BRAF V600E mutation and KRAS codon 13 mutations predict poor survival in Chinese colorectal cancer patients

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Abstract

Background: Mutations in KRAS, BRAF and PIK3CA are the most common somatic alterations found in the colorectal cancer (CRC) patients from Western countries; but their prevalence and prognostic value have not been adequately assessed in Asian patients. The aim of this study was to determine the mutation frequencies of these genes in Chinese CRC patients and to investigate their impact on prognosis.

Methods: The sequences of exon 2 of KRAS, exon 15 of BRAF and exons 9 and 20 of PIK3CA were evaluated by PCR and direct sequencing using DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues from primary CRC tumors of 214 patients (colon/rectum: 126/88).

Results: KRAS, BRAF and PIK3CA mutations were identified in 44.9% (96/214), 4.2% (9/214) and 12.3% (26/212) CRCs, respectively. The most frequent mutations in KRAS, BRAF and PIK3CA were G12D, V600E and H1047R, respectively. All BRAF and 80.8% PIK3CA mutations were from colon cancer patients. BRAF V600E was associated with advanced TNM (P < 0.001), more distant metastases (P = 0.025), and worse overall survival (OS, P < 0.001; multivariate HR = 4.2, P = 0.004) in colon cancer patients. Compared with KRAS wt/BRAF wt CRC patients (N = 109), those with KRAS codon 13 mutations (N = 25) had significantly worse OS (P = 0.016; multivariate HR = 2.7, P = 0.011), whereas KRAS codon 12-mutated cases were not significantly associated with survival. Among the three most common KRAS mutations, G13D (N = 23) showed significant association with poor OS (P = 0.024; multivariate HR = 2.6, P = 0.016) compared with KRAS wt/BRAF wt patients.

Conclusion: Our findings indicate that PI3K/RAS-RAF signaling pathway genes are frequently mutated in Chinese CRC patients, but have different characteristics than found in Western patients. BRAF V600E is an independent prognostic factor for Chinese patients. Our finding that KRAS codon 13 mutations (in particular G13D) are associated with inferior survival in BRAF wild-type CRCs in Chinese patients was not reported thus far. Our data emphasizes the importance of prospective evaluation of molecular features in CRC patients, because a single mutation type may represent a distinct biologic effect and clinical implication.

Keywords: Colorectal cancer, BRAF, KRAS, Survival, Prognosis
Background

Colonorectal cancer (CRC) is one of the most common malignancies both in Western and in Asian countries [1]. In recent years, the morbidity and mortality of CRC have increased rapidly in the Chinese population, so that CRC has become the third leading cause of cancer deaths in China [2]. CRC arises through a multistep carcinogenic process with an accumulation of epigenetic and genetic alterations. Activation of two main EGFR-dependent signaling pathways, the RAS-RAF and the PI3K-PTEN-AKT pathways through mutations was considered to be one of the most common mechanisms involved in colorectal carcinogenesis. Numerous studies have indeed observed that KRAS, BRAF and PIK3CA mutations are commonly present in CRC, with frequencies of 30-50%, 10-15% and 10-20%, respectively. KRAS mutations occur 90% in exon 2 at codons 12 and 13. BRAF mutations are mostly located at codon 600 with a conversion of valine to glutamic acid (V600E) [3].

Although the predictive role of KRAS mutations, and more recently also BRAF mutations to recognize resistance to anti-EGFR therapy in advanced CRC patients has been accepted widely [3-7], the prognostic role of KRAS mutations in CRCs for survival is still controversial [8-12]. For the BRAF V600E mutation, many studies have shown its association with a poor clinical outcome [9,10,12,13]. Given that mutations in KRAS and BRAF are mutually exclusive, BRAF mutations may have potential confounding effect when estimating the prognostic value of KRAS mutations. It was recognized that the KRAS mutation can be better examined in BRAF wild-type CRCs, because almost all BRAF mutant patients are KRAS wild-type.

Other studies have shown that different KRAS mutations in CRCs may have different biological characteristics and may consequently have variable effects in patients. Firstly, an in vitro study showed that KRAS codon 13 mutations (mainly the p.G13D mutation) exhibited weaker transforming activity than codon 12 mutations [15]. Secondly, several clinical studies compared the prognostic roles of KRAS codon 12 mutations with those of codon 13, but did not yet reach consensus because of the limited results, though most studies agreed that KRAS mutations in codon 13 confer a poorer prognosis and outcome for patients under standard chemotherapy [13,14,16-18]. Thirdly, a recent retrospective study of De Rook et al. analyzed the association between KRAS mutations in codon 13 (G13D) versus codon 12 evaluating response and survival in patients with chemotherapy refractory treated with cetuximab, and showed that patients with the KRAS G13D mutation could benefit from cetuximab therapy, whereas those with a KRAS codon 12 mutation were likely to be resistant to cetuximab [19]. An increasing number of sometimes contradictory studies showed that patients with KRAS mutations in codon 13 could have a poorer outcome, but would significantly benefit clinically from an anti-EGFR therapy [20]. Apparently, the real mechanism by which different KRAS mutations affect tumor biology and lead to different outcomes needs to be further elucidated.

