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KRAS and *PIK3CA* bi-mutations predict a poor prognosis in colorectal cancer patients: A single-site report

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ABSTRACT

Study rationale: The coexistence of *KRAS* and *PIK3CA* mutations in cells implies potential synergistic hyperactivation of the *Ras/MAPK* and *PI3K/Akt* oncogenic pathways. Therefore, it is desirable to investigate the concomitant mutations of *KRAS* and *PIK3CA* in colorectal cancer (CRC) samples and whether the concomitant mutations are associated with a poor prognosis in CRC patients.
Aim: To investigate the clinicpathological characteristics and prognostic value of concomitant mutations of *KRAS* and *PIK3CA* in CRC samples.
Methods: In this study, a total of 655 CRC patients from the Sixth Affiliated Hospital of Sun Yat-sen University were enrolled from January to December 2015. Sanger sequencing was applied to survey the mutational status of hotspot regions in the open reading frames (ORFs) of the *KRAS* and *PIK3CA* genes. Clinicpathological parameters were collected and analyzed. The Kaplan-Meier method and Cox regression model were applied to determine the correlation between the *KRAS* and *PIK3CA* mutation statuses and survival.
Results: We found that *KRAS* and *PIK3CA* bi-mutations were significantly associated with aggressive clinicpathological features. Among the studied CRC patients, those with either *KRAS* mutations ($P = 0.004$) or *KRAS* and *PIK3CA* bi-mutations ($P = 0.033$) had poor overall survival (OS). In the multivariable analysis, *KRAS* mutations in exons 3 and 4 but not exon 2 with concomitant *PIK3CA* mutations were associated with a high risk of death (univariate HR = 8.05; 95% CI, 1.926–33.64, $P = 0.004$; multivariate HR = 10.505; 95% CI, 2.304–47.905, $P = 0.002$).
Conclusion: The concomitant mutation statuses of *KRAS* and *PIK3CA* should be considered when the prognostic value of gene mutations is consulted in CRC patients.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide, including China, with morbidity and mortality rates ranking in the top five among all tumors [1]. It has generally been accepted that the occurrence and development of tumors are related to the abnormal activation of many signaling pathways, among which the classic pathways include the mitogen-activated protein kinase (*MAPK*) and phosphatidylinositol-3 kinase (*PI3K*) signaling pathways [2].

Rat sarcoma viral oncogene homolog (*RAS*) genes, including v-Ki-ras2-Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and neuroblastoma rat sarcoma viral oncogene homolog (*NRAS*), were the first confirmed human proto-oncogenes that can transform into oncogenes. *KRAS* is the most frequently mutated *RAS* member in most cancers and is altered at high frequencies (30–50%) in CRC patients [3]. Most *KRAS* mutations are detected in codon 12 or 13 of exon 2 and account for nearly 90% of all mutation types; other mutations in codon 59 or 61 of exon 3 and codon 117 or 146 of exon 4 occur less frequently [4]. *KRAS* mutation often leads to the guanosine

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triphosphate (*GTP)*-bound form of the coded protein. This then results in the persistent activation of downstream signaling pathways such as the *MAPK* and *PI3K* pathways. Although *KRAS* has been validated as a molecular biomarker for anti- epidermal growth factor receptor (*EGFR*) therapy [5], the prognostic value of *KRAS* mutation is still controversial.

Recently, an analysis from the National Cancer Database involving 19,877 nonmetastatic colon cancer patients showed that *KRAS*-mutated tumors were more frequently observed in right sided colon and late-stage tumors and associated with a poor prognosis in stage III patients [6]. A similar result was obtained in a cohort of 2720 patients by Sinicrope et al. [7], the 5-year disease-free survival (DFS) rate was 70.7% for patients with wild-type (WT) *KRAS* and 61% for patients with mutant *KRAS*. In metastatic patients, *KRAS* has been reported to be associated with poor recurrence-free survival and overall survival (OS) after curative resection [8]. Indeed, Ye et al. [9]; studied a large cohort of 1190 Chinese CRC patients and found no significant difference in OS between *KRAS WT* and mutant patients.

Several studies have revealed a strong association of *KRAS* and Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA)* gene mutations in CRC [10,11]. *PIK3CA* is involved in the *PI3K/Akt* signaling pathway and is associated with high mutation rates in CRC (10–20%), second only to *KRAS* [12], and its somatic activating mutation plays an important role in tumorigenesis [13]. In 2007, Kato et al. [14]; found that the OS time for stage II and III CRC patients with mutant *PIK3CA* was significantly shorter than that for CRC patients with *WT PIK3CA* ($P = 0.043$, $HR = 2.478$). Successive studies have also found that *PIK3CA* mutation is related to postoperative recurrence, distant lung or liver metastases, chemotherapy resistance and other adverse prognostic events in CRC patients [15–21]. Multiple studies have found that stage IV CRC patients with *PIK3CA* mutation are resistant to *EGFR* monoclonal antibody therapy [22–25].

