Comparison of KRAS and PIK3CA gene status between primary tumors and paired metastases in colorectal cancer

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Purpose: In metastatic or recurrent colorectal cancer (MRCRC), the concordance of Kirsten rat sarcoma viral oncogene homolog (KRAS) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutation status between the primary tumors and metastases is still controversial. The purpose of this study was to evaluate the association between KRAS and PIK3CA mutational status and various clinicopathologic features, and compare their genotype in primary tumors with that of the paired metastatic tumors.

Method: We compared the mutation status of KRAS and PIK3CA between the primary tumors and the paired metastases of 59 MRCRC patients with available tissues (resection or biopsy). The presence of KRAS and PIK3CA mutations were determined by direct sequencing analysis.

Results: Seventeen patients (28.8%) had the KRAS mutation and 46 patients (80.0%) had the PIK3CA mutation when considering both the primary and metastatic sites. KRAS mutation was observed in ten primary tumors and eleven related metastases (16.9% vs 18.6%), while PIK3CA mutation was found in 26 primary tumors and 32 related metastases (44.1% vs 54.2%). KRAS status was concordant between primary and metastatic sites in 45 patients (76.3%, kappa =0.157), while the concordance of PIK3CA status was only found in 25 patients (42.4%, kappa =-0.141).

The PIK3CA status discordance rate was significantly higher in 40 patients undergoing metachronous resection of primary tumor or metastasis, compared with that in 19 patients with synchronous resection of primary tumor or metastasis (67.5% [27/40] vs 36.8% [7/19]; P=0.026).

Conclusion: Our results demonstrate that low concordance of KRAS and high discordance of PIK3CA mutational status exist between the primary tumors and paired metastasis, and these findings remind us to have second thoughts about the need to evaluate metastatic tumors separately rather than only based on the primary tumor data when targeted therapy is considered.

Keywords: KRAS, PIK3CA, colorectal cancer, primary tumor, metastatic site

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. Despite improvements made in therapy refinement, 25% of patients are diagnosed at IV stage of the disease, and approximately 50% of patients develop into metastatic or recurrent colorectal cancer (MRCRC) even though they received curative resection of primary CRC during the first 5 years from diagnosis.¹ For MRCRC patients, systematic therapy containing chemotherapy, radiotherapy, and targeted therapy was considered to be the major treatment. As for approximately 60%–70% Kirsten rat sarcoma viral oncogene homolog (KRAS) wild-type MRCRC, anti-epidermal growth factor receptor (anti-EGFR) inhibitors, such as cetuximab and...
panitumumab might enhance antitumor effects combined with chemotherapy according to recent guidelines. However, the response rate is not high, nearly 50% KRAS wild-type MRCRC patients cannot benefit from these combined therapies with anti-EGFR inhibitors. Emerging data have proposed phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutation might account for nonresponders to EGFR targeting in CRC. For example, De Roock et al found that patients with PIK3CA mutations had a significantly lower response rate in KRAS wild-type patient.

Furthermore, in clinical practice, most results of gene tests were carried out mainly on primary tumors due to the difficulties to obtain tissues of metastatic tumors. However, some research results raise concerns that genetic profiling of primary tumors may not be representative of metastatic disease. In MRCRC, the concordance of gene mutation status between the primary tumors and metastases is controversial. Jones et al found a high degree of discordance between primary tumors and metastases. In contrast, Vermaat et al reported a high degree of mutational discordance between primary and metastatic samples using next-generation sequencing. Some study reports have shown gene mutations, such as KRAS, NRAS, and BRAF were highly concordant between primary tumors and metastases, while discordant mutations were observed in genes of the phosphoinositide 3-kinase pathway.

Consideration of the above phenomenon, the heterogeneity between primary tumors and metastases seemed as an additional reason for the failure of targeted therapies in MRCRCs. Thus, our study was aimed to evaluate the genetic relationship between primary MRCRCs and their matched metastases that will consequently help in targeted therapy.

