Original Article
Correlation between clinicopathological features and KRAS, NRAS, and BRAF mutation status in Chinese colorectal cancer patients

Xiaoli Zhu¹³*, Xianke Meng²³*, Wenqiang Xiang²³*, Weixing Dai²³, Qianming Bai¹³, Weiqi Sheng¹³, Yongming Lu¹³, Peng Qi¹³, Lijing Wu¹³, Guoxiang Cai²³, Xiaoyan Zhou¹³

¹Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai, China; ²Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, Shanghai, China; ³Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China. *Equal contributors.

Received February 28, 2018; Accepted March 28, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: This study was retrospectively performed to analyze correlations between clinicopathological features of colorectal cancer (CRC) and mutations in KRAS, NRAS, and BRAF in Chinese patients, and to assess the importance of detecting additional mutations in KRAS exons 3 and 4 and NRAS in patients with CRC. RAS (KRAS and NRAS) and BRAF mutations were detected in 715 and 655 patients respectively. The mutation rate of RAS (KRAS or NRAS) was 45.6% (326/715). KRAS exon 2 mutations were evaluated in 36.6% of patients (262/715). Additional mutations in RAS exons occurred in 9.0% of patients (64/715), including KRAS exons 3 and 4 in 5.6% (40/715) and NRAS exons 2, 3, or 4 in 3.4% (24/715). Among 453 patients with wild-type KRAS exon 2, 14.1% (64/453) had other mutations in RAS exons. The most frequent sites of mutations were codons 12, 13, 61, and 146 in KRAS and codons 12 and 61 in NRAS. The mutation rate of BRAF (exon 15) was 4.0% (26/655), and the most frequent mutation site was codon 600. Among 440 patients with CRC who had a primary tumor resection at our center, those with mucinous or signet ring cell CRC were more likely to harbor KRAS mutations than those with adenocarcinoma (62.7% vs. 43.6%, P=0.006 and 59.3% vs. 39.6%, P=0.004, respectively). Female patients had a higher BRAF (exon 15) mutation rate than male patients (5.1% vs. 1.1%, P=0.017). Detection of both KRAS and NRAS mutations is useful for selecting patients who will benefit from anti-EGFR monoclonal antibody therapy. KRAS mutations were more frequent in patients with mucinous adenocarcinoma/signet ring cell CRC, whereas BRAF mutations were more common in female patients with CRC.

Keywords: KRAS, NRAS, BRAF, mutations, colorectal cancer

Introduction
KRAS is frequently mutated in metastatic colorectal cancer (mCRC). Previous randomized, controlled trials indicated that patients with mutations in KRAS exon 2 do not benefit from anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibody (mAb) therapy [1, 2]. Recently, the retrospective PRIME trial showed that other mutations in RAS (exons 2, 3, and 4 in NRAS and exons 3 and 4 in KRAS) are also associated with decreased responses to anti-EGFR mAb therapy. Thus, detection of RAS family mutations (KRAS and NRAS) is recommended in patients with mCRC [3, 4]. Patients with CRC who do not have mutations in both KRAS and NRAS appear to benefit from anti-EGFR mAb therapy [3, 5]. Therefore, analysis of the biological features of CRC specimens may be important prior to cetuximab treatment. Moreover, mutations in KRAS, NRAS, and BRAF have been reported in large cohorts of Chinese CRC patients. In this study, we retrospectively analyzed mutations in KRAS, NRAS, and BRAF in 715 Chinese patients with CRC to explore the distribution of these gene mutations and their correlations with clinical pathological features.

Materials and methods
Patient specimens
Patients (N=715) diagnosed with CRC and underwent RAS mutation analysis from January
KRAS, NRAS, and BRAF mutation in colorectal cancer

Clinical characteristics of patients harboring RAS and BRAF mutations

Clinical characteristics of all the 715 patients are shown in Table 2. The mean age of the 715 patients included in this study was 58 years old (range, 15-87 years), with 419 (58.6%, 419/715) men and 296 (41.4%, 296/715) women. The stage of disease was recorded for 440 patients who received primary tumor resection at FUSCC based on the American Joint Committee on Cancer (AJCC) tumor-node-metastasis staging (TNM) system (7th edition, 2010). Our study was approved by the Ethical Committee and Institutional Review Board of FUSCC. All patients signed informed consent forms before inclusion in this study.

