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NGS-based oncogenic mutations analysis in advanced colorectal cancer patients improves targeted therapy prediction

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ABSTRACT

Background: Characterization of genetic alterations has been revealed to be important to predict the outcomes of targeted therapy in cancer. We here aimed to assess the mutation profiling of 526 colorectal cancer (CRC) patients by next-generation sequencing (NGS) to enable a more personalized anti-EGFR treatment.
Methods: Tumors were analyzed using NGS to determine hotspot mutations in 22 cancer-related genes.
Results: Mutations were observed in 13 genes in 436 of 526 (82.9%) tumors, and the most common mutations occurred in *TP53* and *KRAS*. *PIK3CA* mutations usually coexisted with *KRAS, NRAS* or *BRAF* mutations. A higher frequency of concomitant *PIK3CA* mutations was observed in tumors with *KRAS* outside codon 13 mutations, with *NRAS* codon 61 mutations and with *BRAF* kinase-activated mutations. Moreover, *KRAS*, *PIK3CA*, *AKT1* and *FBXW7* mutations were statistically associated with some clinicopathological features, including location, age or metastasis of CRC patients. For *RAS* wild-type patients treated with cetuximab, longer progression-free survival (PFS) was observed in patients identified as wild type in all 22 genes compared with patients with mutations in one or more genes.
Conclusions: A wild-type result in all 22 cancer-related genes detected by NGS is associated with a better outcome of cetuximab treatment. Determining mutation patterns by NGS may aid to understand the molecular mechanisms of CRC and improve targeted therapy prediction.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, and ranks fourth in cancer incidence and fifth in cancer mortality in China [1]. Metastasis accounts for the major reason of cancer-related death. It is reported that metastasis presents in approximately 25% of newly diagnosed cases, and will develop in about 50% of cases with CRC [2]. Accumulation of genetic alterations, which subsequently lead to deregulation of key oncogenic signaling pathways, contributes to the progression and metastasis of CRC [3]. Thus, identification of the mutation profiling is important to uncover the molecular mechanisms and explore novel therapeutics for metastatic CRC (mCRC).

Mutation-derived target therapy has been introduced into clinical practice in these years. Anti-EGFR monoclonal antibodies, cetuximab and panitumumab, have greatly improved clinical outcomes of mCRC patients [4]. However, *KRAS* and *NRAS* mutations (*RAS* mutations), which can activate the MAPK pathway downstream of EGFR, are validated as predictive biomarkers of resistance to anti-EGFR therapy [5]. Further researches show that *BRAF* and *PIK3CA* mutations may be associated with lack of response to anti-EGFR therapy, although the conclusion has not been confirmed in all studies [6,7]. The prevalence of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations has been investigated in lots of reports [8,9], but published data about the frequency of subtype mutations in these four genes among CRC patients are few. Besides mutations in these four key oncogenes, other mutated genes accounted for deregulation of CRC-related pathways are also identified in the pathogenesis of CRC [10]. These gene mutations may be also important predictive and prognostic markers in anti-EGFR treatment. However, little is known about the prevalence and clinical values of these oncogenic mutations in CRC patients.

Next-generation sequencing (NGS) has been rapidly developed in

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these 10 years [11], and its application has been broadened from research to the clinic [12,13]. NGS can screen multiple gene mutations with high accuracy and limited time [14]. Thus, it makes detecting numerous mutations in one test achievable for CRC patients. In this study, we systematically detected 507 hotspot mutations of 22 cancer-related genes using NGS platform, and explored the correlation of oncogenic mutations with clinicopathological characteristics and anti-EGFR response.

2. Materials and methods

2.1. Patients and specimens

A total of 526 CRC specimens (including 189 biopsy specimens and 337 surgical resection specimens) that were submitted to the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences (CAMS), Beijing, China from June 2015 to October 2016, were retrospectively collected in this study. The study has been approved by the Institute Review Board of the Cancer Hospital, CAMS. The methods were carried out in accordance with the approved guidelines. Informed consent was obtained from all patients. Clinicopathologic characteristics of these CRC patients were obtained from the medical records. For patients who received anti-EGFR treatment, clinical response was assessed by Dr. Aiping Zhou through computing tomography scan, following the Response Evaluation Criteria In Solid Tumors criteria (RECIST, version 1.1). The patients who received anti-EGFR treatment were followed up until 1st October 2017. Objective response was defined as complete response (CR) or partial response (PR), whereas the non-response was defined as stable disease (SD) or progressive disease (PD).