PIK3CA mutations cluster 90% in hotspots of exons 9 and 20, and affect the functionally important helical and kinase domains. PIK3CA mutations are likely to be associated with a poor prognosis [21,22] and clinical resistance to anti-EGFR targeted therapy [23].

Most of the studies that investigated the frequencies and prognostic values of KRAS, BRAF, PIK3CA mutations, and in particular, the efficacies of targeted therapies were performed in Western countries. There is not yet agreement on mutation frequencies in Chinese CRC patients, especially for BRAF and PIK3CA, because the frequencies of such mutations were reported differently in the few data published (Table 1). Furthermore, little is known about their prognostic value in Chinese CRC patients, since few studies had follow-up data. In our study, we aimed to identify the mutation frequencies of KRAS, BRAF and PIK3CA in primary tumors of a cohort of 214 Chinese CRC patients, and to assess their correlations with the clinicopathological characteristics. In addition, follow-up data were collected from all patients to determine their potential prognostic roles in survival.

Methods

Patients and tumor samples

Among the 436 consecutive patients diagnosed with colorectal cancer at Zhongda Hospital Affiliated to Southeast University (Nanjing, China) from 2007 to 2012, 35 were excluded because no surgery was performed. An additional 140 patients were excluded, as they were lost during follow-up period. Among the 261 patients eligible for the genetic testing, 38 patients were excluded because no tissue blocks were available. An extra 9 patients were excluded from the remaining 223 patients because of poor DNA quality. At last 214 patients were included in our study (Figure 1). There was no difference in the major clinicopathological characteristics between the included and excluded patients (see Additional file 1). All of these patients were histologically confirmed colorectal cancer by two experienced pathologists. None of the patients received any adjuvant therapy before resection. The median follow-up time of surviving patients was 34 months. The patients’ demographic and clinicopathological data are presented in Table 2. The collection of materials and patient data was approved by the Institutional Ethics Committee of Zhongda Hospital and written informed consent was obtained from the participants. The study
DNA extraction and mutation analysis
Genomic DNA was extracted from 5 sections of 10 μm thickness of macro-dissected formalin-fixed paraffin-embedded (FFPE) tumor samples, containing at least 50% tumor epithelium, as determined by an experienced pathologist in H&E-stained paraffin sections. The QIAamp DNA Mini Kits (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer’s instructions. For each sample, exons 9 and 20 of *PIK3CA*, exon 20 of *KRAS*, and exon 15 of *BRAF* were amplified by PCR. The presence of mutations was detected by direct sequencing at Beijing Genomic Institute (BGI, ABI 3730xL Genetic analyzer, Shenzhen, China) using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). For all PCR products with sequence variants, both forward and reverse sequence reactions were repeated for confirmation. Primers used for the amplification are listed in Table 3.

Statistical analysis
All statistical analyses were carried out with SPSS statistical software (version 18.0 for Windows, SPSS, Inc.). Data were analyzed with the Mann–Whitney test to compare quantitative and ordered variables and with Student’s t test to compare normally distributed data between two groups. χ² test and Fisher’s exact test were used to compare proportions. Survival analyses were done using the Kaplan-Meier (KM) method with time of surgery as entry date. Overall survival (OS) was defined as the period from the date of surgery until death from any cause or last follow-up. Log rank testing was used for comparison of groups.