Nevertheless, some experts have noted that *PIK3CA* is a favorable biomarker for the prognosis of stage I-III patients [26]. CRC patients with a somatic mutation in *PIK3CA* may benefit from aspirin administration [27]. Zuo et al. [28]; showed that somatic *PIK3CA* mutations might be related to radiotherapy sensitivity, suggesting that CRC patients with liver metastasis who are resistant to *EGFR* monoclonal antibodies could benefit from artery radiotherapy embolization therapy. Therefore, the prognostic value of *PIK3CA* mutation in CRC remains controversial.

At face value, *KRAS* and *PIK3CA* bi-mutations may represent the co-activation of *MAPK* and *PI3K* signaling pathways. Therefore, it is reasonable to conjecture that the coexistence of *KRAS* and *PIK3CA* mutations may have synergistic or additive effects on survival in CRC patients. Some scholars have even noted that the prognostic and predictive value of *PIK3CA* may actually depend on the mutational status of *KRAS* [29–31]. Therefore, whether the concomitant mutations of *KRAS* and *PIK3CA* are potential markers for a poor prognosis in CRC patients requires further exploration.

Materials and methods

Patients

In total, 655 CRC patients who underwent surgical resection at the Sixth Affiliated Hospital of Sun Yat-sen University (SYSU) between January and December 2015 and met the criteria below were included in this retrospective study. All patients signed an informed consent form, which was approved by the Institutional Review Board of the hospital.

The inclusion criteria were as follows: (1) the resected CRC tumor was histologically confirmed; (2) case data and follow-up records were complete; and (3) formalin-fixed, paraffin-embedded specimens were available. The exclusion criterion was as follows: accompanied by other types of cancer or severe diseases such as infection or organ dysfunction.

Detection of KRAS and PIK3CA mutations

Sanger sequencing was performed in the Molecular Diagnostic Laboratory of the Sixth Affiliated Hospital of Sun Yat-sen University. *KRAS* and *PIK3CA* mutation testing was performed with Sanger sequencing and HRM method developed by the Lab. And the detail procedure of the mutation testing can refer to previous studies [32,33].

Statistical analysis

The correlation between *KRAS* and *PIK3CA* mutation statuses and clinicpathological characteristics was evaluated using a chi square test (or Fisher's exact test) for categorical data. OS was defined as the period from the date of surgery to death from any cause. DFS was defined as the period from the date of surgery to tumor recurrence or death. The Kaplan-Meier method was performed to compare OS and DFS between groups. Univariate and multivariate Cox proportional hazards models were used to explore the associations of patient characteristics and gene mutations with OS and DFS. Two-sided *p*-values are reported, and, in general, *P*-values < 0.05 were considered statistically significant. Analyses were performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA).

Transcriptomic analysis

We also looked at the TCGA CRC portal with attention for cases with gene mutation and expression data matched/available (233 cases). The mutational and expression data and related clinical records were downloaded. Differentially expressed genes among different groups were identified by using the R language limma package (version 3.6; https://www.r-project.org/) ($P < 0.05$ and $|\log 2\ FC| > 1$). GO function enrichment analysis and KEGG pathway analysis were performed using the clusterProfiler package.

Results

A total of 655 patients were included in this study, and their clinicpathological characteristics are shown in Supplementary Table 1. The CRC incidence rates were higher in males than in females (59.5% vs 40.5%, respectively). Patients with CRC had a similar age distribution at the time of surgery. Abnormal levels of tumor markers were observed in less than half of CRC patients. The rectum was the most common primary tumor site, followed by the left sided colon and right sided colon (47.5%, 30.7%, and 19.7%, respectively). Nearly half of the CRC patients had advanced tumors (48.7%, 319/655). In total, 20.5%, 60.9%, and 18.5% of tumors were graded as 1, 2 and 3, respectively. A small proportion of patients overexpressed the HER2 oncogene (36.5%, 234/641). High KI-67 expression was observed in 26.6% (174/645) of patients with CRC. Perineural invasion (6.6%, 43/649) and lymph vascular invasion (8.2%, 53/647) were rarely observed in this cohort. Deficient mismatch repair (dMMR) was observed in 6.4% of CRC patients (42/651). The clinicpathological characteristics of this TCGA cohort are summarized in Supplementary Table 2.

