KRAS Codon 12 Mutation is Associated with More Aggressive Invasiveness in Synchronous Metastatic Colorectal Cancer (mCRC): Retrospective Research

Objective: To investigate the connection between mutant KRAS/NRAS/BRAF and clinicopathological characteristics in therapy-naïve synchronous metastatic colorectal cancer (mCRC) in Chinese populations when compared with all wild type (KRAS/NRAS/BRAF wild type).

Patients and Methods: A total of 200 patients with therapy-naïve synchronous mCRC (TNM stage: TanyNanyM1) were retrospectively collected as study objects. Primary tumor tissues from 200 mCRC patients were analyzed through next-generation sequencing panel to assess the mutated regions of KRAS/NRAS/BRAF.

Results: The distribution frequency of gene mutation in our study was 41% KRAS, 4% NRAS, 11.5% BRAF, 0.5% both KRAS and BRAF. Tumors with any gene mutations (any gene mutations in KRAS/NRAS/BRAF), KRAS and KRAS codon 12 mutation were more likely to be located in right-sided colon (P=0.007, P=0.008, P=0.026, respectively). For metastasis, tumors with any gene mutations, KRAS and KRAS codon 12 mutation were significantly correlated with peritoneal metastasis (P=0.019, P=0.017, P=0.014, respectively), liver-peritoneum metastases (P=0.004, P=0.003, P=0.002, respectively) and multi-organ metastases (P=0.002, P=0.008, P=0.001, respectively). Tumors with all wild type were significantly correlated with distant lymph node-only metastasis. No statistically significant differences were found between clinicopathological characteristics and KRAS codon 13 and NRAS mutations.

Conclusion: Our study suggests that clinicopathological characteristics (specifically for metastasis) are related to KRAS/NRAS/BRAF mutations in therapy-naïve synchronous mCRC population in China. We demonstrated that distant lymph node-only metastasis is visibly linked to all wild-type tumors. We found that patients with any gene mutations, KRAS mutation are more likely to carry peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases than those with all wild type. After stratification, KRAS codon 12 mutation, but not codon 13 mutation, was remarkably associated with peritoneal metastasis, liver-peritoneum metastases, and multi-organ metastases compared to all wild type. These results may be useful for aiding in the prediction of prognosis and choosing the appropriate regimen for therapy.

Keywords: synchronous metastatic colorectal cancer, KRAS mutation, NRAS mutation, BRAF mutation, KRAS codon 12 mutation, KRAS codon 13 mutation

Background
Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth most frequent cause of cancer-related mortality worldwide.1 In China, with...
the rapid development of the economy, people’s living standard and food spectrum are gradually westernized, which leads to an increase in the incidence of CRC over time. According to the latest data, China had 521,000 new colorectal cancer cases and 248,000 deaths due to colorectal cancer in 2018 that accounted for approximately 30% of the incidence and mortality in the same periods worldwide. There are no obvious symptoms in early stages of colorectal cancer, so almost 15% to 25% of patients present with synchronous metastasis at diagnosis.2