To identify factors associated with OS, we evaluated the following clinicopathological variables in a univariate Cox
| Table 2 Clinicopathological characteristics according to PI3K/RAS-RAF pathway gene mutation status in 214 (212) colorectal cancer patients |
|---------------------------------------------------------------|
| **Sex** | **KRAS exon 2** | **BRAF exon 15** | **PIK3CA exon 9&20** | **PI3K/RAS-RAF pathway** |
| | No. patients (214/212) | No (%) | Yes (%) | P | No (%) | Yes (%) | P | No (%) | Yes (%) | P | No (%) | Yes (%) | P |
| **Sex** | **KRAS exon 2** | **BRAF exon 15** | **PIK3CA exon 9&20** | **PI3K/RAS-RAF pathway** |
| | No. patients (214/212) | No (%) | Yes (%) | P | No (%) | Yes (%) | P | No (%) | Yes (%) | P | No (%) | Yes (%) | P |
| **Age** | 68.0 | 67.1 | 69.1 | 0.286 | 68.0 | 66.9 | 0.801 | 68.7 | 64.6 | 0.133 | 67.8 | 68.4 | 0.728 |
| **Location** | colon | 126 (124) | 73 (61.9) | 54 (55.6) | 0.406 | 122 (59.5) | 5 (55.6) | 1.000 | 109 (58.6) | 17 (65.4) | 0.509 | 60 (61.2) | 66 (57.9) | 0.623 |
| | rectum | 88 (88) | 45 (38.1) | 42 (44.4) | 83 (40.5) | 4 (44.4) | 77 (41.4) | 9 (34.6) | 38 (38.8) | 48 (42.1) |
| **Differentiation** | 80 (2) | 1 (0.8) | 1 (0.9) | 0 (0) | 2 (1.1) | 1 (3.8) | 1 (1.0) | 2 (1.0) |
| | T1 | 5 (5) | 4 (3.4) | 1 (1.0) | 0.236 | 5 (2.4) | 0 (0) | 0.057 | 5 (2.7) | 0 (0) | 0.806 | 4 (4.1) | 1 (0.9) | 0.724 |
| | T2 | 35 (35) | 15 (12.7) | 20 (20.8) | 35 (17.1) | 0 (0) | 0 (0) | 29 (15.6) | 6 (23.1) | 0.433 | 13 (13.3) | 19 (16.7) | 0.231 |
| | T3 | 167 (166) | 93 (78.8) | 74 (71.7) | 159 (77.6) | 8 (88.9) | 147 (79.0) | 19 (73.1) | 77 (78.6) | 89 (78.1) |
| | T4 | 5 (4) | 4 (3.4) | 1 (1.0) | 4 (2.0) | 1 (11.1) | 3 (1.6) | 1 (3.8) | 2 (2.0) | 1 (0.9) |
| | N(−) | 115 (114) | 70 (59.3) | 45 (46.9) | 0.050 | 113 (55.1) | 2 (22.2) | 0.083 | 99 (53.2) | 15 (57.7) | 0.710 | 61 (62.2) | 53 (46.5) | 0.013 |
| | N(+) | 97 (96) | 46 (39.0) | 51 (53.1) | 90 (43.9) | 7 (77.8) | 85 (45.7) | 11 (42.3) | 35 (35.7) | 61 (53.5) |
| | M(−) | 163 (161) | 88 (74.6) | 75 (78.1) | 0.367 | 159 (77.6) | 4 (44.4) | 0.037 | 144 (77.4) | 17 (65.4) | 1.808 | 77 (78.6) | 84 (73.7) | 0.689 |
| | M(+) | 51 (51) | 30 (25.4) | 21 (21.9) | 46 (22.4) | 5 (55.6) | 42 (22.6) | 9 (34.6) | 21 (21.4) | 30 (26.3) |
| | M(−) | 193 (191) | 104 (88.1) | 89 (92.7) | 0.224 | 188 (91.7) | 5 (55.6) | 0.004 | 166 (89.2) | 25 (96.2) | 0.479 | 89 (90.8) | 102 (89.5) | 0.708 |
| | M(+) | 19 (19) | 13 (11.0) | 6 (6.3) | 15 (7.3) | 4 (44.4) | 18 (9.7) | 1 (3.8) | 8 (8.2) | 11 (9.6) |
| | Metachronous metastases | 176 (174) | 95 (80.5) | 81 (84.4) | 0.062 | 171 (83.4) | 5 (55.6) | 0.055 | 156 (83.9) | 18 (69.2) | 0.097 | 82 (83.7) | 92 (80.7) | 0.574 |
| | Metachronous metastases | 38 (38) | 23 (19.5) | 15 (15.6) | 34 (16.6) | 4 (44.4) | 30 (16.1) | 8 (30.8) | 16 (16.3) | 22 (19.3) |

*a*chi-square test; *b*Fisher exact test; *c*Mann–Whitney test; *d*t test; *DNA of two samples were not available for PIK3CA exon 20. P-values ≤ 0.05 are in bold.
regression model: age (>65 vs ≤65), sex (male vs female), tumor location (colon vs rectum), tumor differentiation grade, tumor diameter (<5 cm vs ≥5 cm), number of lymph nodes examined (<12 vs ≥12), TNM stage, KRAS status (mutant vs wild-type (wt)), BRAF status (mutant vs wt) and PIK3CA status (mutant vs wt). All variables associated with OS with \( P < 0.1 \) in the univariate analysis were entered into a Cox multivariate regression model with backward elimination. A two-sided \( P \) value of \( \leq 0.05 \) was considered statistically significant.

Results

Frequency and distribution of KRAS, BRAF and PIK3CA mutations

KRAS mutation status in exon 2 was detected in 96 out of 214 (44.9%) tumor samples, of which 70 (32.7%) had a single mutation and one had two mutations in codon 12, and 25 (11.7%) had a single mutation in codon 13. The most frequent mutation was 35G > A (G12D), which represented 35.4% of all KRAS mutations, followed by 38G > A (G13D, 24.0%). BRAF mutations in exon 15 were found in 9 out of 214 (4.2%) tumor samples. Only one case was 1801A > G (K601E), whereas the rest were 1799 T > A (V600E) mutations. PIK3CA mutations were found in 26 out of 212 patients (12.3%), with 12 cases in exon 9 (5.7%) and 14 cases in exon 20 (6.6%). The most frequently detected mutations were 1633G > A (E545K) in exon 9 and 3140A > G (H1047R) in exon 20 among a total of 11 variants. Mutations are summarized in Table 4. The distribution of the mutations in 212 samples is shown in Figure 2. In total, 114 cases (53.8%) had a mutation in at least one of the three genes, with 97 patients (45.8%) having a mutation in a single gene and 17 patients (8.0%) in two genes. 16 cases had concomitant occurrence of KRAS and PIK3CA mutations, but this association was not statistically significant (\( P = 0.075 \)). Only one patient had a BRAF and a PIK3CA mutation simultaneously. Mutations in KRAS and BRAF were not observed in the same tumor (\( P = 0.005 \)), which is consistent with previous studies stating that they were mutually exclusive [35].

