Effectors of Epidermal Growth Factor Receptor Pathway: The Genetic Profiling of KRAS, BRAF, PIK3CA, NRAS Mutations in Colorectal Cancer Characteristics and Personalized Medicine

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Abstract

Mutations in KRAS oncogene are recognized biomarkers that predict lack of response to anti-epidermal growth factor receptor (EGFR) antibody therapies. However, some patients with KRAS wild-type tumors still do not respond, so other downstream mutations in BRAF, PIK3CA and NRAS should be investigated. Herein we used direct sequencing to analyze mutation status for 676 patients in KRAS (codons 12, 13 and 61), BRAF (exon 11 and exon 15), PIK3CA (exon 9 and exon 20) and NRAS (codons 12, 13 and 61). Clinopathological characteristics associations were analyzed together with overall survival (OS) of metastatic colorectal cancer patients (mCRC). We found 35.9% (242/674) tumors harbored a KRAS mutation, 6.96% (47/675) harbored a BRAF mutation, 9.9% (62/625) harbored a PIK3CA mutation and 4.19% (26/621) harbored a NRAS mutation. KRAS mutation coexisted with BRAF, PIK3CA and NRAS mutation, PIK3CA exon 9 mutation appeared more frequently in KRAS mutant tumors (P = 0.027) while NRAS mutation almost existed in KRAS wild-types (P < 0.001). Female patients and older group harbored a higher KRAS mutation (P = 0.018 and P = 0.031, respectively); BRAF (V600E) mutation showed a higher frequency in colon cancer and poor differentiation tumors (P = 0.020 and P = 0.030, respectively); proximal tumors appeared a higher PIK3CA mutation (P < 0.001) and distant metastatic tumors shared a higher NRAS mutation (P = 0.010). However, in this study no significant result was found between OS and gene mutation in mCRC group. To our knowledge, the first large-scale retrospective study on comprehensive genetic profile which associated with anti-EGFR MoAbs treatment selection in East Asian CRC population, appeared a specific genotype distribution picture, and the results provided a better understanding between clinicopathological characteristics and gene mutations in CRC patients.

Introduction

Colorectal cancer (CRC) still causes majority of mortality in the world [1]. In mCRC tumors, exceedingly poor prognosis was observed. Fortunately, the rapid development in biological agents appears a promising future in treatment. Cetuximab or panitumumab, the monoclonal antibody (MoAb) targeted on epidermal growth factor receptor (EGFR), has been implemented in clinical practice, however, major data about the frequency of mutations in downstream effectors of the EGFR signaling pathway, such as BRAF, PIK3CA and NRAS, may induce a negative effect on the response in anti-EGFR targeted treatment [9,10,11].

Although previous clinical trials have indicated that patients who carry KRAS mutations in codons 12 and 13 are non-responsive to the EGFR-targeted therapy [4,5,6,7], and the wild-type status seems a response condition, some wild-type patients still fail to respond to anti-EGFR monoclonal antibody therapy [8], and the mechanism remains unclear. It is possible that mutations in the downstream effectors of the EGFR signaling pathway, such as BRAF, PIK3CA and NRAS, may induce a negative effect on the response in anti-EGFR targeted treatment [9,10,11].

To date, genetic profiling of individual tumors affect the selection of therapy and treatment response have been proven in clinical practice, however, major data about the frequency of
oncogenes mutations were presented in Western populations and few data are available for the Chinese. Since gene mutation status alters with ethnic differences [12], we design this study to investigate the ethnicity-specific role of mutations in development and progression of CRC. KRAS, BRAF, PIK3CA and NRAS mutations in primary tumors from Chinese CRC patients were detected and their potential correlations with clinicopathological factors were analyzed. Furthermore, we collected the survival data of mCRC subgroup patients, in order to obtain an appropriate insight between gene mutation and survival status. We intended that these data could benefit the design of future clinical trials and individualized therapy in CRC patients.