2.2. Isolation of genomic DNA

All specimens were fixed in 10% neutral buffered formalin for 16–48 h, and then were embedded in paraffin. Tissue blocks with adequate tumor cellularity (≥ 20%) were assessed by an experted pathologist (Dr. Jianming Ying), and then were selected to obtain genomic DNA. DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. DNA quality was evaluated based on the absorbance ratios of 260/280 nm using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher, MA, USA). The DNA quantity was evaluated using the Quantus™ Fluorometer 2.0 with the Qubit® dsDNA HS Assay Kit (Thermo Fisher).

2.3. DNA library construction and sequencing

Ten ng of DNA was used to construct amplicon DNA library with the Ion Ampliseq Colon and Lung Cancer Panel (Thermo Fisher). The panel was used to detect 507 hotspot mutations in 22 cancer-related genes, including *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *EGFR*, *AKT1*, *ERBB2*, *PTEN*, *STK11*, *MAP2K1*, *ALK*, *DDR2*, *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBXW7*, *FGFR3*, *NOTCH1*, *ERBB4*, *FGFR1* and *FGFR2*. The genomic DNA was amplified by PCR, and then was ligated to different barcodes. The library of each sample was mixed. Next, the mixed library was clonally amplified onto the IonSpheres (ISP), and then enriched on the Ion OneTouch system to prepare the DNA template, using the Ion PGM(TM) Hi-Q(TM) OT2 Kit (Thermo Fisher). Finally, the enriched ISPs were added onto a 318 Chip, and sequencing was carried out on the Ion Torrent PGM platform using Ion PGM HI-Q SEQ Kit (Thermo Fisher), following the manufacturer's instructions.

2.4. Sequence data analysis

Sequence data from PGM runs were generated using Torrent Suite Software. Initial variant calling was filtered through comparing to the alignments of the 1000 Genomes data to GRCh 37, and mutations were designated and annotated through Torrent Variant Caller. Mutations were identified when the coverage depth ≥1000 reads and mutant allele frequency ≥5%. All mutations were further visually examined using the Integrative Genomics Viewer.

2.5. Statistical analysis

Chi-square test or Fisher's exact test was used to observe the associations of mutated genes with clinicopathological variables (univariate analysis). Multivariate analysis of clinicopathological factors associated with mutation status was determined using logistic regression models. PFS was analyzed using the Kaplan-Meier method, and Log-rank tests were used to compare the PFS between different groups. Cox's proportional hazard models were used to estimate the hazard ratio (HR) and the corresponding 95% Confidence Intervals (CI). Statistical analysis was performed with the SPSS 18.0 software. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Patient characteristics

A total of 526 CRC patients were collected. Among these patients, 316 were male and 210 were female, with a median age of 57 years. Patients with distant metastasis included liver metastasis in 238 patients (45.2%), lung metastasis in 113 patients (21.5%) and other metastasis (peritoneum, bone, uterus, ovary, adrenal gland and so on) in 98 patients (18.6%). All patient characteristics were listed in Table 1.

3.2. Analysis of oncogenic mutations

Data from Ion Torrent sequencing showed that 1 to 4 somatic mutations were observed in 13 genes in 436 of 526 (82.9%) tumors. The

Table 1
Patient characteristics in 526 CRC patients.

Clinicopathologic characteristics	Cases (n = 526)
Gender	
Male	316
Female	210
Age (years)	
Median (range)	57 (15-83)
≤60	338
> 60	188
Location	
Left	164
Right	101
Rectum	259
Missing	2
Smoking	
Never	325
Former/Current	153
Missing	48
Drinking	
Never	307
Former/Current	171
Missing	48
BMI (kg/m^2)	
≤25	282
> 25	135
Missing	109
Mismatch repair (MMR) status	
Proficient	249
Deficient	15
Missing	177
Metastasis	
Liver	238
Lung	113
Others	98
Missing	45