PI3K/RAS-RAF pathway mutations and clinicopathological characteristics

We did not find any significant associations between KRAS mutations and patients’ clinicopathological characteristics, except that KRAS mutations were associated with more lymph node involvement (53.1% vs 46.9%, \( P = 0.050 \)). Data are shown in Table 2. Mutations in BRAF or PIK3CA showed a significant correlation with tumor location. All mutations in BRAF were from colon cancer patients and almost all were localized in the proximal colon (8/9). Likewise, most mutations in PIK3CA were from colon cancer patients (21/26, \( P = 0.014 \)). Compared

Table 3 The primers used in PCR amplification and sequencing

| Genes | Primers (sequence 5’–3’) |
|-------|--------------------------|
| KRAS  | F: TTAACCTTATGTGTGACATGCTAA  
|       | R: ATCAAAGAATGGTCCTGCAC |
|       | Exon 2 |
|       | F: CTTACTCTACACCTCAG  
|       | R: TAACCTACGACGATCTCGGG |
|       | Exon 15 |
|       | F: AGTACAGACTAGCTAGAGCAAT  
|       | R: CATGGTGAGATCAGCCAAAT |
|       | Exon 9 |
|       | F: ATGATGGCTGGCTCGGAAT  
|       | R: TGGGAATCCAGAGTGGCTT |
|       | Exon 20 |

Table 4 KRAS, BRAF and PIK3CA mutations identified in 214 colorectal cancer patients

| Genes | Nucleotide | Amino acid | Case (total) | % |
|-------|------------|------------|--------------|---|
| KRAS  | exon 2     | 34G > A    | G12S         | 2 |
|       |            | 34G > C    | G12R         | 1 |
|       |            | 34G > T    | G12C         | 5 |
|       |            | 35G > A    | G12D         | 34|
|       |            | 35G > C    | G12A         | 8 |
|       |            | 35G > T    | G12V         | 20|
|       |            | 35G > T & 35G > A | G12V & G12D | 1 |
|       | exon 15    | 1799 T > A | V600E        | 8 |
|       |            | 1801A > G  | K601E        | 1 |
| BRAF  | exon 9     | 1624G > A  | E542K        | 1 |
|       |            | 1633G > A  | E545K        | 7 |
|       |            | 1634A > C  | E545A        | 1 |
|       |            | 1636C > A  | Q546K        | 2 |
|       |            | 1637A > G  | Q546R        | 1 |
|       | exon 20    | 3062A > T  | Y1021F       | 2 |
|       |            | 3139C > T  | H1047Y       | 1 |
|       |            | 3140A > G  | H1047R       | 8 |
|       |            | 3140A > T  | H1047L       | 1 |
|       |            | 3145G > C  | G1049R       | 1 |
|       |            | 3155C > A  | T1052K       | 1 |
| PIK3CA| exon 9     | 1624G > A  | E542K        | 1 |
|       |            | 1633G > A  | E545K        | 7 |
|       |            | 1634A > C  | E545A        | 1 |
|       |            | 1636C > A  | Q546K        | 2 |
|       |            | 1637A > G  | Q546R        | 1 |
|       | exon 20    | 3062A > T  | Y1021F       | 2 |
|       |            | 3139C > T  | H1047Y       | 1 |
|       |            | 3140A > G  | H1047R       | 8 |
|       |            | 3140A > T  | H1047L       | 1 |
|       |            | 3145G > C  | G1049R       | 1 |
|       |            | 3155C > A  | T1052K       | 1 |

*DNA of 2 samples was not available for PIK3CA exon 20.
to patients without mutation (wild-type patients), those who harbored at least one mutation in any of the three genes were not different in any of the listed features except lymph-node involvement when admitted (53.5% vs 35.7%, \(P = 0.013\)). There was no significant difference in listed features between those carrying two gene mutations and the wild-type patients (data not shown).

We further analyzed the impact of \textit{BRAF} mutation in the 126 colon-cancer patients. Among the 9 patients with a \textit{BRAF} mutation, 8 were V600E and 1 was K601E. As mutation in codon 601 does not have a clear biological function, we only took the V600E mutation into further analysis (Table 5). The V600E mutation was correlated with significantly higher TNM stage (\(P = 0.014\)). Furthermore, patients with this \textit{BRAF} mutation had a >2.5-fold higher risk for distant metastases than patients without this mutation (62.5% vs 22.9%, \(P = 0.025\)). The risk for synchronous metastases was >8-fold higher in patients with than without this \textit{BRAF} mutation (50.0% vs 5.9%, \(P = 0.002\)). Notably, 3 out of 8 patients with the V600E mutation developed both synchronous and metachronous metastases.