Materials and methods
Study population
Among patients with histologically confirmed colorectal adenocarcinoma who had been treated or followed up at Zhejiang Cancer Hospital between June 2004 and July 2013, 59 MRCRC patients who had undergone surgical resection or biopsy of both primary tumors and related metastatic sites were enrolled. The retrospective study was performed using the stored samples at the Department of Pathology, and all of the samples were from patients who had received surgical resection or biopsy of both primary and related metastatic tumors with their consent, and anonymized before the study. The patient did not provide written informed consent in our study. The study was approved by the medical ethics committee at Zhejiang Cancer Hospital.

Table 1 The PCR primers for KRAS and PIK3CA gene amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tr>
<td>KRAS</td>
<td>5′-AGGTACTGGTGAGTATTTGATGT-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-CCCTATTGTTGATCATTTCGTC-3′ (reverse)</td>
</tr>
<tr>
<td></td>
<td>5′-GTTGCACCTGTAATACCGACT-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-CATGGCATTGACAAAGACT-3′ (reverse)</td>
</tr>
<tr>
<td></td>
<td>5′-AGCAACAAAAACAGCGTCAGGA-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TTGAAGAAAAACTGTATATATAATGAC-3′ (reverse)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>5′-AGACACAAAAACAGCGTCAGGA-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TTGAAGAAAAACTGTATATATAATGAC-3′ (reverse)</td>
</tr>
</tbody>
</table>

Abbreviations: KRAS, Kirsten rat sarcoma viral oncogene homolog; PCR, polymerase chain reaction; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

Sequence analysis of KRAS and PIK3CA gene
Formalin-fixed paraffin-embedded primary tumor and metastatic tissue specimens were microdissected manually under the supervision of experienced gastrointestinal pathologists. The DNA was extracted according to the manufacturer’s instructions of E.Z.N.A.FFPE DNA Kit (Lot. D3399-01, OMEGA Bio-Tek, Norcross, GA, USA). For mutation analyses, extracted tumor DNA samples were amplified by polymerase chain reaction (PCR) using primers (Table 1). The PCR conditions were as follows: one cycle of 95°C for 5 minutes; 34 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and one cycle of 72°C for 10 minutes. The PCR products were determined by 3% agarose gel electrophoresis and then sequenced using the same forward primer of each gene by Invitrogen 3730XL genetic analyzer (Life Technologies, Carlsbad, CA, USA). The sequencing results were analyzed with Chromas software under the condition of signal-to-noise ratio >98%.

Statistical analysis
The concordant rate of KRAS and PIK3CA mutational status in primary tumors and related metastases was evaluated, the Kappa index was measured using Cohen’s kappa coefficient, which can assess the concordance between categorical variables of the same individuals. The effect of KRAS and PIK3CA mutational status on clinicopathologic features was assessed using Pearson’s chi-square or Fisher’s exact tests. The relationship between the discordant rates of the KRAS and PIK3CA mutation status and various clinicopathologic
features was also evaluated using univariate analyses (Pearson’s chi-square or Fisher’s exact tests). \( P < 0.05 \) was considered significant. All analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Patient characteristics (N=59) and the association between KRAS or PIK3CA mutational status and clinicopathologic features**

Of the 59 MRCRC in this study, 30 patients (50.8%) were male. The median age was 46 years (range, 18–80 years). Related metastatic sites for gene analysis were liver (N=33), lung (N=9), peritoneum (N=4), pelvic (N=4), ovary/uterus (N=6), groin (N=1), stomas (N=1), and pancreas (N=1), respectively. The primary tumor sites were right colon in 13 patients (22.0%), left colon in 15 patients (25.4%), rectum in 29 patients (49.2%), and others in two patients (3.4%). Other patient characteristics are shown in Table 2. Age in MRCRC patients was different according to the KRAS mutational status. Age \( \geq 60 \) years was more frequent in wild-type KRAS patients than in mutant KRAS patients (83.3% vs 16.7%; \( P = 0.010 \)). There was no association between the KRAS or PIK3CA status and other clinicopathologic features (sex, primary tumor location, histology and grade, clinical situations for the development of systemic metastasis, and metastasis sites) (Table 2).