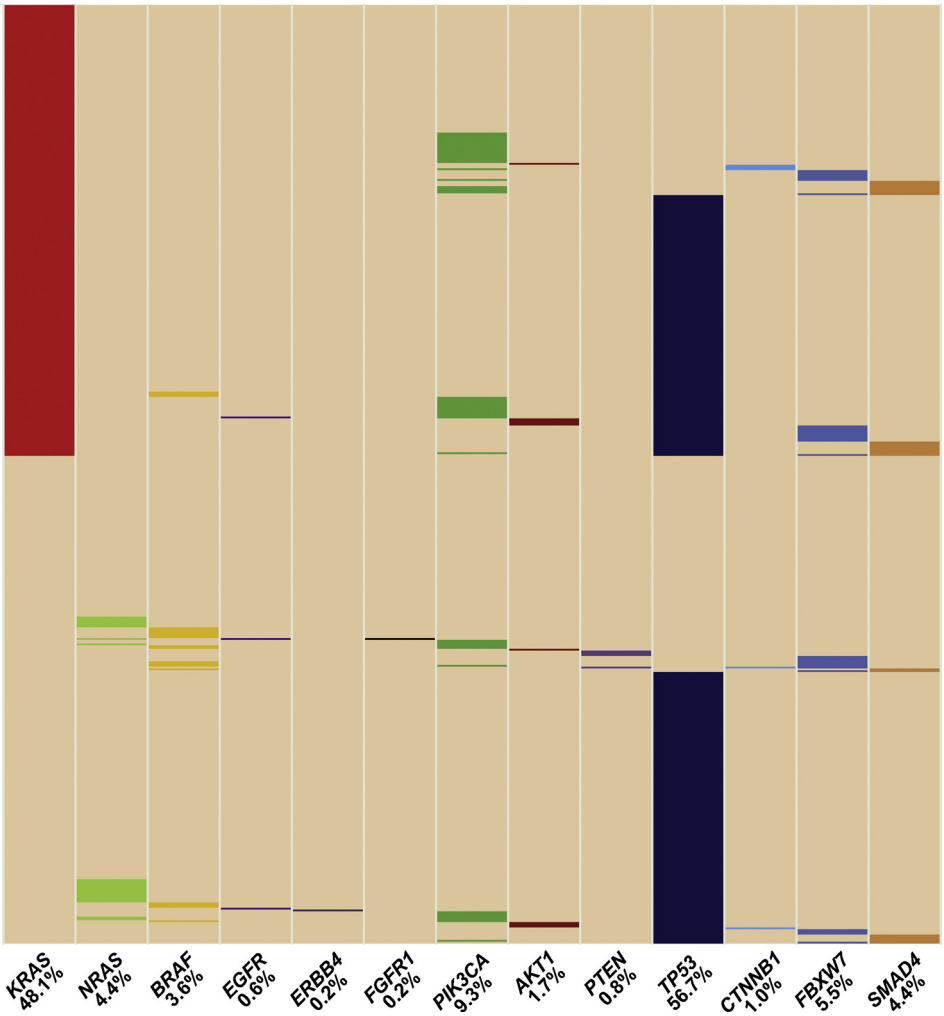

Fig. 1. Mutation profiling of 526 CRC patients detected by NGS. A total of 526 tumor samples were tested by Ion Torrent sequencing with the Ampliseq Colon and Lung Cancer Panel. Mutations were observed in 13 genes. Each column represented a gene, and mutations were marked with different colors.

most common mutations occurred in *TP53* (56.7%) and *KRAS* (48.1%), followed by *PIK3CA* (9.3%), *FBXW7* (5.5%), *SMAD4* (4.4%), *NRAS* (4.4%), *BRAF* (3.6%), *AKT1* (1.7%), *CTNNB1* (1.0%), *PTEN* (0.8%), *EGFR* (0.6%), *ERBB4* (0.2%) and *FGFR1* (0.2%) mutations (Fig. 1). However, nine genes (*MAP2K1*, *NOTCH1*, *STK11*, *FGFR2*, *FGFR3*, *DDR2*, *MET*, *ALK* and *ERBB2*) did not harbor any somatic mutation.

There were 298 of 526 (56.7%) tumors that harbored mutations in *KRAS*, *NRAS*, *BRAF* or *PIK3CA*. As shown in Supplementary Fig. 1, nineteen unique *KRAS* mutations were observed in 253 of 526 (48.1%) tumors. Double *KRAS* mutations were detected in 3 tumors. The most common *KRAS* mutations were p.G12D, p.G13D and p.G12 V mutations. Nine unique *NRAS* mutations were observed in 23 of 526 (4.4%) tumors. The most common *NRAS* mutation was p.Q61 K mutation. Seven unique *BRAF* mutations were observed in 19 of 526 (3.6%) tumors, and the most common mutation was p.V600E mutation. Twelve unique *PIK3CA* mutations were observed in 49 of 526 (9.3%) tumors. The most common *PIK3CA* mutations were p.H1047R, p.E545 K and p.E542 K mutations.