Materials and Methods

Patients

We investigated 676 consecutive patients who underwent surgery for colorectal cancer at the Cancer Institute/Hospital of the Chinese Academy of Medical Sciences (Beijing, China) between August 2010 and December 2011, all the patients were carried out primary resection in our hospital, and no patient had received chemotherapy before surgery. Each patient was contact- to provide available formalin-fixed, paraffin-embedded (FFPE) CRC tissues. Written informed consent was obtained from individual patients, and the experimental protocol was approved by the Institutional Review Board (IRB) in Cancer Institute/ Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College. CRC diagnosis was confirmed by hematoxylin and eosin (HE) staining and histological analysis. Overall survival was defined as the period from the start of diagnosed CRC until death from any cause or last follow-up. The patients’ demographic and clinicopathological data are presented in Table 1.

DNA Extraction and Mutation Analysis

Before the extraction of genomic DNA, all CRC samples were identified by two pathologists in order to ensure the representative malignant cells exist in each sample, the tissue blocks were cut into 5 μm sections, then microdissection was performed to guarantee each tissue sample tested contained >90% cancer cells. DNA was extracted by the QiAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and stored at −80°C until use.

We detected the mutation hotspots in KRAS (codons 12 and 13), BRAF (exon15), PIK3CA (exon 9 and exon 20) and NRAS (codon 61), where the most mutations occur in these genes [13,14], besides, rare types of mutations for KRAS (codon 61), BRAF (exon 11) and NRAS (codons 12 and 13) were also included. The program for the PCR amplification in KRAS, BRAF, NRAS and PIK3CA exon 20 was as follows: 1 min of initial denaturation at 95°C, 35 cycles of amplification consisting of 30 s at 94°C, 40 s at 57°C, and 30 s at 72°C, with a final additional elongation at 72°C for 7 min. PIK3CA exon 9 amplification was carried out with a touchdown PCR program; 94°C (2 min); 3 cycles of 94°C (30 sec), 64°C (30 sec), 70°C (30 sec); 3 cycles of 94°C (30 sec), 61°C (30 sec), 70°C (30 sec); 3 cycles of 94°C (30 sec), 58°C (30 sec), 70°C (30 sec); 32 cycles of 94°C (30 sec), 57°C (30 sec), 70°C (40 sec); 1 cycle of 70°C (5 min). When performing the PCR, a non-template control was included in each batch. After PCR reaction, the products were purified and subjected to direct sequencing (ABI 3500xL Genetic Analyzer; Applied Biosystems, Carlsbad, CA, USA).

Statistical Analysis

Statistical analysis was carried out by the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). The Chi-square (χ²) test was used to compare the proportion of gene mutations among groups with different clinicopathologic factors. Multiple logistic regression analysis was done to investigate the effects of covariates on gene mutations, using a backward stepwise (likelihood ratio) method with odds ratio (OR) calculated, and variables which showed statistically significant association with gene mutations were subjected to final regression analysis. Survival analysis was done with the Kaplan-Meier survival function with the method of log-rank test. The two-sided significance level was set at P<0.05.

Results

KRAS Mutation

KRAS mutation status could not be assigned to 2 of 676 (0.30%) samples, 35.9% (242/674) harbored a KRAS mutation, 25.7% (173/674) in codon12, 6.8% (46/674) in codon13, and 2.1% (14/672) in codon61. Moreover, one patient harbored a double KRAS mutation in both codon12 and 13 (GGT>GTT, GGC>GAC). The corresponding order for KRAS codon12 mutation frequency was G12D, G12V, G12A, G12C, G12S and G12R; in KRAS codon13, the most frequent mutation was G13D, followed by G13C and G13S. The major mutation subtype in codon61 was Q61H, and Q61L, Q61R were also found in this study (Figure 1). KRAS mutation appeared more frequently in female than male (41.3% vs 32.3%, P = 0.02), and patients older than 60 years also showed a higher rate of KRAS mutation (39.9% vs 32.0%, P = 0.03). We did not find other significant associations between KRAS mutation and patients’ clinicopathological characteristics (Table 1).