**Frequency and types of KRAS and PIK3CA mutation**

Ten (16.9%) patients with KRAS mutation and 26 (44.1%) patients with PIK3CA mutation were observed in primary tumors, with eleven (18.6%) and 32 (54.2%) in related metastatic sites, respectively. Seventeen patients (28.8%) had the KRAS mutation and 46 patients (80.0%) had PIK3CA mutation in any place of the primary or metastatic sites. Of those 17 patients with KRAS mutation, two patients had a KRAS12 codon GGT-AGT mutation, five had a KRAS12 codon GGT-GCT mutation, four had a KRAS12 codon GGT-GAT mutation, three had a KRAS12 codon GGT-GTT mutation, two had a KRAS61 codon CAA-CAT mutation, and one patient had a KRAS117 codon AAA-ATA mutation. In addition, of those 46 patients with PIK3CA mutation, 45 patients had a PIK3CA545 codon

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**Table 2** Patient characteristics and the association between KRAS or PIK3CA status and clinicopathologic parameters

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients</th>
<th>All</th>
<th>KRAS</th>
<th>KRAS</th>
<th>P-value</th>
<th>PIK3CA</th>
<th>PIK3CA</th>
<th>P-value</th>
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<td>Sex</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>18 (60.0)</td>
<td>12 (40.0)</td>
<td></td>
<td>6 (20.0)</td>
<td>24 (80.0)</td>
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</tr>
<tr>
<td>Female</td>
<td>29</td>
<td>24 (82.8)</td>
<td>5 (17.2)</td>
<td></td>
<td>7 (24.1)</td>
<td>22 (75.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt; 60 ) years</td>
<td>36</td>
<td>30 (83.3)</td>
<td>6 (16.7)</td>
<td></td>
<td>7 (19.4)</td>
<td>29 (80.6)</td>
<td></td>
<td>0.010</td>
</tr>
<tr>
<td>( \geq 60 ) years</td>
<td>23</td>
<td>12 (52.2)</td>
<td>11 (47.8)</td>
<td></td>
<td>6 (26.1)</td>
<td>17 (73.9)</td>
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</tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Right</td>
<td>13</td>
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<td>4 (30.8)</td>
<td>2 (15.4)</td>
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<td>0.777</td>
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<td>3 (20.0)</td>
<td>5 (33.3)</td>
<td>10 (66.7)</td>
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<tr>
<td>Rectum</td>
<td>29</td>
<td>20 (70.0)</td>
<td>9 (30.0)</td>
<td>6 (20.7)</td>
<td>23 (79.3)</td>
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<td>0 (0)</td>
<td>2 (100)</td>
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<tr>
<td>Synchronous</td>
<td>24</td>
<td>20 (83.3)</td>
<td>4 (16.7)</td>
<td>4 (16.7)</td>
<td>20 (83.3)</td>
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<td>Metachronous</td>
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<td>26 (74.3)</td>
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<td>Histology and grade</td>
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<td></td>
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<tr>
<td>Well/moderately differentiated</td>
<td>39</td>
<td>28 (71.8)</td>
<td>11 (28.2)</td>
<td>9 (23.1)</td>
<td>30 (76.9)</td>
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<td>0.885</td>
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<td>Poorly differentiated, mucinous</td>
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<td>4 (20.0)</td>
<td>16 (80.0)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concurrent</td>
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<td>14 (73.7)</td>
<td>5 (26.3)</td>
<td>5 (26.3)</td>
<td>14 (73.7)</td>
<td></td>
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<td>0.770</td>
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<tr>
<td>Subsequent</td>
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<td>12 (30.0)</td>
<td>8 (20.0)</td>
<td>32 (80.0)</td>
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</table>