**Prognostic value of \textit{BRAF} and \textit{KARS} codon 13 mutations**

In a KM analysis of the \textit{BRAF} V600E mutation in 126 colon patients, V600E was strongly associated with a poorer OS (log-rank \(P < 0.001\); 3-year OS: 16.7% in the \textit{BRAF} V600E mutant vs 73.2% in the \textit{BRAF} wild-type (wt); Figure 3A). No differences were found between patients with and without \textit{KRAS} mutations (log-rank \(P = 0.133\); 3-year OS: 64.6% in the \textit{KRAS} mutant vs 72.4% in the \textit{KRAS} wt; Figure 3B) in the survival analysis. Similarly, no differences were found for \textit{PIK3CA} mutations or at least one mutation in any of the three genes (data not shown). However, several recent studies suggested to exclude the confounding effect of \textit{BRAF} mutation from \textit{KRAS} wt patients when evaluating the prognostic value of \textit{KRAS}, as \textit{BRAF} mutation is associated with a poorer prognosis [9,13,14]. We then selected \textit{BRAF} wt cases only and compared \textit{KRAS}-mutants/\textit{BRAF} wt cases with \textit{KRAS} wt/\textit{BRAF} wt cases to assess the prognostic value of \textit{KRAS} mutations. A total of 205 cases (214 cases - 9 \textit{BRAF} mutants) remained in the analysis (Figure 4) with 52 death events. Intriguingly, \textit{KRAS} mutations showed its prognostic value when \textit{BRAF} mutations were excluded in the KM analysis (log-rank \(P = 0.035\); 3-year OS: 64.6% in \textit{KRAS} mutants/\textit{BRAF} wt vs 76.3% in \textit{KRAS} wt/\textit{BRAF} wt; Figure 3C). We

![Figure 2 The distribution of mutations is illustrated in a pie chart of 212 colorectal cancer samples.](http://www.biomedcentral.com/1471-2407/14/802)

### Table 5 Clinicopathological characteristics according to \textit{BRAF} V600E mutation status in 126 colon cancer patients

| \textit{BRAF} codon 600 mutation | No (%) | Yes (%) | \(P\) |
|---------------------------------|--------|---------|------|
| **Sex**                         |        |         |      |
| Male                            | 71 (60.2) | 5 (62.5) | 1.000<sup>b</sup> |
| Female                          | 47 (39.8) | 3 (37.5) |      |
| **Age**                         |        |         |      |
| 69.4                             | 65.4    | 0.664<sup>a</sup> |
| **Differentiation**             |        |         |      |
| well                             | 15 (12.7) | 1 (12.5) | 0.192<sup>c</sup> |
| moderate                        | 91 (77.1) | 3 (37.5) |      |
| poor                            | 5 (4.2)  | 2 (25.0) |      |
| missing                         | 7 (5.9)  | 2 (25.0) |      |
| **Tumor diameter**              |        |         |      |
| <5 cm                           | 49 (41.5) | 2 (25.0) | 0.469<sup>b</sup> |
| \(\geq 5\) cm                    | 67 (56.8) | 6 (75.0) |      |
| missing                         | 2 (1.7)  | 0 (0)    |      |
| **TNM-stage**                   |        |         |      |
| I                               | 9 (7.6)  | 0 (0)    | 0.014<sup>c</sup> |
| II                              | 50 (42.4) | 2 (25.0) |      |
| III                             | 49 (42.4) | 2 (25.0) |      |
| IV                              | 7 (5.9)  | 4 (50.0) |      |
| missing                         | 2 (1.7)  | 0 (0)    |      |
| **T**                           |        |         |      |
| T1                              | 0 (0)    | 0 (0)    | 0.106<sup>c</sup> |
| T2                              | 9 (7.6)  | 0 (0)    |      |
| T3                              | 105 (89.0) | 7 (87.5) |      |
| T4                              | 2 (1.7)  | 1 (12.5) |      |
| missing                         | 2 (1.7)  | 0 (0)    |      |
| **N**                           |        |         |      |
| N(−)                            | 62 (52.5) | 2 (25.0) | 0.157<sup>b</sup> |
| N(+)                            | 55 (46.6) | 6 (75.0) |      |
| missing                         | 1 (0.8)  | 0 (0)    |      |
| **Metastases**                  |        |         |      |
| M(−)                            | 91 (77.1) | 3 (37.5) | 0.025<sup>b</sup> |
| M(+)                            | 27 (22.9) | 5 (62.5) |      |
| **Synchronous metastases**      |        |         |      |
| M(−)                            | 110 (93.2) | 4 (50.0) | 0.002<sup>b</sup> |
| M(+)                            | 7 (5.9)  | 4 (50.0) |      |
| missing                         | 1 (0.8)  | 0 (0)    |      |
| **Metachronous metastases**     |        |         |      |
| M(−)                            | 96 (81.4) | 4 (50.0) | 0.056<sup>b</sup> |
| M(+)                            | 22 (18.6) | 4 (50.0) |      |

<sup>a</sup>Fisher exact test; <sup>b</sup>Mann–Whitney test; <sup>c</sup>t test. P-values \(\leq 0.05\) are in bold.
Figure 3 Kaplan-Meier curves. Panel A shows OS according to BRAF V600E mutation status in 126 colon cancer patients. Panel B shows OS according to KRAS mutation status in 214 colorectal cancer patients. Panels C, D and E show OS according to KRAS, KRAS codon 13 and KRAS c.38G > A (G13D) mutation status in 205 BRAF wild-type colorectal cancer patients, respectively.
further analyzed the prognostic roles of two subtypes of KRAS mutations. Interestingly, patients with a KRAS codon 13 mutation experienced a significant decrease in OS in KM analysis compared with patients with a KRAS wt/BRAF wt genotype (log-rank P = 0.016; 3-year OS: 53.4% in KRAS codon 13 mutants/BRAF wt vs 76.3% in KRAS wt/BRAF wt; Figure 3D), while KRAS codon 12 mutations did not show this effect. Among the 3 most common KRAS codon 12 and 13 mutations analyzed, c.38G > A (p.G13D; N = 23) was significantly associated with worse OS compared with KRAS wt/BRAF wt (log-rank P = 0.024; 3-year OS: 55.8% in KRAS c.38G > A mutants/BRAF wt vs 76.3% in KRAS wt/BRAF wt; Figure 3E).