BRAF Mutation

The status of BRAF mutation was detected in 99.8% (675/676) samples, 6.96% (47/675) harbored a BRAF mutation, 4.1% (30/675) in exon15 and 2.5% (17/676) in exon11. The V600E mutation in exon15 was the most frequent subtype (1.8%,12/675), and followed by V600M mutation and other types. In exon11, the mutations distributed widely, R461K and G463E were relatively more common in these mutations (Figure 1). BRAF and KRAS mutations were not mutually exclusive, 4.55% (11/242) of KRAS tumors harbored a BRAF mutation (of which 7/242[2.89%] exon15 and 4/242[1.66%] exon 11 mutations). However, BRAF (V600E) only existed in KRAS wild types (0.0% vs 2.78% [12/431], P = 0.005). In this group, V600E mutation showed a strong association with primary tumor site, tumor in colon appeared more frequently to harbor a V600E mutation (9/285[3.2%] in colon vs 3/390[0.8%] in rectum; P = 0.020), besides, with the tumor differentiation getting worse, a higher V600E mutation rate emerged (5/87[5.7%] in poor differentiation vs 7/555[1.3%] in moderate differentiation; P = 0.030). No other significant association was found between BRAF mutation and patients’ characteristics (Table 1).

PIK3CA Mutation

PIK3CA1 mutation status could not be assigned to 7.54% (51/676) samples, 9.9% (62/625) harbored a PIK3CA1 mutation, 7.0% (45/643) in exon9 and 2.67% (17/636) in exon20. The E545K in exon9 appeared more frequently than any other mutation subtype, followed by E342K, E345G, Q346E and others. By contrast, nearly all mutations in exon20 was H1047R, only one sample was H1047Y mutation, the spectrum of these mutations was showed in Figure 2. We also detected one sample harbored a double

![Figure 1](https://example.com/figure1.png)

![Figure 2](https://example.com/figure2.png)
mutation in exon 9 (L540V and Q546E), besides, this sample also had a KRAS mutation (G13D). There was a strong significant association between PIK3CA exon 9 and KRAS mutations (23/230[10.0%] in KRAS mutant vs 22/412[5.3%] in KRAS wild types, \( P = 0.027 \)), whereas this association was not found in PIK3CA exon 20 with KRAS mutation (\( P = 0.673 \)). BRAF and PIK3CA