3.3. Concomitant mutations

Concomitant mutations in *KRAS*, *NRAS*, *BRAF* and *PIK3CA* were detected in 49 of 298 (16.4%) mutant tumors. There are 3 tumors with two different *KRAS* mutations, 3 tumors with a *KRAS* mutation and a *BRAF* mutation, 35 tumors with a *KRAS* mutation and a *PIK3CA* mutation, 3 tumors with a *NRAS* mutation and a *PIK3CA* mutation, 4 tumors with a *BRAF* mutation and a *PIK3CA* mutation, and 1 tumor with a *KRAS* mutation and two *PIK3CA* mutations (Fig. 2A). *NRAS* mutations were mutually exclusive to *KRAS* or *BRAF* mutations. Forty-three *PIK3CA* mutant tumors were concomitant with *KRAS*, *NRAS* or *BRAF* mutations. There was a moderate correlation between *PIK3CA* mutant allele frequencies and *KRAS*, *NRAS* or *BRAF* mutant allele frequencies in tumors with concomitant mutations ($r = 0.55$, $P < 0.01$) (Fig. 2B). Concomitant *KRAS* and *PIK3CA* mutations were observed in 29 of 159 (18.2%) tumors with *KRAS* codon 12 mutations, in 2 of 58 (3.4%) tumors with *KRAS* codon 13 mutations and in 5 of 38 (13.2%) tumors with non-*KRAS* codon 12/13 mutations, suggesting that concomitant *KRAS* and *PIK3CA* mutations were less likely to be observed in tumors with *KRAS* codon 13 mutations. Concomitant *NRAS* and *PIK3CA* mutations were detected in 0 of 10 tumors with *NRAS* codon 12/13 mutations and in 3 of 13 (23.1%) tumors with *NRAS* codon 61 mutations. Concomitant *BRAF* and *PIK3CA* mutations were detected in 4 of 14 (28.6%) tumors with *BRAF* kinase-activated mutations and in 0 of 5 tumors with *BRAF* kinase-impaired or unknown mutations (Fig. 2C). Concomitant *KRAS* and *BRAF* mutations were detected in 0 of 14 tumors with *BRAF* kinase-activated mutations and in 3 of 5 (60%) tumors with *BRAF* kinase-impaired or unknown mutations (Fig. 2D).