**Univariate and multivariate analysis of outcome predictors**

To correct for significant prognostic factors, variables including age, sex, differentiation grade, tumor diameter, number of lymph nodes examined, TNM stage and KRAS/BRAF/PIK3CA genotype were first examined in colon cancer patients with the univariate Cox regression model (Table 6). Besides sex (P = 0.009) and TNM stage (P ≤ 0.000), BRAF V600E mutation showed a significant association with a higher risk of overall mortality (hazard ratio (HR), 5.1; 95% confidence interval (CI), 2.1-12.4; P ≤ 0.001). The independent prognostic value of the BRAF V600E mutation was further tested in multivariate analysis with backward stepwise elimination, including the following variables: sex, TNM stage and BRAF V600E mutation. No significant interactions were observed between the variables. The BRAF V600E mutation remained as an independent predictor for poor prognosis in patients with colon cancer (HR, 4.2; 95% CI, 1.6-11.0; P = 0.004) (Table 6). Compared with the KRAS wt/BRAF wt cases, those with a KRAS codon 13 mutation experienced a significant decrease in OS in the Cox regression analysis (univariate: HR, 2.4, 95% CI, 1.1-5.3; P = 0.026; multivariate HR, 2.6, 95% CI, 1.2-5.8; P = 0.016; Table 8).

**Discussion**

In this study, we determined mutation frequencies of KRAS, BRAF and PIK3CA in 214 Chinese CRC patients with resectable tumors and examined the correlations between their genotypes and clinicopathological

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**Table 6 Analysis of OS in 126 colon cancer patients by Cox regression analysis**

| Variables       | Univariate analysis | Multivariate analysis |
|-----------------|---------------------|----------------------|
|                 | HR (95% CI)         | P        | HR (95% CI) | P           |
| Age             | 0.995               |          |            |             |
| <=65            | 1.0                 |          |            |             |
| >65             | 1.0 (0.5-2.108)     | 0.009    | 1.0         | 0.009       |
| Sex             | 0.160               |          |            |             |
| Female          | 1.0                 |          |            |             |
| Male            | 3.0 (1.3-7.0)       | 0.009    | 3.3 (1.3-7.8) | 0.009      |
| Differentiation |                     |          |            |             |
| well            | 1.0                 |          |            |             |
| moderate        | 0.5 (0.2-1.1)       | 0.032    | 0.962       |             |
| poor            | 1.0 (0.3-3.9)       | 0.032    | 1.0 (0.3-3.9) | 0.032     |
| Lymphnode examined |                 |          |            |             |
| >12             | 1.0                 |          |            |             |
| <=12            | 2.0 (1.0-4.1)       | 0.032    | 2.0 (1.0-4.1) | 0.032     |
| Tumor diameter  | 0.188               |          |            |             |
| <5 cm           | 1.0                 |          |            |             |
| >= 5 cm         | 1.7 (0.8-3.5)       | 0.032    | 1.7 (0.8-3.5) | 0.032     |
| TNM-stage       | <0.001              | 0.017    |            |             |
| I               | 1.0                 |          | 1.0         |             |
| II              | 1.4 (0.2-10.8)      | 0.749    | 1.3 (0.2-9.9) | 0.749     |
| III             | 1.8 (0.2-13.9)      | 0.568    | 2.1 (0.3-15.9) | 0.568    |
| IV              | 9.7 (1.2-78.3)      | 0.032    | 5.8 (0.7-47.8) | 0.032    |
| KRAS status     | 0.795               |          |            |             |
| wt              | 1.0                 |          | 1.0         |             |
| mutant          | 1.1 (0.5-2.2)       | 0.032    | 1.1 (0.5-2.2) | 0.032     |
| BRAF V600E status |                 |          |            |             |
| wt              | 1.0                 |          | 1.0         |             |
| mutant          | 5.1 (2.1-12.4)      | 0.42     | 4.2 (1.6-11.0) | 0.42    |
| PIK3CA status   | 0.727               |          |            |             |
| wt              | 1.0                 |          | 1.0         |             |
| mutant          | 1.2 (0.5-2.8)       | 0.032    | 1.2 (0.5-2.8) | 0.032     |

P-values ≤ 0.05 are in bold.
characteristics. Our data showed that BRAF and PIK3CA mutations were related to tumor site. In addition, we clarified the prognostic values of BRAF V600E mutation and KRAS mutations in codon 13. To the best of our knowledge, we assessed for the first time the impact of KRAS mutations, including distinguished mutation subtypes, on prognosis in Chinese CRC patients when the confounding effect of a BRAF mutation was controlled.