| Characteristics | Number (%) | KRAS Mutations (%) | P | BRAF Mutations (%) | P | PIK3CA Mutations (%) | P | NRAS Mutations (%) | P |
|-----------------|------------|--------------------|---|--------------------|---|----------------------|---|--------------------|---|
| Sex Female      | 269 (39.8) | 111 (41.3)         |   | 19 (7.1)           |   | 28 (11.3)            |   | 10 (4.0)           |   |
| Age \( \leq 60 \) | 342 (50.6) | 109 (32.0)         | 0.03| 23 (6.7)           | 0.81| 25 (8.0)            | 0.11| 15 (4.9)           | 0.38|
| Age \( > 60 \)  | 334 (49.4) | 133 (39.9)         |   | 24 (7.2)           |   | 37 (11.9)            |   | 11 (3.5)           |   |
| Mean 60±11      |            |                    |   |                    |   |                      |   |                    |   |
| Age Range       | 23–86      |                    |   |                    |   |                      |   |                    |   |
| Primary tumor site Rectum | 391 (57.8) | 138 (35.4)         | 0.74| 27 (6.9)           | 0.96| 22 (6.1)            | <0.001| 19 (5.4)           | 0.09|
| Primary tumor site Colon | 285 (42.2) | 104 (36.6)         |   | 20 (7.0)           |   | 40 (15.1)            |   | 7 (2.6)           |   |
| Tumor location* Proximal | 133 (19.7) | 52 (39.1)          | 0.38| 9 (6.8)            | 0.92| 25 (19.8)           | <0.001| 4 (3.1)           | 0.51|
| Tumor location* Distal | 542 (80.2) | 189 (35.0)         |   | 38 (7.0)           |   | 37 (7.4)            |   | 22 (4.5)           |   |
| Missing data    | 1 (0.1)    |                    |   |                    |   |                      |   |                    |   |
| Tumor differentiation Well | 31 (4.6)  | 17 (54.8)          | 0.06| 1 (3.2)            | 0.18| 4 (14.3)            | 0.21| 2 (6.9)           | 0.07|
| Tumor differentiation Moderate | 556 (82.2) | 197 (35.6)        |   | 36 (6.5)           |   | 46 (9.0)            |   | 17 (3.4)           |   |
| Tumor differentiation Poor | 87 (12.9)  | 27 (31.0)          |   | 10 (11.5)          |   | 12 (14.5)           |   | 7 (8.4)           |   |
| Missing data    | 2 (0.3)    |                    |   |                    |   |                      |   |                    |   |
| Tumor stage I   | 92 (13.6)  | 33 (35.9)          | 0.56| 5 (5.4)            | 0.61| 8 (9.9)             | 0.29| 4 (4.9)           | 0.03|
| Tumor stage II  | 238 (35.2) | 86 (36.1)          |   | 17 (7.1)           |   | 20 (9.0)            |   | 8 (3.6)           |   |
| Tumor stage III | 288 (42.6) | 107 (37.4)         |   | 19 (6.6)           |   | 25 (9.3)            |   | 7 (2.6)           |   |
| Tumor stage IV  | 55 (8.1)   | 15 (27.3)          |   | 6 (10.9)           |   | 9 (18.0)            |   | 6 (12.2)           |   |
| Missing data    | 3 (0.5)    |                    |   |                    |   |                      |   |                    |   |
| Depth of invasion T1 | 18 (2.7)  | 5 (27.8)           | 0.21| 1 (5.6)            | 0.57| 0 (0.0)             | 0.34| 1 (6.3)           | 0.69|
| Depth of invasion T2 | 105 (15.4) | 37 (35.6)          |   | 4 (3.8)           |   | 9 (9.7)             |   | 4 (4.2)           |   |
| Depth of invasion T3 | 321 (77.1) | 184 (35.4)         |   | 40 (7.7)           |   | 48 (9.9)            |   | 20 (4.2)           |   |
| Depth of invasion T4 | 30 (4.5)   | 16 (53.3)          |   | 2 (6.7)            |   | 5 (17.2)            |   | 0 (0.0)           |   |
| Missing data    | 2 (0.3)    |                    |   |                    |   |                      |   |                    |   |
| Lymph node N0   | 342 (50.8) | 123 (36.0)         | 0.88| 23 (6.7)           | 0.48| 30 (9.5)            | 0.75| 12 (3.8)           | 0.89|
| Lymph node N1   | 190 (28.0) | 66 (34.9)          |   | 11 (5.8)           |   | 16 (9.4)            |   | 8 (4.7)           |   |
| Lymph node N2   | 142 (20.9) | 53 (37.6)          |   | 13 (9.2)           |   | 16 (11.7)           |   | 5 (3.8)           |   |
| Missing data    | 3 (0.3)    |                    |   |                    |   |                      |   |                    |   |
| Distant metastasis Yes | 55 (8.1)  | 15 (27.3)          | 0.16| 6 (10.9)           | 0.26| 9 (18.0)            | 0.08| 6 (12.2)           | 0.01|
| Distant metastasis No  | 619 (91.6) | 227 (36.8)         |   | 41 (6.6)           |   | 53 (9.2)            |   | 19 (3.3)           |   |
| Missing data    | 2 (0.3)    |                    |   |                    |   |                      |   |                    |   |

Colon*: Colon is defined as right colon, transverse colon, left colon, sigmoid colon, rectosigmoid transition zone.
Tumor location*: Proximal tumor is defined as right colon and transverse colon; distal tumor is defined as left colon, sigmoid colon, rectosigmoid transition zone and rectum.

Table 1. Characteristics of 676 CRC patients and association of gene mutations with clinicopathological parameters.

Colon: Colon is defined as right colon, transverse colon, left colon, sigmoid colon, rectosigmoid transition zone.
Tumor location*: Proximal tumor is defined as right colon and transverse colon; distal tumor is defined as left colon, sigmoid colon, rectosigmoid transition zone and rectum.