DNA extraction

Genomic DNA was extracted from formalin-fixed paraffin-embedded CRC tissue. A standard xylene-phenol protocol was used to dissolve paraffin. Tissue specimens (4-5 mm) were digested with proteinase K. Genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration and quality were determined on a Nanodrop spectrophotometer (ND-1000, Thermo-Fisher Scientific, Wilmington, DE, USA).

Direct sequencing of RAS and BRAF

PCR amplification and direct sequencing of exons 2, 3, and 4 of KRAS, exons 2, 3, and 4 of NRAS, and exon 15 of BRAF were performed. Primers for KRAS, NRAS, and BRAF are shown in Table 1. The following PCR conditions were used: 94°C for 10 minutes, then 38 cycles for denaturing at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 7 minutes. PCR products were purified using a QIAquick gel extraction kit (Qiagen, Germany) and were used to prepare sequencing reactions. Sequencing was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the following PCR conditions: 94°C for 1 minute, 24 cycles of denaturing at 94°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 1 minute, and final extension at 72°C for 5 minutes. Sequenced PCR products were purified and analyzed on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Statistical analyses

Chi-square or Fisher’s exact tests were performed for categorical variables. All statistical analyses were performed with SPSS for Windows version 22 (IBM Corp, Armonk, NY, USA). Two-sided P<0.05 was recognized as being statistically significant.

Results

Clinical characteristics of patients harboring RAS and BRAF mutations

Clinical characteristics of all the 715 patients are shown in Table 2. The mean age of the 715 patients included in this study was 58 years old (range, 15-87 years), with 419 (58.6%, 419/715) men and 296 (41.4%, 296/715) women. The stage of disease was recorded for 440 patients who received primary tumor resection at FUSCC based on the American Joint Committee on Cancer (AJCC) tumor-node-metastasis staging (TNM) system (7th edition, 2010). Our study was approved by the Ethical Committee and Institutional Review Board of FUSCC. All patients signed informed consent forms before inclusion in this study.
women. The RAS mutation rate was 45.6% (326/715), with KRAS mutations more common (42.2%, 302/715) than NRAS mutations (3.4% 24/715). Among 655 patients who were analyzed for BRAF mutations, mutation rate found in BRAF exon 15 was 4.0% (26/655).

KRAS, NRAS, and BRAF mutations in patients with CRC

The distribution of KRAS mutations among 715 CRCs is displayed in Table 3. The most common mutation was in KRAS exon 2 (36.6%, 262/715) at codons 12 and 13. Among patients who did not have a mutation in KRAS exon 2, 14.1% (64/453) had mutations in KRAS exons 3 or 4 or in NRAS. Forty patients had mutations in KRAS exons 3 and 4. The most common mutation sites of KRAS exons 3 and 4 were codon 61 (47.5%, 19/40) and codon 146 (32.5%, 13/40) respectively. Common amino acid changes were Q61H>Q61L>Q61R<Q61K in codon 61 of KRAS exon 3 and A146T>A146V in codon 146 of KRAS exon 4.

The distribution of NRAS and BRAF mutations in 715 CRCs is displayed in Table 4. Twenty-four patients had mutations in NRAS. The most common sites of mutations were codon 61 in exon 3 (37.5%, 9/24) and codon 12 in exon 2 (29.2%, 7/24). Common amino acid changes were Q61K>Q61L>Q61R and Q61H in codon 61 of NRAS exon 3 and G12D and G12V>G12C in codon 12 of NRAS exon 4.

NRAS exon 4 mutations were rare (8.3%, 2/24) compared with mutations in exons 2 and 3. Other uncommon mutations in KRAS exons 3 and 4 and in NRAS are presented in Table 3.

Among the 655 patients with BRAF mutations, 26 had a mutation in BRAF exon 15. The most common site of mutation was codon 600 (76.9%, 20/26). Other sites of mutations included codons 601, 594, and 559 (23.1%, 6/26).