Gene mutations

Of the 655 patients, the mutation rate of *KRAS* was 46.6% (305 of 655; exon 2 (n = 259) and exon 3 or 4 (n = 46)), and that of *PIK3CA* was 12.8% (84 of 655; exon 9 (n = 61) and exon 20 (n = 22)) (Supplementary Table 3). *KRAS* and *PIK3CA* concomitant mutations were commonly observed in *PIK3CA*-mutated tumors (55/84, 65.5%) (Table 1). Only one patient harbored a *PIK3CA* mutation in both exons 9 and 20 concomitant with a *KRAS* mutation in exon 4. We also performed similar analysis on the TCGA CRC dataset, 223 cases of patients with *KRAS* or *PIK3CA* mutation information available. The mutation rate of *KRAS* and *PIK3CA* were 43% (97 of 223; exon 2 (n = 79), exon 3 or 4 (n = 17)) and 15.2% (34 of 223; exon 10(n = 13), exon 21(n = 6) and other exons (n = 15)), respectively. *KRAS* and *PIK3CA* concomitant mutations were commonly observed in *PIK3CA*-mutated tumors (25/34, 73.5%). Five cases were detected more than one exon mutations in *PIK3CA*, and all of them were concurrent mutations with *KRAS*. However, we could not find the coexistence of *PIK3CA* mutations in exon 9 or 20 and *KRAS* mutations in exon 3 or 4 (Supplementary Tables 4, 5).

Table 1
Distribution of KRAS and PIK3CA concomitant mutations among 655 CRC patients.

	KRAS	Exon 2								Exon 3			Exon 4
PIK3CA		p.G12S	p.G12D	p.G12A	p.G12V	p.G12C	p.G12P	p.G13D	p.G13C	p.A59T	p.Q61K	p.Q61H	p.A146T
Exon 9	p.E542K				6	1	1				1		
	p.E545K	1	11		4	1		6	1	1			1
	p.E545D				1								
	p.E545A	1	1	1									
	p.E545G							1					
Exon 20	p.H1047L	1	5	1	2			2				1	
	p.H1047R		1		1								
	p.E545G, p.H1047Y									1			

Correlations with clinical characteristics

As showed in Table 2, there was a significant difference categorized either by sex, age, primary tumor site, levels of CA-199 and CA125, microsatellite instability (MSI) status, TNM stage, and tumor grade (all with $P < 0.05$). Tumors with *KRAS* and *PIK3CA* bi-mutations tended to be located in a proximal location ($P < 0.001$), poorly differentiated ($P = 0.019$), and have elevated CA-199 ($P = 0.001$) and CA125 levels ($P = 0.033$). CRC patients were more likely to be diagnosed in the late stage than in the early stage ($P = 0.002$) at the initial diagnosis. Compared with individual *KRAS* mutations, additional *PIK3CA* mutations were associated with more advanced tumors ($P = 0.008$). There were more cases with dMMR in the individual *PIK3CA* mutation group than in the bi-mutation group ($P = 0.032$). However, we found no significant association between mutation type and clinicpathological characteristics in TCGA dataset (Supplementary Table 6).

Survival analysis

To clarify whether *KRAS/PIK3CA* gene mutations are associated with longer or shorter OS and DFS, we used the Kaplan-Meier method to compare OS and DFS between patients with *KRAS* or *PIK3CA* mutant and *WT* tumors, and found that patients with mutant *KRAS* experienced shorter OS than patients with *WT KRAS* (*Log-rank* $P = 0.004$) (Fig. 1A). Furthermore, patients with *KRAS* mutations in exon 2 experienced significantly poor OS than *KRAS* mutations in exon 3 and 4 (*KRAS* exon 2 vs. *KRAS* exon 3 and 4*; Log-rank* $P = 0.018$) (Fig. 1D). No significant difference in OS was found between patients with mutant *PIK3CA* and those with *WT PIK3CA* (*Log-rank* $P = 0.061$) (Fig. 1B). However, subgroup analysis showed that patients with *PIK3CA* mutations in exon 9 (vs. *WT, Log-rank* $P = 0.006$) (Fig. 1E) experienced worse OS than patients with *PIK3CA* mutations in exon 20 (vs. *WT*, *Log-rank* $P = 0.365$). The Kaplan–Meier plots of TCGA dataset shown that neither *KRAS* or *PIK3CA* mutations had significant impact on OS status (Supplementary Fig. 1A, B, D).