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mutations were not mutually exclusive, 10% (4/40) of BRAF mutation coexists with PIK3CA mutation (of which 3/40 [7.5%] exon 9 and 1/40 [2.5%] exon 20). For the clinicopathological characteristics analysis, patients with tumor located in rectum had a significantly lower PIK3CA mutation rate than other sites in colon and rectosigmoid transition zone (6.1% vs 15.1%, P < 0.001) and proximal tumors appeared a higher PIK3CA mutation rate (19.8% vs 7.4%, P < 0.001). No other significant association was found in this analysis (Table 1).

NRAS Mutation
We detected NRAS mutation in 92.0% (621/676) samples, and 4.19% (26/621) harbored a NRAS mutation. Although NRAS is closely to KRAS which also included in Ras gene [13], unlike KRAS, most NRAS mutation occurred in codon61 (2.02%, 13/643), rather than in codon12 or 13 (1.75%, 11/630). The most frequently mutation subtype in codon61 was Q61R, and G12D in codon12/13 (Figure 2). Besides, one G13W and one G60E mutation were also detected in these samples. Moreover, we still found that NRAS mutation appeared a strong significant association with KRAS wild types (1/227 [0.44%] in KRAS mutant vs 25/394 [6.3%] in KRAS wild types, P < 0.001). Interestingly, NRAS codon61 mutation only harbored in KRAS wild types (0.0% vs 3.2% [13/410], P = 0.006), whereas NRAS codon12 and 13 did not share this association (P = 0.063). Only one sample harbored a BRAF mutation (V600E) with a NRAS mutation (G15W), and 6.78% (4/59) PIK3CA mutation harbored a NRAS mutation (of which 2/59 [3.9%] in codons 12 and 13, 2/61 [3.28%] in codon61). Furthermore, NRAS mutation occurred more frequently in distant metastasis tumors (12.2% vs 3.3%, P = 0.010), and different tumor stage showed a different NRAS mutation rate (P = 0.030). (Table 1).

In the multivariate logistic regression analysis, we selected sex, age, primary tumor site, tumor differentiation, tumor stage and distant metastasis as covariates, and KRAS mutants appeared more frequently in patients older than 60 (P = 0.023), as well in female patients (P = 0.016). BRAF mutations did not show any significant association with characteristics (data not shown), while BRAF (V600E) mutants shared significant association with tumor differentiation (P = 0.016). As for PIK3CA mutations, colon cancer appeared a higher mutation rate than rectum cancer (P < 0.001), however, NRAS mutations showed more frequently in rectum cancer (P = 0.031), although no significant association was found in univariate analysis (P = 0.09). Moreover, a strong significant association still existed between NRAS mutants and distant metastasis in the multivariate analysis (Table 2).

Analysis of Gene Mutation in mCRC Patients
Fifty-five of 676 patients were confirmed as mCRC, and all these 55 samples were collected before chemotherapy. We further investigated the mutants distribution and clinicopathological characteristics association in this group. 27.3% (15/55) harbored a KRAS mutation, of which 93.3% (14/15) in codon12 and 6.7% (1/15) in codon13, respectively. The BRAF mutation rate was 10.9% (6/55), 66.7% (4/6) in exon15 and 33.3% (2/6) in exon11. PIK3CA mutation was detected in 18.0% (9/50) tumors, 66.7% (6/9) was detected in exon9 and 33.3% (3/9) in exon20. 12.24% (6/49) tumors were detected as NRAS mutants, of which 50% (3/6) in codons12 and 13, 33.3% (2/6) in codon61, besides, one sample harbored a G13W mutation. Statistical analysis indicated that KRAS mutation was significantly higher in the deeper invasion stage (5/7 [71.4%] in T4 vs 10/48 [20.8%] in T3; OR 9.500, 95% CI 1.599–56.426; P = 0.013), and tumor with poor differentiation showed a higher NRAS mutation rate than moderate differentiation (5/19 [26.3%] vs 1/30 [3.3%]; OR 10.357, 95% CI 1.103–97.266; P = 0.027). We did not find any other significant association between gene mutation (included subgroup analysis) and clinicopathological characteristics (data not shown).