Two patients harbored mutations in both KRAS and NRAS, and only one patient harbored mutations in both KRAS and BRAF.

Associations between RAS or BRAF mutations and clinicopathological features

Associations between KRAS, NRAS, or BRAF mutations and the clinicopathological features of patients are presented in Table 5. Patients with mucinous or signet ring cell CRC were more likely to harbor KRAS mutations compared with patients with adenocarcinoma (mucinous or signet ring cell cancer vs. adenocarcinoma cancer, 62.7% vs. 43.6%, P=0.006 and 59.3% vs. 39.6%, P=0.004, respectively). No statistical significance was observed between KRAS mutations and other clinicopathological features. All clinicopathological fea-
mutations appeared to be unrelated to \( NRAS \) mutations.

Female patients had a higher \( BRAF \) mutation rate compared with male patients (female vs. male, 5.1% vs. 1.1%, \( P=0.017 \)). However, age, histological type, tumor location, and TNM stage did not significantly correlate with the presence of a \( BRAF \) mutation.

**Discussion**

Recent studies showed that mutations in \( RAS \) family members (\( NRAS \) mutations and \( KRAS \) mutations outside exon 2) are associated with resistance to anti-EGFR mAb therapy [6]. Sorich et al. analyzed nine randomized, controlled trials comprising a total of 5948 patients with CRC, finding that approximately 20% patients with wild-type \( KRAS \) exon 2 harbored another \( RAS \) mutation [7]. They concluded that patients with CRC and any type of \( RAS \) mutation are unlikely to benefit from anti-EGFR mAb therapy [7]. In the PRIME trial, 17% (108/512) of patients had a wild-type \( KRAS \) exon 2 but had other mutations at other sites within \( RAS \) [6]. The effects of anti-EGFR mAb therapy differ between patients who lack \( RAS \) mutations and those who lack mutations in \( KRAS \) exon 2 but have other mutations at other sites within \( RAS \) [6]. These studies suggested that \( KRAS \) exon 2 and other \( RAS \) mutations serve as a negative predictive factor of response to anti-EGFR mAb treatment. Therefore, detection of multiple \( RAS \) mutations is necessary in patients with CRC before anti-EGFR mAb treatment. In our cases,
**KRAS, NRAS, and BRAF mutation in colorectal cancer**

**Table 5.** Association between KRAS, NRAS, and BRAF mutations and clinicopathological features of 440 patients who received primary tumor resection at FUSCC.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Total number (N=440)</th>
<th>RAS n (%)</th>
<th>KRAS n (%)</th>
<th>NRAS n (%)</th>
<th>BRAF n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mutation</td>
<td>Wild-type</td>
<td>P value</td>
<td>Mutation</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>0.116</td>
<td>0.153</td>
<td>1.000*</td>
<td>1.000*</td>
</tr>
<tr>
<td>&lt;70</td>
<td>373</td>
<td>178 (47.7)</td>
<td>195 (52.3)</td>
<td></td>
<td>162 (43.4)</td>
</tr>
<tr>
<td>≥70</td>
<td>67</td>
<td>25 (37.3)</td>
<td>42 (62.7)</td>
<td></td>
<td>23 (34.3)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>0.216</td>
<td>0.224</td>
<td>0.709</td>
<td>0.017*</td>
</tr>
<tr>
<td>Male</td>
<td>263</td>
<td>115 (43.7)</td>
<td>148 (56.3)</td>
<td></td>
<td>105 (39.9)</td>
</tr>
<tr>
<td>Female</td>
<td>177</td>
<td>88 (49.7)</td>
<td>89 (50.3)</td>
<td></td>
<td>81 (45.8)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>0.247</td>
<td>0.118</td>
<td></td>
<td>0.215</td>
</tr>
<tr>
<td>Colon</td>
<td>234</td>
<td>114 (48.7)</td>
<td>120 (51.3)</td>
<td></td>
<td>107 (45.7)</td>
</tr>
<tr>
<td>Rectal</td>
<td>206</td>
<td>89 (43.2)</td>
<td>117 (56.8)</td>
<td></td>
<td>79 (38.3)</td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
<td>0.006</td>
<td>0.004</td>
<td>1.000*</td>
<td>1.000*</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>381</td>
<td>166 (43.6)</td>
<td>215 (56.4)</td>
<td></td>
<td>151 (39.6)</td>
</tr>
<tr>
<td>Mucinous/signet ring cell</td>
<td>59</td>
<td>37 (62.7)</td>
<td>22 (37.3)</td>
<td></td>
<td>35 (59.3)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td>0.467</td>
<td>0.917</td>
<td>0.113*</td>
<td>1.000*</td>
</tr>
<tr>
<td>I/II</td>
<td>134</td>
<td>58 (43.3)</td>
<td>76 (56.7)</td>
<td></td>
<td>56 (41.8)</td>
</tr>
<tr>
<td>III/IV</td>
<td>306</td>
<td>145 (47.4)</td>
<td>161 (52.6)</td>
<td></td>
<td>130 (42.5)</td>
</tr>
</tbody>
</table>