3.4. Correlation of oncogenic mutations with clinicopathological characteristics

The association of oncogenic mutations with clinicopathologic

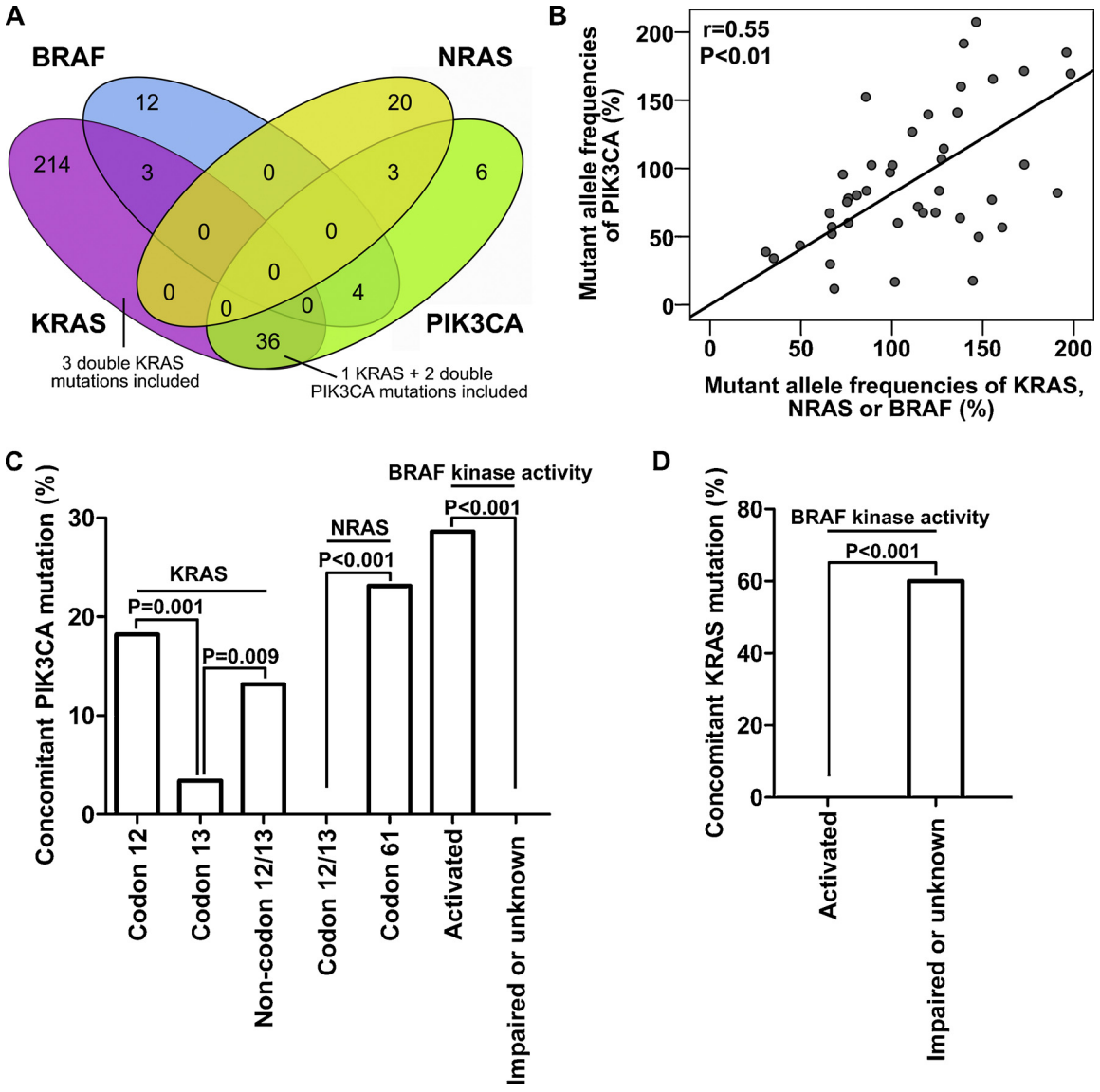

Fig. 2. Analysis of concomitant mutations in *KRAS*, *NRAS*, *BRAF* and *PIK3CA*. (a) Venn diagram showing the distribution of single and concomitant mutations in *KRAS*, *NRAS*, *BRAF* and *PIK3CA* genes. (b) Correlation between *PIK3CA* mutant allele frequencies and *KRAS*, *NRAS* or *BRAF* mutant allele frequencies in tumors with concomitant mutations. (c) Higher incidence rate of concomitant *PIK3CA* mutations was observed in tumors with *KRAS* outside exon 13 mutations, with *NRAS* codon 61 mutations and with *BRAF* kinase-activated mutations. (d) Higher incidence rate of concomitant *KRAS* and *BRAF* mutations was observed in tumors with *BRAF* kinase-impaired/unknown mutations.

features was analyzed and summarized. As shown in Table 2, the frequency of *KRAS* mutations was significantly higher in patients with older age (58.5% vs. 42.3%, $P < 0.001$), right-sided colon and rectum cancers (right-sided colon 55.4%, left-sided colon 39.6%, rectum 50.6%, $P = 0.023$), proficient MMR (pMMR) status (pMMR 51.4%, dMMR, 20%, $P = 0.018$) and lung metastasis (59.3% vs. 46.2%, $P = 0.015$). *PIK3CA* mutations were more likely to occur in patients with right-sided colon cancer (right-sided colon 17.8%, left-sided colon 8.5%, rectum 6.6%, $P = 0.004$). *AKT1* mutations were more frequently to occur in patients who were female (4.3% vs. 0%, $P = 0.001$) and with right-sided colon cancer (right-sided colon 5.9%, left-sided colon 1.2%, rectum 0.4%, $P = 0.001$). *FBXW7* mutations showed a higher frequency in patients who never drank alcohol (6.8% vs. 2.3%, $P = 0.035$), and had a BMI equal to or lower than 25 kg/m^2 (7.1% vs. 1.5%, $P = 0.016$). No association of other gene mutations with clinicopathological features was observed (Supplementary Table 1).