Overall Survival Analysis in mCRC Patients
Overall survival of patients in this subgroup was analyzed with the Kaplan-Meier method, in this relative small subgroup (n = 55), survival information was collected successfully in only 45 patients, of whom 37 had received chemotherapy after surgery, either with...
infusional fluorouracil, leucovorin and irinotecan (FOLFIRI) or infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4). However, the relative small sample size did not present any significant result between gene mutation and OS, including gene subsets analysis (data not shown).

**Discussion**

During the past decades, large amounts of research data emerged from molecular basis [15], drug investigation and usage [3], genetic profiling effects [16] studies have led to the thriving research on identification of multiple molecular subsets and targeted therapy in colorectal cancer. Following the discovery that

**Table 2.** Multivariate logistic regression in CRC patients between gene mutations and clinicopathological characteristics.

| Characteristics          | KRAS                                      | B BRAF (V600E)                                        |
|--------------------------|-------------------------------------------|------------------------------------------------------|
|                          | Adjusted odds ratio (95% CI)               | LRT p value                                          | Adjusted odds ratio (95% CI) | LRT p value |
| Sex                      | 0.671(0.485–0.928)                        | 0.016                                                | 0.627(0.197–1.994)          | 0.429       |
| Age                      | 1.450(1.052–1.999)                        | 0.023                                                | 1.241(0.381–4.040)          | 0.720       |
| Primary tumor site       | 1.066(0.768–1.480)                        | 0.702                                                | 3.587(0.947–13.588)         | 0.060       |
| Tumor differentiation    | 0.675(0.453–1.006)                        | 0.053                                                | 4.101(1.298–12.957)         | 0.016       |
| Tumor stage              | 1.098(0.864–1.396)                        | 0.443                                                | 1.310(0.601–2.855)          | 0.496       |
| Distant metastasis       | 0.681(0.362–1.280)                        | 0.232                                                | 0.637(0.069-5.891)          | 0.691       |

| Characteristics          | PIK3CA                                     | NRAS                                                |
|--------------------------|--------------------------------------------|------------------------------------------------------|
|                          | Adjusted odds ratio (95% CI)               | LRT p value                                          | Adjusted odds ratio (95% CI) | LRT p value |
| Sex                      | 0.766(0.447–1.315)                        | 0.334                                                | 1.394(0.587–3.311)          | 0.452       |
| Age                      | 1.491(0.869–2.559)                        | 0.147                                                | 1.700(0.305–1.609)          | 0.401       |
| Primary tumor site       | 2.773(1.604–4.792)                        | <0.001                                               | 0.348(0.134–0.907)          | 0.031       |
| Tumor differentiation    | 1.025(0.534–1.970)                        | 0.940                                                | 1.587(0.609–4.138)          | 0.345       |
| Tumor stage              | 0.939(0.616–1.432)                        | 0.771                                                | 0.708(0.384–1.305)          | 0.268       |
| Distant metastasis       | 1.802(0.814–3.989)                        | 0.146                                                | 4.930(1.817–13.375)         | 0.002       |

**Figure 2. Frequency of the various PIK3CA and NRAS mutations.** Panel A: PIK3CA mutations (exon9: n = 643; exon20: n = 636). Panel B: NRAS mutations (codons12 & 13: n = 630; codon61: n = 643). The data are presented as percentages (number of total samples).

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mutant KRAS tumors were resistant to anti-EGFR antibodies, patients with metastatic colorectal cancer are now recommended to detect the KRAS codons12 and 13 mutation status before MoAbs therapy [8,17,18]. However, even in KRAS wild-type tumors, up to 65% patients were still resistant to anti-EGFR monoclonal antibodies [8]. Besides, although the detection of KRAS mutation status before MoAbs therapy is widely accepted, there is little agreement on its predictive and prognostic role, for published studies provided different results in the relationship between KRAS mutation and clinical outcomes in CRC, and the main effectors in downstream signaling pathway of KRAS, such as BRAF, PIK3CA and NRAS were already studied in many clinical trials, which showed the capability to present as potential predictive or prognostic biomarkers [14,19].