Then, the group with *KRAS* and *PIK3CA* bi-mutations was examined. As shown in Fig. 1H, *KRAS* exon 2 group has poorer OS (vs. *WT*, *Log-rank* $P < 0.001$), with a similar trend like the *PIK3CA* exon 9 and *KRAS* exon 2 group (vs. *WT*, *Log-rank* $P = 0.042$), but not the *PIK3CA* exon 20 and *KRAS* exon 2 group (vs. *WT Log-rank* $P = 0.739$). Fig. 1I shown that although *PIK3CA* exon 9 and *KRAS* exon 2 has poorer OS, but there have no statistically significant difference than other groups including the *PIK3CA* exon 9, the *PIK3CA* exon 20 group and the *PIK3CA* exon 20 and *KRAS* exon 2 group (*Log-rank* $P > 0.05$). As shown in Fig. 1C and Supplementary Table 7, we observed a trend toward worse OS in patients with concomitant mutations, but that trend was not significant in the multivariate analysis (*Log-rank* $P = 0.033$, univariate HR = 2.078; 95% CI, 1.059–4.079, $P = 0.034$; multivariate HR = 1.281; 95% CI, 0.609–2.692, $P = 0.514$). Only if the *PIK3CA* mutation coexisted with the *KRAS* mutation in exons 3 and 4 did patients experience much shorter OS than other patients (*Log-rank* $P < 0.001$, univariate HR = 8.05; 95% CI, 1.926–33.64, $P = 0.004$; multivariate HR = 10.505; 95% CI, 2.304–47.905, $P = 0.002$) (Fig. 1F, G,Table 3). The Kaplan–Meier plots of TCGA dataset shown that, the *KRAS* and *PIK3CA* bi-mutation group had no statistical difference on OS when compare to other groups (Supplementary Fig. 1C). Whereas, the Cox regression model shown that in the multivariate but not univariate analysis, the *KRAS* and *PIK3CA* bi-mutation group had poor OS than *WT* (univariate HR = 1.204; 95% CI, 0.517, 2.805, $P = 0.667$; multivariate HR = 3.010 95% CI, 0.991, 9.149, $P = 0.052$) (Supplementary Table 8). When referring to specific KRAS mutation types, the *PIK3CA* and *KRAS* exon 2 group have poorer OS than the *KRAS* exon group (*Log-rank* $P = 0.041$, Supplementary Fig. 1E); when compared to *WT*, the *PIK3CA* and *KRAS* exon 2 group have a poorer OS (univariate HR = 1.575; 95% CI, 0.675,3.675, $P = 0.293$; multivariate HR = 3.864 95% CI, 1.236 12.080, $P = 0.020$) (Supplementary Table 9).

With the same setting of groups and analysis procedures, we also compared the association between mutational status and DFS. However, no significant difference in DFS was found between these groups, although the *KRAS* mutations group, especially the *KRAS* exon 2 group have poorer DFS than *WT*, but no significant different were found in further analysis (Fig. 2, Supplementary Fig. 2).

Transcriptomic analysis

We used downloaded data from TCGA portal with gene mutation information and expression data matched for the transcriptomic analysis. Differentially expressed genes (DEGs) between various groups were identified first as described in methods. Then the list of DEGs were subjected to enrichment analysis in terms of molecular functions, biological processes, and cellular components, or KEGG pathways (Supplementary Figs. 3–5, Supplementary Table 10). We speculate that different subtype might lead to different tumor metabolism and this phenomenon should be considered in stratification of chemotherapy and future drug development accordingly. Subgroup analysis were also performed (Supplementary Figs. 6–12). Given the small size of cohort and detailed clinical information partially missed, solid conclusion is yet to be explored and open to question.

Discussion

Hyperactivation of the *MAPK* and *PI3K* signaling pathways can lead to uncontrolled cell proliferation and apoptosis, further transformation of carcinogenesis and invasion and metastasis [2]. In this study, a strong association of *KRAS* and *PIK3CA* mutations was revealed. Simultaneous mutations in *PIK3CA* exon 9 and *KRAS* exon 2 were the most common (37/55,67.2%), consistent with the findings by Li et al. [15]. Compared with *WT* tumors, tumors with *KRAS* and *PIK3CA* bi-mutations were significantly associated with aggressive clinicpathological features. For example, increased proportions of proximal colon cancer, were prone to metastasize to distal organs at initial surgery, and high-grade tumors tended to have elevated CA19-9 and CA125 levels. Compared to CRC patients with individual *KRAS* mutations, those with bi-mutations had more advanced tumors that were more frequently located in the right sided colon than in the rectum and left sided colon (44.4% vs 21.5%, 37% vs 53.7%, and 18.5% vs 24.8%, respectively,

Table 2
Correlations between gene mutation types and clinicpathological characteristics.