*Fisher test. FUSCC, Fudan University Shanghai Cancer Center.*
14.1% (64/453) of patients with wild-type KRAS exon 2 had mutations in KRAS exons 3 or 4 or in NRAS. Detection of other RAS mutations in patients with CRC who lack mutations in KRAS exon 2 may help avoid unnecessary toxicities and costs related to anti-EGFR mAb therapy. We compared our data with other studies (Table 6) and found a similar rate of KRAS mutations [8-11]. However, the NRAS mutation rate in the United States (1.2%) was lower than that reported in other studies, including our study [11]. The total RAS mutation rate was similar among studies [8-11]. Therefore, the total RAS mutation rate in patients with CRC may not exhibit significant geographic or racial differences.

We found that the most common sites of mutations were in codons 12 (27.4%) and 13 (9.0%) in KRAS exon 2. In KRAS exons 3 and 4, the most common sites for mutations were codons 61 (2.7%), 146 (1.8%), and 117 (0.7%). Mutations in KRAS codons 14 (V14I), 22 (Q22K), 59, and 117 were rare (0.1%). The most common sites of mutations in NRAS were codon 12 in exon 2 and codon 61 in exon 3. Mutations in codons 13, 22, 59, 60, and in exon 4 (codons 117 and 146) were rare.

In the present study, we found that mucinous tumors harbored a higher KRAS mutation rate than did the adenocarcinomas subtype, consistent with findings from a previous study [12]. Other clinicopathological features, such as sex, age, and tumor location did not exhibit associations with KRAS mutations, which further supports previous findings [13].

The BRAF mutation rate was 4% (26/715) in our study. Compared with western studies, we found that the BRAF mutation rate in CRC was higher in Germany or Greece, and Romania (7% and 7.3%, respectively) than in China [8, 9, 11]. The most common mutation in BRAF was in codon 600. In addition, we found that seven cases harbored mutations in codons 601, 594, and 559. Interestingly, BRAF mutations tended to be more frequent in female patients than in male patients, which is in line with previous western population-based studies [14-16]. However, the association between BRAF mutations and gender was not found in other Chinese studies [17, 18]. The different results might be caused by case selection bias or regional differences.

In conclusion, detection of mutations in both KRAS and NRAS could be used to select patients who will benefit from anti-EGFR mAb therapy. This test should be a routine molecular assay performed in patients with CRC before anti-EGFR mAb therapy.

Acknowledgements

The present study was supported by the National Key R&D Program of China (No. 2016YFC0905300 and 2016YFC0905301), the Grant of Science and Technology Commission of Shanghai Municipality (No. 164019-70502), the Grant of National Natural Science Foundation of China (No. 81572351), and the Shanghai Shenkang Program (No. SHDC120-14206).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoyan Zhou, Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai 200032, China; Department of Oncology, Shanghai Medical College, Fudan University, 270 Dong’an Road, Xuhui District, Shanghai 200032, China. Tel: +86 21-64175590; Fax: +86 21-64170067; E-mail: xyzhou100@163.com; Dr. Guoxiang Cai, Department of Colorectal Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China; Department of Oncology, Shanghai Medical College, Fudan University, 270
References