Comparing our results with the studies from Western countries, differences in mutation distribution and frequency were observed. We identified a frequency of ~45% for a KRAS mutation, which is in the same range as found in earlier studies of Chinese and Western CRC patients [3,27]. The distribution of KRAS mutations in the Western population showed that G12D was the most frequent mutation subtype in codon 12, followed by G12V/C/S/A/R or G12V/S/C/A/R [27,36]. In contrast, the corresponding order of KRAS codon 12 mutation frequency in our data was G12D/V/A/C/S/R, as was found in another study of Chinese CRC patients [31]. For codon 13, the order of two mutation subtypes (38G > A and 37G > T) was not different from that found by others. The BRAF mutation frequency in CRC patients from Western countries is 10-15% [37-39]. In our study, the BRAF mutation frequency was ~4%, that is, in the same range as in Japanese and other Chinese reports from different regions including Taiwan (1-7%) [13,24,28,31]. This finding suggests that the BRAF mutation frequency in Asian CRC patients is lower than in Western patients.

The PIK3CA gene encodes the PI110 catalytic subunit of PI3K that regulates the pathway. In agreement with earlier studies, the PIK3CA mutation frequency was ~12% in our samples and could co-occur with KRAS or BRAF mutations [21,37,40]. And, in the 17 cases with concomitant mutations, 16 of them had PIK3CA and KRAS mutations (P = 0.075), while only one case had PIK3CA and BRAF mutations. The concomitant occurrence of PIK3CA and KRAS mutations was reported previously in CRC and other human cancer types [21,35]. The coexistence of KRAS and BRAF mutations was not observed in our patient cohort, consistent with earlier studies. The mutual exclusive occurrence of KRAS and BRAF mutations suggests they occur in different tumor subtypes [12].

We also investigated the clinicopathological characteristics of CRC patients with respect to KRAS, BRAF, PIK3CA mutations. We found that the frequencies of BRAF and PIK3CA mutations were significantly lower in rectal than in colon cancer. A lower frequency in rectal cancer was also observed in a few Western studies [35]. This observation emphasizes the difference between colon and rectal cancers, which may result in distinct treatment responses and prognosis [41,42].

In this Chinese cohort of 126 sporadic colon cancer patients, we found that the BRAF V600E mutation was significantly associated with a higher metastatic rate and a poorer OS. In the multivariate analysis, BRAF V600E was an independent prognostic factor for OS in colon cancer, next to sex and TNM (Table 6). Actually, together with another case harboring a BRAF K601E mutation, BRAF mutations was also associated with a poorer OS (log rank, P = 0.002, data not shown) in our study cohort. It has been

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Table 7 Analysis of OS according to KRAS mutation status in 205 BRAF wt colorectal cancer patients by cox regression analysis

| KRAS   | BRAF | Total N | No. of events | Univariate analysis | Multivariate analysis |
|--------|------|---------|---------------|---------------------|----------------------|
|        |      |         |               | HR (95% CI)         | P                    |
| wt     | wt   | 109     | 22            | 1.0                 | 1.0                  |
| All codon 12 mutants | wt   | 71      | 20            | 1.6 (0.9-2.9)       | 0.139                |
| All codon 13 mutants   | wt   | 25      | 10            | 2.5 (1.2-5.2)       | 0.019                |

NOTE: We tested KRAS codon 12 and 13 mutations among BRAF wild type cases. The multivariate Cox regression model initially included age, sex, tumor location, tumor differentiation, tumor diameter, number of lymph nodes examined, TNM stage, KRAS and PIK3CA status. A backward stepwise elimination with a threshold of P = 0.1 was used to select variables in the final model. TNM stage and KRAS status were finally entered the multivariate analysis. P-values ≤ 0.05 are in bold.

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Table 8 Analysis of OS according to the 3 most common KRAS codon 12 and 13 mutations in 205 BRAF wt colorectal cancer patients by cox regression analysis

| KRAS   | BRAF | Total N | No. of events | Univariate analysis | Multivariate analysis |
|--------|------|---------|---------------|---------------------|----------------------|
|        |      |         |               | HR (95% CI)         | P                    |
| wt     | wt   | 109     | 22            | 1.0                 | 1.0                  |
| c.38G > A | wt | 23      | 9             | 2.4 (1.1-5.3)       | 0.026                |
| c.35G > A | wt | 34      | 9             | 1.4 (0.6-3.0)       | 0.425                |
| c.35G > T | wt | 20      | 5             | 1.4 (0.5-3.6)       | 0.546                |

NOTE: The multivariate Cox regression model included the same set of covariates selected in Table 7. P-values ≤ 0.05 are in bold.
well recognized that BRAF V600E mutation confers a poor prognosis in Western CRC patients [9,10,12,13]. However, among the limited number of BRAF mutation studies in Chinese patients, only one study performed a survival analysis in a sample of 314 patients, including colon and rectum cancers. Although they reported the same conclusion as we do, they did not clarify which mutation types contributed to this effect [26]. As our patients were treated with the same chemotherapy and none of them received targeted therapy after surgery, our result may be interpreted as that the BRAF V600E mutation is a sensitive prognostic indicator independent of treatment regimen and disease progression. Obviously, this observation needs to be confirmed in a larger population of Chinese patients. Nevertheless, our findings suggest that prospective evaluation of the BRAF mutation status is equally important in Chinese patients with colon cancer, even though its mutation frequency (4.7%) is lower than Western patients and no effective therapy available. The manifest adverse effects of this mutation require more vigorous treatment and surveillance in this group of high-risk patients.