	KRAS and *PIK3CA* wild-type		*KRAS* mutations alone		*PIK3CA* mutations alone		*KRAS* and *PIK3CA* bi-mutations		*P* value	*P* value	*P* value	*P* value
	N	%	N	%	N	%	N	%		(bi-mutations vs wild-type)	(bi-mutations vs *KRAS* mutations alone)	(bi-mutations vs *PIK3CA* mutations alone)
Sex												
Female	113	35.20	112	44.80	17	58.60	23	41.80	0.022*	0.345	0.687	0.143
Male	208	64.80	138	55.20	12	41.40	32	58.20				
Age, years												
< 60 years	175	54.50	109	43.60	14	48.30	23	41.80	0.047*	0.081	0.809	0.571
≥ 60 years	146	45.50	141	56.40	15	51.70	32	58.20				
CEA level												
0–5 ng/ml	197	62.30	128	53.10	18	62.10	33	60.00	0.171	0.741	0.355	0.854
>5 ng/ml	119	37.70	113	46.90	11	37.90	22	40.00				
CA-199 level												
0–37 ng/ml	285	89.90	192	79	25	86.20	41	74.50	0.001*	0.001*	0.469	0.216
> 37 ng/ml	32	10.10	51	21.00	4	13.80	14	25.50				
CA-125 level												
0–35 ng/ml	296	93.70	220	90.50	22	75.90	47	85.50	0.005*	0.033*	0.265	0.275
>35 ng/ml	20	6.30	23	9.50	7	24.10	8	14.50				
HER2												
Positive	122	39.00	91	37.00	7	24.10	14	26.40	0.165	0.08	0.143	0.821
Negative	191	61.00	155	63.00	22	75.90	39	73.60				
KI67												
Positive	99	31.30	60	24.40	5	17.20	10	18.50	0.065	0.056	0.356	0.885
Negative	217	68.70	186	75.60	24	82.80	44	81.50				
Tumor location												
Proximal colon	43	13.60	52	21.50	10	34.50	24	44.40	< 0.001*	< 0.001*	0.002*	0.013*
Distal colon	117	37.00	60	24.80	14	48.30	10	18.50				
Rectum	156	49.40	130	53.70	5	17.20	20	37.00				
MSI status												
MSI-L/MSI-H	20	6.30	13	5.20	6	20.70	3	5.50	0.015*	0.812	0.944	0.032*
MSS	298	93.70	236	94.80	23	79.30	52	94.50				
T												
Tis-T1	20	6.20	10	4.00	0	0.00	2	3.60	0.055	0.124	0.518	0.685
T2	40	12.50	31	12.40	1	3.40	3	5.50				
T3	242	75.40	178	72.10	23	79.30	43	78.20				
T4	19	5.90	31	12.40	5	17.20	7	12.70				
N												
N0	178	55.50	136	54.40	17	58.60	32	58.20	0.353	0.656	0.175	0.934
N1	94	29.30	86	34.40	6	20.70	13	23.60				
M												
M0	271	84.16	212	84.80	23	79.30	37	67.30	0.012*	0.002*	0.002*	0.247
M1	50	15.53	38	15.20	6	20.70	18	32.70				
TNM staging												
I	39	12.10	28	11.20	0	0.00	4	7.30	0.023*	0.007*	0.008*	0.246
II	126	39.30	100	40.00	16	55.20	23	41.80				
III	106	33.00	84	33.60	7	24.10	10	18.20				
IV	50	15.60	38	15.20	6	20.70	18	32.70				
Tumor grade												
Grade 3	46	14.40	49	19.70	10	34.50	16	29.10	0.009*	0.019*	0.074	0.871
Grade 2	200	62.50	159	63.90	13	44.80	26	47.30				
Grade 1	74	23.10	41	16.50	6	20.70	13	23.60				
Perineural invasion												
No	293	92.70	234	94.40	26	89.70	52	94.50	0.712	0.625	0.956	0.408
Yes	23	7.30	14	5.60	3	10.30	3	5.50				
Lymphovascular invasion												
No	290	92.40	229	92.00	25	86.20	50	90.90	0.705	0.713	0.796	0.508
Yes	24	7.60	20	8.00	4	13.80	5	9.10				

* *P*-values ≤ 0.05.

$P = 0.002$). The major differences observed between single *PIK3CA* mutations and *KRAS* and *PIK3CA* bi-mutations were the MSI status and tumor location, consistent with previous studies in which the *PIK3CA* mutation was reported to be closely related to the distal colon and dMMR status [19]. We also try to validated our findings in CRC cohort from TCGA. However, we found no significant association between mutation type and

