Patient consent was waived due to the retrospective nature of this study.

Author contributions

Xiang Zhang: Conceptualization; Data curation; Formal analysis; Methodology; Software; Writing – original draft; Writing – review & editing.

Haizhong Ma: Conceptualization; Formal analysis; Methodology; Software; Visualization.

Yinjun He: Conceptualization; Data curation; Resources; Visualization.

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Weixiang Zhong: Data curation; Formal analysis.

Guosheng Wu: Data curation; Supervision.

Xile Zhou: Data curation; Investigation.

Hanju Hua: Data curation; Funding acquisition.

Feng Ye: Data curation; Funding acquisition.

Hui Cai: Conceptualization; Methodology.

Weiqin Jiang: Conceptualization; Data curation; Funding acquisition; Methodology; Writing – original draft; Writing – review & editing.

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Competing interests
The authors declare that there is no conflict of interest.

Availability of data and materials
To protect the privacy of the patients, individual data are only available upon reasonable request in accordance to corresponding regulatory.

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