Moreover, multivariate analysis revealed that *KRAS* mutations were associated with older age (odds ratio [OR], 1.542; 95% CI, 1.065–2.235; $P = 0.022$) and lung metastasis (OR, 1.570; 95% CI, 1.014–2.432; $P = 0.043$). *PIK3CA* mutations were associated with right-sided colon cancer (OR, 2.159; 95% CI, 1.122–4.157; $P = 0.021$). *AKT1* mutations were associated with right-sided colon cancer (OR, 7.745; 95% CI, 1.840–32.605; $P = 0.005$). *FBXW7* mutations were associated with patients with a BMI equal to or lower than 25 kg/m^2 (OR, 7.771; 95% CI, 1.018–59.294; $P = 0.048$) (Supplementary Table 2).

3.5. The clinical outcomes of anti-EGFR treatment

Among the 179 *RAS* wild-type mCRC patients, 56 (31.3%) patients received the anti-EGFR antibody cetuximab in combination with chemotherapy. Clinical response was evaluated in 54 mCRC patients. PR was observed in 24 (44.4%) patients, and 17/24 (70.8%) patients had response duration for at least 6 months. SD was observed in 23 (42.6%) patients, and 13/23 (56.5%) patients showed stable duration for at least 6 months. PD was observed in 7 (13.0%) patients. There were 22 patients identified as wild type in all the detected 22 genes, while 32

Table 2
Association of oncogenic gene mutations with clinicopathologic characteristics.

	Cases	*KRAS* mutations	P	*PIK3CA* mutations	P	*AKT1* mutations	P	*FBXW7* mutations	P
Gender									
Male	316	150 (47.5%)		28 (8.9%)		0 (0%)		18 (5.7%)	
Female	210	103 (49.0%)	0.723	21 (10.0%)	0.660	9 (4.3%)	**0.001**	11 (5.2%)	0.822
Age (years)									
≤60	338	143 (42.3%)		26 (7.7%)		4 (1.2%)		18 (5.3%)	
> 60	188	110 (58.5%)	< **0.0001**	23 (12.2%)	0.086	5 (2.7%)	0.368	11 (5.9%)	0.800
Location									
Left	164	65 (39.6%)		14 (8.5%)		2 (1.2%)		8 (4.9%)	
Right	101	56 (55.4%)		18 (17.8%)		6 (5.9%)		4 (4.0%)	
Rectum	259	131 (50.6%)	**0.023**	17 (6.6%)	**0.004**	1 (0.4%)	**0.001**	17 (6.6%)	0.566
Missing	2	1				2			
Smoking									
Never	325	159 (48.9%)		33 (10.2%)		6 (3.9%)		19 (5.8%)	
Former/Current	153	79 (51.6%)	0.580	13 (8.5%)	0.567	1 (0.3%)	0.546	6 (3.9%)	0.378
Missing	48	15		3		2		4	
Drinking									
Never	307	150 (48.9%)		29 (9.4%)		7 (4.1%)		21 (6.8%)	
Former/Current	171	88 (51.5%)	0.585	17 (9.9%)	0.860	1 (0.3%)	0.313	4 (2.3%)	**0.035**
Missing	48	15		3		1		4	
BMI (kg/m^2)									
≤25	282	133 (47.2%)		25 (8.9%)		4 (1.4%)		20 (7.1%)	
> 25	135	71 (52.6%)	0.299	15 (11.1%)	0.466	4 (3.0%)	0.487	2 (1.5%)	**0.016**
Missing	109	49		9		1		7	
MMR status									
Proficient	249	128 (51.4%)		29 (11.6%)		4 (1.61%)		17 (6.8%)	
Deficient	15	3 (20%)	**0.018**	0	0.329	0	0.553	0	0.614
Missing	262	122		20		5		12	
Liver metastasis									
Yes	238	115 (48.3%)		20 (8.4%)		2 (0.8%)		10 (4.2%)	
No	243	122 (50.2%)	0.679	27 (11.1%)	0.317	6 (2.5%)	0.298	14 (5.8%)	0.432
Missing	45	16		2		1		5	
Lung metastasis									
Yes	113	67 (59.3%)		11 (9.7%)		1 (0.9%)		9 (8.0%)	
No	368	170 (46.2%)	**0.015**	36 (9.8%)	0.988	7 (1.9%)	0.750	15 (4.1%)	0.097
Missing	45	16		2		1		5	
Other metastasis									
Yes	98	47 (48.0%)		9 (9.2%)		3 (3.1%)		6 (6.1%)	
No	383	190 (49.6%)	0.771	38 (9.9%)	0.826	5 (1.3%)	0.441	18 (4.7%)	0.751
Missing	45	16		2		1		5	