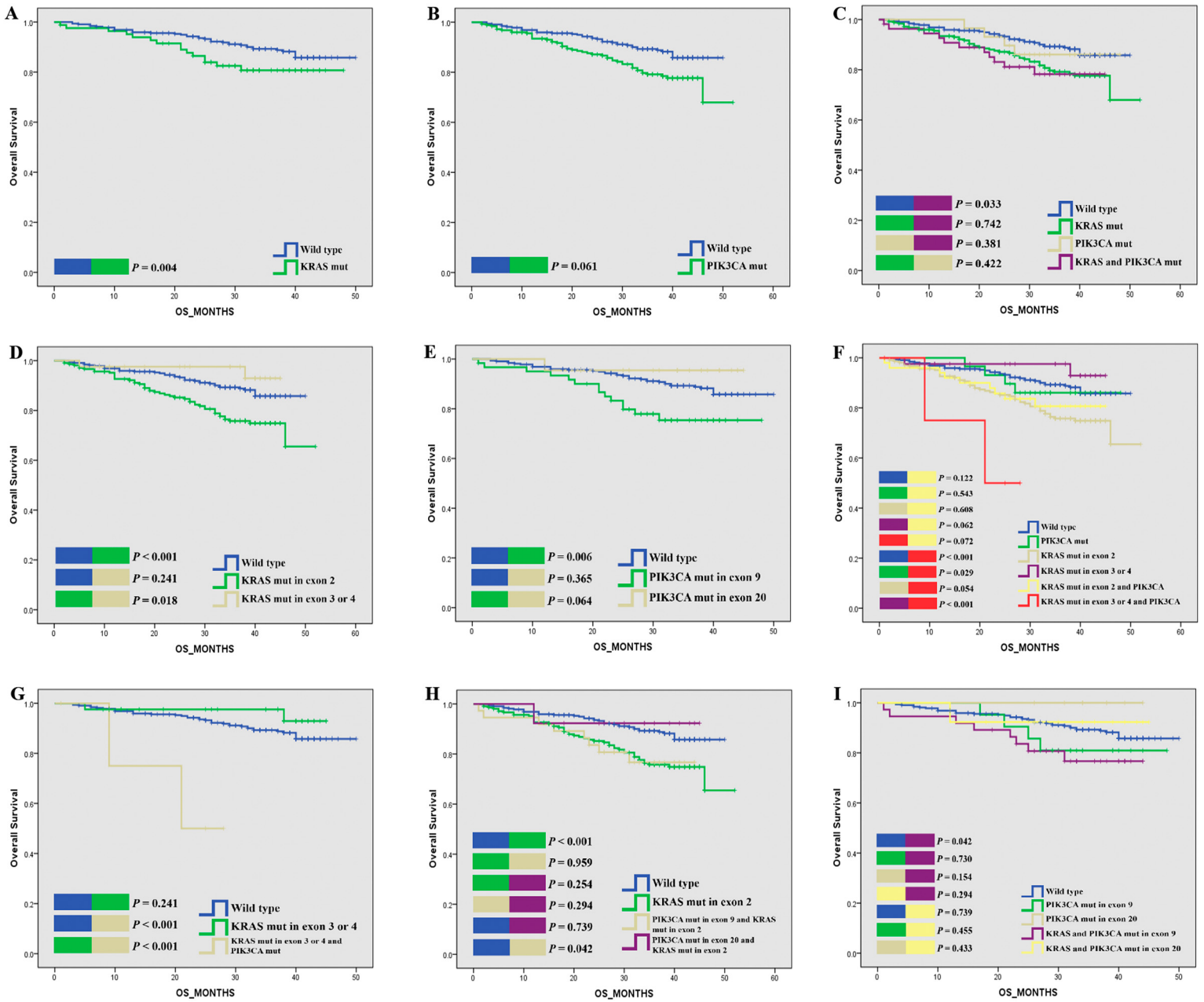

Fig. 1. Kaplan-Meier plots of OS for CRC patients according to gene mutation status.

Table 3
Univariate and multivariate analyses of different prognostic parameters regarding to OS of the CRC patients.

	Univariate analysis		Multivariate analysis	
Parameters	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (< 60 vs. ≥ 60 years)	0.690 (0.464,1.027)	0.068		
Gender (Female vs. Male)	0.911 (0.609,1.363)	0.649		
Histological grade	0.610 (0.446,0.834)	0.002*	0.754 (0.517,1.100)	0.142
CEA level (>5 ng/ml vs. ≤5 ng/ml)	3.512 (2.280,5.408)	<0.001*	1.510 (0.914,2.496)	0.108
CA19–9 level (>37 ng/ml vs. ≤37 ng/ml)	5.137 (3.440,7.672)	<0.001*	2.130 (1.285,3.532)	0.003*
CA12–5 level (>35 ng/ml vs. ≤35 ng/ml)	3.251 (2.006,5.271)	<0.001*	1.401 (0.783,2.505)	0.256
Tumor location		0.003*		0.405
Proximal colon	2.281 (1.359,3.830)	0.002*	1.203 (0.663,2.183)	0.544
Distal colon	1.963 (1.230,3.133)	0.005*	1.419 (0.852,2.365)	0.179
Rectum	1		1	
TNM stage	3.731 (2.864,4.861)	<0.001*	2.153 (1.459,3.178)	<0.001*
HER2 (Negative vs Positive)	1.31 (0.854,2.008)	0.216		
KI-67 (Negative vs Positive)	1.485 (0.91,2.424)	0.114		
MSI status (dMMR vs pMMR)	0.286 (0.07,1.158)	0.079		
Perineural invasion (Yes vs no)	3.109 (1.864,5.184)	<0.001*	0.974 (0.485,1.958)	0.941
Lymphovascular invasion (Yes vs no)	2.650 (1.504,4.668)	0.001*	1.952 (1.068,3.565)	0.03*
Chemotherapy or radiotherapy (Yes vs No)	1.426 (0.953,2.133)	0.085	0.476 (0.294,0.771)	0.003*
Radical Surgery (No vs Yes)	11.592(7.804,17.217)	<0.001*	3.526 (1.891,6.576)	<0.001*
Mutations status		0.001*		0.001*
KRAS/PIK3CA wild-type	1		1	
PIK3CA mut alone	1.246 (0.444,3.496)	0.677	0.660 (0.219,1.994)	0.462
KRAS mutation in exon 2	2.147 (1.395,3.303)	0.001*	2.185 (1.338,3.569)	0.002*
KRAS mutation in exon 3 or 4	0.451 (0.109,1.870)	0.272	0.868 (0.206,3.669)	0.848
Coexistence of *PIK3CA* mutant and *KRAS* mutation in exon 2	1.786 (0.861–3.704)	0.119	1.080 (0.486–2.398)	0.850
Coexistence of *PIK3CA* mutant and *KRAS* mutation in exon 3 or 4	8.050 (1.926,33.644)	0.004*	10.505 (2.304–47.905)	0.002*

Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using univariate or multivariate Cox proportional hazards regression in SPSS 20.0. *P*-values were calculated using univariate or multivariate Cox proportional hazards regression in SPSS 20.0. *P*-values < 0.05 were considered to indicate statistical significance.
* $P < 0.05$.

clinicpathological characteristics. Although please keep in mind that the small sample size might give rise to statistical bias (Supplementary Tables 2, 6).

In our study, we found that *KRAS* mutations are consistently associated with poor prognosis in both univariate and multivariate analysis (univariate HR = 2.147; 95% CI, 1.395,3.303, $P = 0.001$; multivariate HR = 2.185; 95% CI, 1.338,3.569, $P = 0.002$) (Table 3). Among the specific mutations in *KRAS*, those in exon 2 but not exons 3 and 4 showed a significant association with OS (*Log-rank* $P < 0.001$) (Fig. 1D). Regarding the potential co-selection of *KRAS* and *PIK3CA*, we observed a trend toward worse OS in patients with concomitant mutations, but not indicated in multivariate analysis (univariate HR = 2.078; 95% CI, 1.059–4.079, $P = 0.034$, multivariate HR = 1.281; 95% CI, 0.609–2.692, $P = 0.514$) (Supplementary Table 7). Therefore, we initially speculated that the prognosis of *PIK3CA* and *KRAS* concomitant mutations may depend on the *KRAS* mutational status. However, further study shown that patients with *KRAS* mutation in exon 3 and 4 as well as *PIK3CA* mutation have much shorter OS than other patients (univariate HR = 8.05; 95% CI, 1.926–33.64, $P = 0.004$; multivariate HR = 10.505; 95% CI, 2.304–47.905, $P = 0.002$,Table 3). Not only that, multivariate analysis shown the HR of this particular group are much higher than the *KRAS* exon 2 mutations group (univariate HR = 2.147;95% CI,1.395–3.303, $P = 0.001$; multivariate HR =2.185;95% CI,1.338–3.569, $P = 0.002$), also much higher than the PIK3CA and KRAS exon 2 group (univariate HR = 1.786;95% CI, 0.861–3.704, $P = 0.119$; multivariate HR =1.080; 95% CI, 0.486–2.398, $P = 0.850$). We performed similar analysis on the CRC cohort from TCGA, the results were consistent with our conclusion that the bi-mutations have poorer OS, but only refer to *KRAS* mutations in exon 2 (univariate HR = 1.575; 95% CI, 0.675–3.675, $P = 0.293$, multivariate HR = 3.864, 95% CI, 1.236–12.080, $P = 0.020$) (Supplementary Tables 8, 9). In summary, the concomitant mutations did have synergistic effect on survival status in CRC patients. We also found that the *KRAS* and *PIK3CA* mutation status may influence OS but not DFS (Fig. 2). We speculate that this observation is mainly because we performed radical surgery early after the initial diagnosis in stage I-III CRC patients.

Currently, many studies have suggested that the *KRAS* and *PIK3CA* mutation status is significantly associated with the chemotherapy response. When compare to others, the bi-mutations tumor may lead to distinct tumor metabolism and tumor immune response which hints that the corresponding therapy need in-depth study (Supplementary Figs. 3–12, Supplementary Table 10). *EGFR* monoclonal antibody therapy combined with the *FOLFIRI* or *FOLFOX* regimen has shown significant advantages in selected patients with stage IV CRC. However, many large clinical trials have found that CRC tumors with *KRAS* or *PIK3CA* gene mutations do not respond to *EGFR* monoclonal antibody therapy [34,35]. Jhawer et al. [36]; found that cetuximab had no effect on cell lines with *KRAS* exon 2 and *PIK3CA* exon 20 mutations but *WT* or individual *KRAS/PIK3CA* mutations. *MEK* inhibitors are potential drugs for the treatment of CRC; however, the efficacy of a single therapy on CRC tumors with *KRAS* mutations varies greatly [37]. Studies have shown that *PIK3CA* mutations can reduce the sensitivity of *KRAS*-mutant CRC cells to *MEK* inhibitors, but *MEK* inhibitors combined with *PI3K* signaling pathway inhibitors can improve the treatment effect [38]. However, due to the high cost of targeted therapy drugs, fluorouracil-based chemotherapy is still the major treatment option for advanced CRC treatment in China. In this study, among the 112 metastatic CRC patients, 87 chose postoperative chemotherapy, and a few were treated with *EGFR* or *VEGF* monoclonal antibody therapy after surgery. Therefore, the prognostic impact of targeting drugs on this small cohort could not be adequately evaluated.