**Abbreviations:** KRAS, Kirsten rat sarcoma viral oncogene homolog; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; WT, wild-type; MT, mutation type.
with various clinicopathologic factors such as primary tumor location, metastatic organs, type of primary or metastatic tumor specimens (biopsied vs resected), and any chemotherapy/radiotherapy between resection of primary and metastasis before obtaining primary or metastatic tumor specimens or resection style. The lung was the most frequent site showing KRAS discordance, however, no difference was seen in the discordance rate of KRAS status for the metastatic organs. Two patients with tissues acquired from primary tumor (left colon and rectal colon) and related stomas and groin metastatic site showed discordance of KRAS status (mutation type [P]-wild-type [M]). One patient with tissues acquired from primary tumor (transverse colon) and related pancreatic metastatic site showed concordance of PIK3CA status (mutation type [P]-mutation type [M]). Otherwise, the discordant rate of PIK3CA status was frequent (liver, 60.6%; lung, 55.5%; and peritoneum, 50.0%) regardless of related metastatic site. Two patients with tissues acquired from primary tumor (transverse colon and left colon) and related pancreatic and stomas metastatic site showed discordance of PIK3CA status (mutation type [P]-wild-type [M]). One patient with tissues acquired from primary tumor (rectal colon) and related groin metastatic site showed concordance of PIK3CA status (mutation type [P]-mutation type [M]). Additional analyses were performed to find whether the discordance rates of KRAS and PIK3CA status were influenced by other various clinicopathologic factors (67.5% vs 36.8%; \( P=0.026 \)). However, there was no difference in the discordant rate of KRAS and PIK3CA status for other clinicopathologic factors (Table 5).

**Discussion**

EGFR monoclonal antibodies, such as cetuximab and panitumumab are currently approved for the treatment of metastatic CRC patients with KRAS wild-type tumors. However, their antitumor activity has been limited by intrinsic and acquired drug resistance. One explanation for drug resistance is cancer genetic heterogeneity, which contains content of two aspects.13,17 One refers CRC patients can harbor different gene mutations between primary tumors and paired metastatic sites, another means that even gene discordance existed within different regions of the same tissues. Heterogeneous in genes have been reported to play a role in resistance to anti-EGFR drugs in CRC, including activating mutations in KRAS, NRAS, BRAF, and PIK3CA.9 Genetic heterogeneity makes it more difficult to decide to use the anti-EGFR drugs only based on the results of gene test either from primary or metastatic tumor. Therefore, we conducted the study to compare these gene statuses between
Comparison of KRAS and PIK3CA in colorectal cancer

primary tumors and paired metastasis in CRC for developing effective therapeutic strategies. We evaluated all the genes statuses, such as KRAS, NRAS, BRAF, and PIK3CA in primary CRC and their matched metastasis, and compared the discordance between the two sites. However, only one patient with NRAS mutation was found in metastatic site and no BRAF mutation was detected due to the relatively small sample size. Subsequently, we performed direct sequencing of KRAS and PIK3CA genes in 59 primary CRC tumors and matched metastases to define the mutational concordance of these genes in primary and metastatic tumors. The KRAS or PIK3CA mutational statuses were regarded as positive if they were mutated in any place of primary tumors or related metastases in our study. Approximately 28.8% of CRCs harbor KRAS mutations, which is almost consistent with prior studies. While PIK3CA mutations were detected in 80% of patients with 44.1% of primary tumors and 54.2% of related metastatic sites, respectively, which showed relatively higher mutational rate than that of prior reports.18,19 Approximately more than 80% KRAS mutations were detected in codons 12, 13 in exon 2, and our results showed 100% mutation analyses in codons 545 of the PIK3CA gene

Table 5 Univariate analyses on the association between clinicopathologic features and the discordance rates of KRAS and PIK3CA mutation status