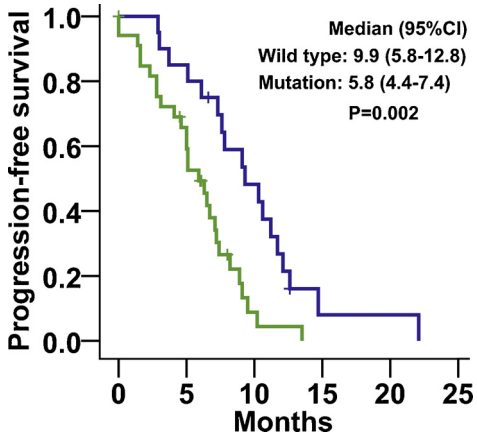

Fig. 3. Survivals by mutational status in *RAS* wild-type mCRC patients treated with anti-EGFR antibody.

patients identified as mutations in 1–2 genes. The clinical outcomes were analyzed according to overall mutation status (wild-type or mutation in one or more genes). The results showed that the PFS of patients identified as wild type in all the detected 22 genes was 9.9 (95% CI 5.8–12.8) months, significantly longer than the 5.8 (95% CI 4.4–7.4) months in patients with mutation in any of the detected genes (Fig. 3, $P = 0.002$).

A multivariate analysis was further performed, adjusting for all covariates with P < 0.10 in the univariate analysis including sex, location, mutation status and *TP53* mutations. The results showed that a wild-type result determined by NGS was an independently good prognostic factor for PFS ($P = 0.012$, Table 3).

4. Discussion

With the development of targeted therapy, molecular testing has become more and more important for cancer patients. In this study, we implemented Ion Torrent PGM platform to detect hotspot mutations of 22 cancer-related genes in 526 CRC patients, and established an analysis pipeline for these mutations. We further summarized the prevalence and clinical values of mutation profiling in a large cohort of CRC patients by NGS.

Our results showed that somatic mutations were observed in 13 genes in 436 of 526 (82.9%) tumors. Among these mutated genes, *KRAS* mutations were detected in 253 of 526 (48.1%) tumors. The *KRAS* mutation rate is slightly higher, as compared to COSMIC database and previous studies in which such alterations are reported to exist in 30–40% of CRC population [15,16]. However, most of those studies detect *KRAS* mutations using Sanger sequencing or allele-specific PCR assay, which are limited by low sensitivity or only detection of common mutations. Our data are consistent with some other NGS results obtained from CRC patients in western countries [17,18], suggesting that NGS provides a high sensitivity and broad screening for mutation detection. Further, we identified that *KRAS* p.G12D mutation was the most common *KRAS* mutation, and double *KRAS* mutations were detected in 3 tumors. A *KRAS* p.D47Y mutation that had not ever been reported was detected in exon 3. Further research is needed to uncover

Table 3
Univariate and multivariate Cox regression analysis of factors associated with progression-free survival in patients after cetuximab treatment.

	Univariable Analysis		Multivariable Analysis	
	Hazard Ratio (95%CI)	*P*	Hazard Ratio (95%CI)	*P*
Age (≥60 vs < 60 years)	0.58 (0.29-1.19)	0.136	–	
Sex (female vs male)	1.76 (0.94-3.30)	**0.078**	2.03 (1.05-3.94)	0.056
Location (right vs left)	2.80 (1.05-7.46)	**0.04**	1.53 (0.58-4.63)	0.457
Smoking (never vs ever)	0.91 (0.50-1.65)	0.754	–	
Drinking (never vs ever)	1.20 (0.67-2.16)	0.547	–	
BMI (kg/m^2)(≤25 vs > 25)	0.81 (0.44-1.49)	0.501	–	
Any mutation (none vs yes)	0.36 (0.19-0.70)	**0.002**	0.23 (0.07-0.73)	**0.012**
***TP53* (wild type vs mutation)**	0.50 (0.27-0.93)	**0.028**	1.15 (0.55-4.19)	0.43

its function in CRC. In accordance with previous work [19], the frequency of *NRAS* mutations in our study was 4.4%, and *NRAS* and *KRAS* mutations were mutually exclusive. The *BRAF* mutation rate was 3.6% in our study, which is lower when compared with that reported in western population [17], suggesting that *BRAF* mutation is less common in Chinese CRC patients. Racial differences in *BRAF* mutation rate are also proved by Yoon et al. in stage III CRC patients [20].