To our knowledge, this study investigated the first time gene mutation type distribution in Chinese CRC population, and involved not only KRAS, but also BRAF, PIK3CA, NRAS together for comprehensive analysis between gene mutation and clinicopathological characteristics, in addition, the overall survival of metastatic colorectal cancer. Previous studies usually focused on KRAS, BRAF, PIK3CA [20,21,22], but not include NRAS mutation, or study sample size was too small to draw confirmed conclusions [20]. Many studies could not collect enough appropriate samples to describe a relative complete outline for Chinese CRC patients in genetic profile, and our investigation aimed to present the key mediated gene mutation of CRC, to some extent, representing the East Asian population.

KRAS gene encodes a small G protein which acts as a key transducer in EGFR pathway, mutations in KRAS gene lead to constitutive signaling through the EGFR pathway and active downstream MAPK and PIK3CA dependent pathways [18,23]. Previous studies have analyzed KRAS mutation distribution from western population, which indicated that G12D was the most frequent mutation subtype in codon12, followed by G12V, G12C, G12S, G12A and G12R [24]. However, in present study, the corresponding order for KRAS codon12 mutation frequency was G12D, G12V, G12A, G12C, G12S and G12R. As for codon13, the difference remained in subtype distribution (G13/D/C/R in western population vs G13/D/C/S in this study). In addition to gaining more information and expanding the recognition of the KRAS mutation, the sample size of our series allowed us to investigated the rare codon61 mutation, since mutant tumors with KRAS codon61 led to significantly lower response rate than wild types (0.0% vs 35.7%, P = 0.0055) [14], while the mutation incidence (2.1%) was even higher than some codon12 and 13 mutations, we then suggested that codon61 detection should be taken into consideration during clinical practice. This study showed a 35.9% KRAS mutation rate, which was similar to previous studies [4,9,14,22], and patients older than 60 appeared more frequently to harbor a KRAS mutation. Meanwhile, in mCRC patients, KRAS mutation was significantly higher in the deeper invasion stage (OR 9.500, 95% CI 1.062–5.330; P = 0.004) and poor differentiation tumor harbored a higher V600E mutation (P = 0.030). These data indicated that colorectal cancer treatment should be regarded from a deeper extent, for colon and rectum cancer required different therapy in different stage.

We confirmed the association between KRAS and PIK3CA mutations in CRC, which was comparable with previous studies [14,20,25,32], and only exon9 (not included exon20) shared a strong association with KRAS mutation [14]. This was consistent with the findings that the gain of function by exon9 mutations (the helical domain) was highly dependent on RAS-GTP binding, especially in E542K and E545K, while exon20 mutations (the kinase domain) active was likely in the absence of RAS-GTP binding [33]. The PIK3CA mutation frequency varies between 13.6%–18.0% in western population [34–35], while we reported a relative low mutation frequency (9.9%). Studied population may lead to this difference mainly, for other studies which based on Chinese population also showed lower mutation frequency (4.9%–8.2%) [20,36]. In the logistic regression analysis, PIK3CA mutation appeared more frequently in colon cancer than rectum at the same time, which was supported by a recent study [37]. Previous studies indicated that PIK3CA mutation existence implied negative prognosis, either a shorter median progression-free survival (PFS) [38], or a shorter median OS [39–40]. However, since the PIK3CA mutation effect seemed to be considered together, the separate effect of each subtype appeared unclear, for several studies had showed that exon9 and exon20 mutation led to different results [14,41]. The large European consortium study indicated that only exon20 mutation was associated with worse clinical outcome [14], and was supported by other research data [16,42]. But because exon20 mutation was relatively low compared with exon9 (2.96% vs 9.96%) [14], and in our study (2.67% vs 7.0%). The reported data should be regarded as clinical related hypothesis and required confirmation, based on further genetic profiling and clinical trials investigation.
The RAS gene (KRAS, NRAS, HRAS) encodes a series of GTP/GDP related switches that convey extracellular signals, resulting in regulating growth and survival of cells [43]. As one of the RAS family, NRAS shared close relations with KRAS [13], while unlike KRAS mutation occupies such a large percentage in colorectal cancer, NRAS mutations were rare. Irahara N and colleagues [44] reported a 2.2% (5/225) mutation incidence, and 2.64% (17/644) mutation rate in another study [14], while we detected 4.19% (26/621) tumors harbored a NRAS mutation. The higher NRAS mutation incidence presented a specific characteristic for Chinese population. As rare data was reported in Chinese patients for NRAS mutation status, our study may provide some original contribution. However, future investigations are needed to draw a better picture in this area. NRAS mutations were not mutually exclusive with BRAF and PIK3CA mutation, although another study did not share this [44]. NRAS mutation coexisted with KRAS wild-type (P<0.001), of note, codon61 mutation only appeared in KRAS wild-type tumors (P = 0.006), and codon12 and 13 had a significantly higher mutation rate in distant metastatic tumors (P = 0.016). These data can partially help explain the anti-EGFR MoAbs resistance in KRAS wild-type patients, as NRAS mutations were significantly associated with lower disease control rate and response rate to MoAbs [14,45], and we recommended NRAS mutation detection should be taken into consideration before MoAbs treatment, especially in KRAS wild-type tumors. However, considering the low mutation incidence, the magnitude of NRAS mutation effect was still confused, larger sample size or preselected patients investigation seemed essential in future design.