Another point worth noting was that sex was an independent predictor for prognosis in our colon cancer patients, with male patients being at a higher risk than female patients. Concordant with our conclusion, the study [43] which looked at the cumulative 10-year incidence and mortality of CRC among men at ages 50, 55, and 60 in US revealed that women reached equivalent levels of disease 4–8 years later than men. This finding indicates the importance of the choice of age at initiation of CRC screening.

Although, the predictive role of KRAS mutation in adopting anti-EGFR antibody therapy has been well recognized, its prognostic value in survival remains controversial. This may be caused by different study size, patient selection, operation options, chemotherapy regimens, sample controlling, material characters, detection method and data analysis. Importantly, few studies realized that KRAS wt samples were mixed with BRAF mutants, which strongly affects the prognostic value of KRAS mutations [13,14]. In our study, the negative prognostic role of KRAS mutations emerged when BRAF mutant patients were separated from the KRAS wt patients (Figure 3C).

Only a small and very recent detailed analysis estimated the prognostic effect of KRAS mutations when codon 12 and 13 are counted separately [13,14,20]. The main finding of these clinical studies is that KRAS mutations in codon 13 confer a poorer prognosis and outcome on patients under standard chemotherapy. In agreement, our KM curves clearly demonstrated that OS in patients with KRAS codon 13 mutations, in particular, c.38G > A (p.G13D), the most frequent codon 13 mutation in our patients (23 out of 25) and in general [19]), was significantly worse than that in patients without KRAS and BRAF mutations (Figure 3D&E). KRAS codon 12 mutations, on the other hand, had no effect on patients’ OS in our study. In both univariate and multivariate analysis, we further confirmed KRAS codon 13 (G13D) mutation as an independent negative prognostic factor for OS. Since our patients had only received standard chemotherapy and none of them had targeted medicine after resection, our findings support KRAS codon 13 (G13D) mutation as a prognostic biomarker in the natural process of colorectal cancer.

In contrast to the clinical findings, the in vitro studies suggested that KRAS codon 13 (G13D) mutations confer a weaker transforming capacity on cells than codon 12 mutations [20]. In addition, recent computational analysis revealed that KRAS protein with a mutation in codon 13 has a similar structure and dynamics as KRAS wt protein. Consequently, patients with this mutation could benefit from anti-EGFR antibody therapy [44]. In fact, several recent studies investigated the efficacy of anti-EGFR therapies for mutations in codon 13 and 12 separately [20] and reported improved PFS and OS for advanced CRC patients with the G13D mutation after receiving cetuximab alone or in combination with chemotherapy. Therefore, KRAS codon (G13D) may not only be a prognostic biomarker but may also be predictive for a positive response to anti-EGFR treatment.

The limitations of this study include its retrospectiveness, relative small sample size (n = 214) and short follow-up time. Nevertheless, we have found that BRAF V600E and KRAS G13D mutations were associated with worse OS in Chinese CRC patients. Moreover, we did not obtain epigenetic status or microsatellite instability (MSI) data, which plays a role in CRCs. However, the frequency of BRAF mutation is low in Chinese CRC patients, with only 9 (~4%) in the present study, so that further subgroup analysis was not feasible in this study. We are enlarging our sample size by recruiting CRC patients from other clinical centers and will have longer follow-up data for further analysis. Furthermore, additional mutations, including KRAS mutations beyond exon 2 and NRAS mutations, will be analyzed in our cohorts, since current studies based on Western CRC patients seem to suggest that they may be prognostic for outcome and predictive for the efficacy of anti-EGFR therapies [45], and few data is available on Chinese patients.

Conclusion

In conclusion, our study demonstrated the BRAF V600E mutation was an independent prognostic factor for colon cancer patients and was the first study on Chinese patients to find that KRAS codon 13 mutations (in particular, c.38G > A, p.G13D), but not codon 12 mutations,
were associated with poor prognosis in BRAF wild-type CRCs. A single mutation type may represent a distinct biologic effect and clinical implication [46], but also appears to convey benefit from a targeted therapy. Our findings show that molecular features in CRC patients are important to avoid confounding effects in future clinical trials.

Additional file

Additional file 1: Word file, a summary table of the major clinicopathological characteristics of the patients included and excluded in this study.

Abbreviations
CI: Confidence interval; CRC: Colorectal cancer; FFPE: Formalin-fixed paraffin-embedded; HR: Hazard ratio; KW method: Kaplan-Meier method; OS: Overall survival; wt: Wild-type.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
YH designed the study. JC, FG and YH conducted the experiments and data analysis. JC and YH interpreted the data and drafted the manuscript. XS, LZ and AZ helped with sample preparation, patients’ data collection and interpretation. HJ helped the statistical analysis. All authors read and approved the final manuscript.

Acknowledgements
The authors thank Pathology and General Surgery departments of Zhongda Hospital affiliated to Southeast University for the preparation of the FFPE sections and the collection of the clinical data, and Professor Wouter H Van Cutsem and Akihiko Miyoshi for the preparation of the FFPE and the collection of the clinical data, respectively. The current study was supported by the National Natural Science Foundation of China (81272400) and Research Fund for the Doctoral Program of Higher Education of China (20130092110052).

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Received: 27 June 2014 Accepted: 17 October 2014

Published: 3 November 2014

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