Previous observations have found that *PIK3CA* mutations are significantly associated with *KRAS* mutations [21], and *PIK3CA* can cooperate with *BRAF* or *KRAS* mutations to promote cancer progression [22,23]. In our study, 43 of 49 concomitant mutant tumors were harboring *PIK3CA* mutations. Interestingly, we found that a higher frequency of concomitant *PIK3CA* mutations was observed in tumors with *KRAS* outside codon 13 mutations, with *NRAS* codon 61 mutations and with *BRAF* kinase-activated mutations. We further found that the mutant allele frequencies of *PIK3CA* mutations were significantly correlated with the mutant allele frequencies of *KRAS*, *NRAS* and *BRAF* mutations, suggesting that concomitant *PIK3CA* and *RAS/BRAF* mutations usually occur in the same tumor population. It is no surprise since *PIK3CA* mutations usually occur after *RAS* or *BRAF* mutations and drive clonal evolution from large adenoma to invasive adenocarcinoma in cooperation with other mutations [24]. Consistent with a previous study in lung cancer [25], we found that *BRAF* kinase-impaired mutations usually coexisted with *KRAS* mutations, but not *PIK3CA* mutations.

Further, we investigated the relationship between the clinicopathological characteristics and the detected gene mutations. Mutations in receptor tyrosine kinase (RTK) growth factor receptors are uncommon in CRC [26]. However, *KRAS*, *NRAS* and *BRAF* mutations occur in more than 50% of CRC [27]. Our data showed that *KRAS* mutations were more frequently to occur in patients with older age and lung metastasis. PI3K pathway is important in cell growth, invasion and other processes. The most common alterations in PI3K pathway are mutations in *PI3KCA*, which encodes the catalytic subunit of PI3K. Other genetic alterations include mutations that inactivate *PTEN* and activate *AKT1* [28]. We here found that *PIK3CA* and *AKT1* mutations were both more frequent to occur in patients with right-sided colon cancer. Absence of functional FBXW7, and constitutively activated β-catenin contribute to the deregulation of Wnt signaling [10], which play a critical role in CRC progression. In our study, we found that *FBXW7* mutations were more likely to occur in patients who were thin.

Although anti-EGFR targeted therapy is an effective treatment, up to 60% of mCRC patients with *RAS* wild-type tumors do not benefit from anti-EGFR treatment [29]. Therefore, identification of biomarkers that can predict the clinical outcomes of anti-EGFR treatment in *RAS* wild-type patients is needed. Studies have shown that additional biomarkers, such as *BRAF* and *PIK3CA* mutations, may also account for the resistance to anti-EGFR treatment [30]. In our study, we found that all patients with *BRAF* or *PIK3CA* mutations did not respond to anti-EGFR treatment. We further found that longer PFS was observed in patients identified as wild type in all the detected 22 genes. These results suggest that use of the 22 gene panel sequencing can further predict the clinical outcomes of anti-EGFR treatment in *RAS* wild-type patients. Detection of oncogenic mutations in multiple genes may better predict the clinical outcomes. However, as more and more molecular alterations are proved to participate in CRC progression, further studies are needed to investigate whether use of whole-exome sequencing or a gene panel with more genes is more helpful.

There are several limitations in our study. First, only 56 patients received anti-EGFR treatment were collected. Larger samples may be needed to further confirm our conclusions. Second, there are some other possible resistance mechanisms that we do not include in our study, including HER2 amplification, EGFR gene polymorphisms and loss of PTEN expression [31–33]. Third, as only 54 patients received anti-EGFR treatment were collected, we could not identify whether the number or quality of mutated genes affected the clinical outcomes of anti-EGFR treatment.

In summary, we assessed the hotspot mutations in a large cohort of 526 Chinese CRC patients by NGS. *KRAS*, *PIK3CA*, *AKT1* and *FBXW7* mutations were associated with some clinicopathological features, including location, age or metastasis of CRC patients. Patients with wild-type tumors in all 22 genes showed better PFS as compared to patients with mutated tumors in any of these oncogenic pathway genes. We hope these discoveries may help to improve individual targeted therapy prediction in advanced CRC patients.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2018.12.037.

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