In conclusion, our study suggests that *KRAS* and *PIK3CA* bi-mutations are associated with more aggressive clinicpathological features and should be regarded as new addition of molecular stratification, especially when referring to specific *KRAS* mutation types. Longer follow-up times and increased sample sizes are required for more stringent findings, and clinical trials are needed to validate the value of these mutations in the comprehensive management of CRC patients.

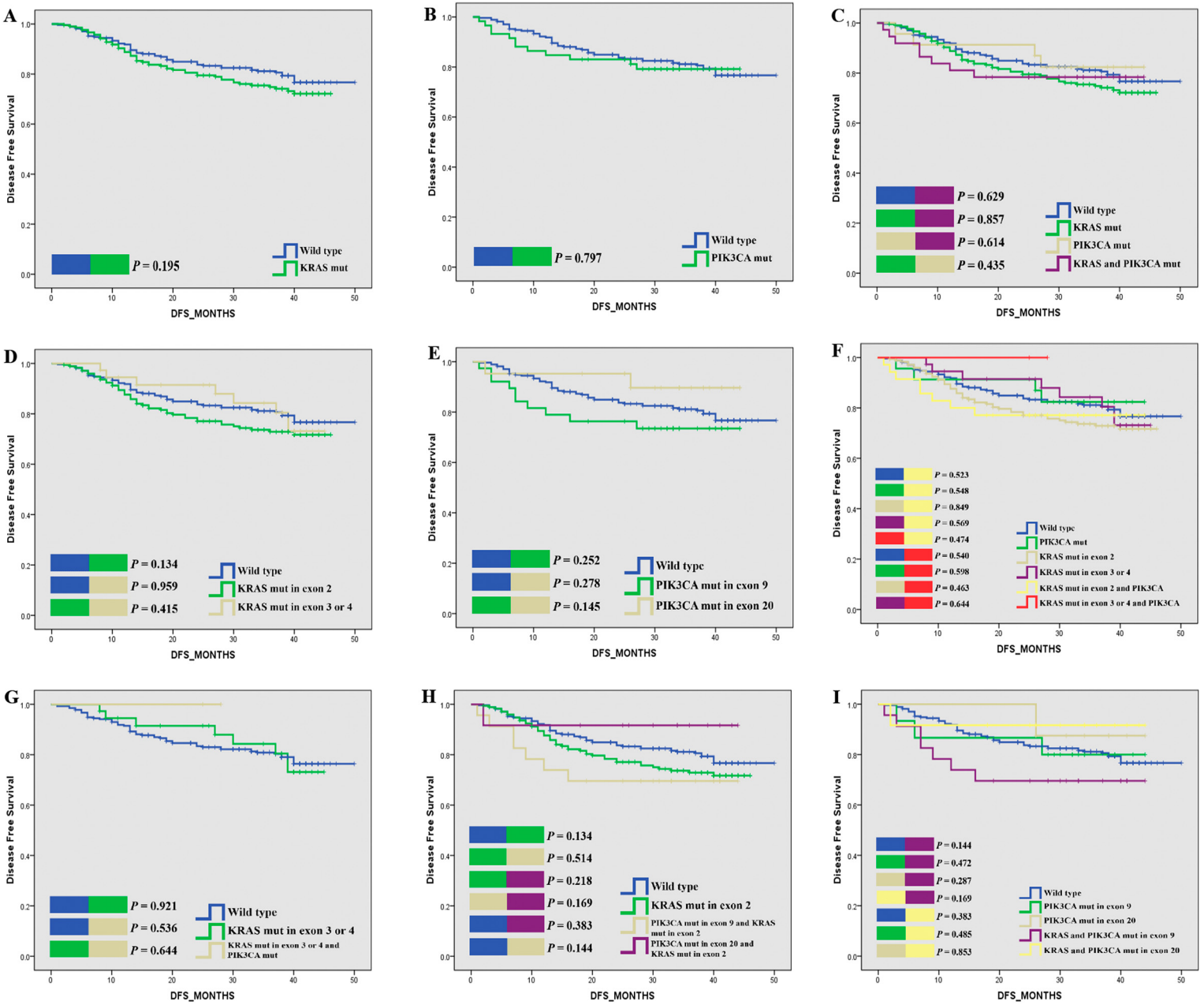

Fig. 2. Kaplan-Meier plots of DFS for CRC patients according to gene mutation status.

CRediT authorship contribution statement

Qianxin Luo: Conceptualization, data collection and analysis, manuscript draft preparation.
Dianke Chen: Conceptualization, methodology guidance.
Xinjuan Fan: Resources.
Xinhui Fu: Resources.
Tenghui Ma: Manuscript editing.
Daici Chen: Conceptualization, supervision, manuscript preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

Our study was approved by the Institutional Ethics Committee of Sixth Affiliated Hospital, Sun Yat-sen University. The related ethical approval code is: 2018–071.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100874.

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