There were several limitations in this retrospective study, including the relatively small number (n = 45) of patients in the survival analysis, then the limited information could not support confirmed conclusions in present study. Additionally, other potentially negative factors such as loss of expression of phosphatase and tensin homologue (PTEN) should be involved, thus essential effects of these biomarkers in clinical practice stayed further validation. Moreover, as epigenetic status or microsatellite instability (MSI) plays a significant role in CRC tumors, these features should be involved into analysis. Besides, gene expression in the key effectors, different tumor locations may provide information for better understanding in CRC, either in carcinogenesis or tumor progression and these should be taken into consideration in future studies. As high throughput detecting method has been implemented in screening gene variants or sequencing, different types of gene alternations have been investigated comprehensively in colorectal cancer [46], these studies have provided potential genes which need further investigations.

In a recent randomised trial [47], patients were preselected for only KRAS codon12, 13 and 61 wild-type tumors, however, therapy with panitumumab to irinotecan did not improve the overall survival compared with irinotecan alone, then refinement of molecular selection was required considering patients’ welfare. Another multicenter randomised placebo-controlled trial tested a novel multikinase inhibitor (Regorafenib) [48], although the study obtained a significant result in prolonging median OS (6.4 vs 3.0 months, hazard ratio 0.77; 95% CI 0.64-0.94; one-sided p = 0.0052), in view of the small incremental survival benefit, potentially exposed to toxic effects and heavy economic burden, the new agent seemed not to be a cost-effective option, while selecting the subset of patients who would really benefit from Regorafenib based on the identification of biomarkers was a high priority. We have already known that genotype subgroup would lead to different clinical outcomes in mCRC MoAbs treatment [14,49,50], all the data indicated that more precise classification of genetic profile should be implemented to enhance the clinical targeted therapy, then our study here was in order to bring us a step closer to personalized medicine.

In conclusion, this study presented a clear genotype distribution picture scroll in East Asian CRC population, involving potential molecular predictors KRAS, BRAF, PIK3CA, NRAS, which showed a specific characteristic. However, prospective randomised trials are needed to provide proposals and validate conclusions. More comprehensive genomics analysis and molecular classification should be performed, to recognize the genetic profile better and to improve the clinical choice smarter.

**Author Contributions**

Conceived and designed the experiments: Y. Shi XH. Performed the experiments: Y. Shen JW. Analyzed the data: Y. Shen XH JW SW. Contributed reagents/materials/analysis tools: HY DL. Wrote the paper: Y. Shen JW XH Y. Shi.

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