<table>
<thead>
<tr>
<th>Gene</th>
<th>Characteristic</th>
<th>Univariate analysis</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Concordant cases, N (%)</td>
<td>Discordant cases, N (%)</td>
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<tr>
<td>Metastatic site</td>
<td></td>
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<td></td>
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<tr>
<td>Liver</td>
<td>25 (75.8)</td>
<td>8 (24.2)</td>
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<td>Lung</td>
<td>6 (66.7)</td>
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<td>1 (7.1)</td>
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</tr>
<tr>
<td>Others</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
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<td>Primary tumor location</td>
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</tr>
<tr>
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<td>9 (90)</td>
<td>1 (10)</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>11 (78.6)</td>
<td>3 (21.4)</td>
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<tr>
<td>Rectum</td>
<td>21 (72.4)</td>
<td>8 (27.6)</td>
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<td>KRAS</td>
<td>Type of tumor specimens</td>
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<td>Biopsied</td>
<td>13 (92.8)</td>
<td>1 (7.1)</td>
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<td>Any chemotherapy/radiotherapy between resection of primary and metastasis</td>
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<td>0.333</td>
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<td>Yes</td>
<td>29 (80.6)</td>
<td>7 (19.4)</td>
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<tr>
<td>No</td>
<td>16 (69.6)</td>
<td>7 (30.4)</td>
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<td>Right</td>
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<td>7 (70)</td>
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</tr>
<tr>
<td>Left</td>
<td>7 (50)</td>
<td>7 (50)</td>
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<tr>
<td>Rectum</td>
<td>13 (44.8)</td>
<td>16 (55.2)</td>
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<td>Type of tumor specimens</td>
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<td>13 (36.1)</td>
<td>23 (63.9)</td>
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<td>7 (36.8)</td>
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<tr>
<td>Metachronous</td>
<td>13 (32.5)</td>
<td>27 (67.5)</td>
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</table>

Abbreviations: KRAS, Kirsten rat sarcoma viral oncogene homolog; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.
showed an increased the paired specimens obtained from metachronous resection 
PIK3CA found no difference in the discordant rate of 
tion style (synchronous or metachronous) (Goswami et al, 
therapy between resection of primary and metastasis or resec 
any 
s. As previously reported, discrepancy may be related to 
the different sites of primary tumor location, the metastatic 
s. Moreover, acquisition of new mutations may be developed 
ous discordance of the status of gene involved in 
KRAS targeted therapy, it could be more appropriate to perform 
patients with wild-type 
cancer. Therefore, our findings indicated that if 
patients with synchronous resection of the two sites, receiving inter 
vening chemotherapy between resection of primary and metastasis and even increasing number of lines of interven 
ning chemotherapy, can be the possibility for the observed discordance in the KRAS and PIK3CA mutation status.23 The rates of KRAS and PIK3CA gene discordance between primary and metastasis vary by the related metastatic sites, with a relatively higher rate of discordance in brain, bone, peritoneum, or lung metastases when compared with other metastatic organs including liver, distant LN, or ovary.24 Therefore, the causes of discordance need to be further evaluated in future larger studies.

Our study shares several limitations common to the majority of published findings in this field. First, our muta 
tional analysis was performed by the traditional sequencing analysis with relatively low sensitivity, and not confirmed 
by other more sensitive methods as amplification-refractory mutation system allele-specific PCR combined with Scorpions probes or peptide nucleic acid-clamp allele-specific reverse transcription-PCR assay. Second, the retros 
pective analysis is prone to bias or error, and the sample is relatively small. Third, we did not conduct the repetitive and multipoint mutational analysis of the same tissue, which 
may lead to the false-negative results. Despite these limita 
tions, our study provides some clinically meaningful sugges 
tions. This study demonstrated the existence of a significant discordance of PIK3CA and relative concordance of KRAS mutations occurring in primary tumors and their correspond 
ing metastases in patients with CRC. These reminded that 
gene test can not only be conducted in primary tissues, but 
the metastatic specimen also needs to be reexamined if the 
tissue is available. Our study also raised the hypothesis that combined analysis of KRAS and PIK3CA to select the proper CRC patients will be an effective strategy for EGFR-targeted therapy, which remains to be proven in well-designed clinical studies.

In conclusion, our findings indicate a concordance of 
KRAS mutation and a discordance of PIK3CA mutation between the primary tumors and the matched metastases in CRC and suggest that status of specific molecules in metastatic tumors need to be reevaluated when the patients with metastases are about to use the EGFR-targeted therapy.

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Author contributions
All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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