Patients with synchronous metastatic colorectal cancer (mCRC) seem to have much poorer prognosis than those with early and middle stages. With the fast progression of medical technology, we have gradually recognized that CRC is a historically and clinically heterogeneous disease and the accumulation of mutated genes results in CRC tumorigenesis.3,4 As is well known, the RAS-RAF-MAPK pathway, as a signaling pathway downstream of the epidermal growth factor receptor (EGFR), which can promote carcinogenesis of colorectum,5,6 can be driven by mutations of oncogenes such as Kirsten rat sarcoma viral oncogene homolog (KRAS) or V-raf murine sarcoma viral oncogene homolog B1 (BRAF) of the EGFR-mediated pathway.7 In recent decades, the presence of monoclonal antibodies targeted at EGFR such as cetuximab and panitumumab, has been shown to be effective for treatment of metastatic colorectal cancer (mCRC). The increasing usage of standard regimens of chemotherapy with or without targeted therapy (including anti-epidermal growth factor receptor antibody and anti-angiogenesis antibody) improves patients’ prognosis and relieves pain.8–10 The combination of chemotherapy and anti-EGFR therapy treatment has resulted in a remarkable improvement compared with chemotherapy-only in several clinical trials,11 the combination therapy as first-line strategy for mCRC patients with all wild type (all of KRAS, NRAS and BRAF genes are wild type) is recommended by the latest American Society of Clinical Oncology (ASCO) guidelines; KRAS mutation can be detected in 30–50% of CRC and can be used as a tool to predict resistance to anti-EGFR treatment. In addition, almost 90% of KRAS mutation present in codon 12 or 13.12,13 3–5% of CRC shows a mutation in neuroblastoma RAS viral oncogene homolog (NRAS), which has similar clinical and pathological characteristics to KRAS mutation.14,15 Patients with NRAS mutation also respond poorly to anti-EGFR treatment.16,17 5%–10% of CRC can harbor mutant BRAF with 90% of its mutation situated in V600E which has also shown a negative response to anti-EGFR therapy as well as unsatisfying outcomes.18–20 In a word, it is concluded that patients with any gene mutations (defined as any gene mutations in KRAS, NRAS or BRAF) probably gain resistance to anti-EGFR treatment, and may have poorer outcomes and different metastatic patterns than those with all wild type. Therefore, there is an urgent need to comprehensively sequence gene status to select suitable candidates for personalized therapy and regular surveillance. Treatment regimens and prognosis of patients with mutant KRAS/NRAS/BRAF have been fully explored. However, the correlation between gene mutations and clinicopathological features (especially for distant organ metastasis) in mCRC has not been fully discussed.

In the present study, we retrospectively enrolled 200 therapy-naïve synchronous mCRC patients at first diagnosis in China. We analyzed the genetic status of KRAS, NRAS and BRAF for each patient with next-generation sequencing to explore whether there is a connection between clinicopathological characteristics and gene mutations in mCRC when compared with all wild type.

Patients and Methods
Ethics Statement
This study was approved by the clinical research ethics committee of the Jiangsu Cancer Hospital and was conducted in accordance with the Declaration of Helsinki. In the context of COVID-19 pandemic and social distancing policy, informed consent from all patients was obtained verbally and confirmed by the clinical research ethics committee of the Jiangsu Cancer Hospital.

Patients and Clinical Data
We retrospectively collected 504 CRC patients in the Affiliated Tumor Hospital of Nanjing Medical University from December 2015 to February 2020. The inclusion criteria were as follows: 1) histologic samples were pathologically demonstrated as colorectal carcinoma; 2) clinical data and genetic test data were completed. The exclusion criteria were: 1) patients with non-metastatic colorectal cancer and non-synchronous metastatic colorectal cancer; 2) preoperative chemotherapy, radiotherapy, targeted therapy and immunotherapy were accepted; 3) primary tumor was located in cecum, appendix and ileocecal junction; 4) samples were histologically confirmed as neuroendocrine carcinoma or containing neuroendocrine components; detailed selection
process is shown in Figure 1. In total, 200 patients with therapy-naïve synchronous mCRC at first diagnosis who underwent primary lesion resection or endoscopic biopsy were included in our study. Clinicopathological data were extracted from medical documents. The pTNM stage system was reviewed according to the 8th edition AJCC cancer staging. Tumor grading and staging were based on the World Health Organization (WHO) criteria. Assessment of distant metastasis was done mainly according to simultaneously confirmed radiological data by two radiologists. Primary lesion of right-sided colon, defined as tumor, was located in ascending colon, hepatic flexure and transverse colon; primary lesion of left-sided colon, defined as tumor, was located in splenic flexure, descending and sigmoid colon; primary lesion of the rectum, defined as large bowel up to the edge of 16 cm from the dentate line.

DNA Extraction and Sequencing
All tissue samples that were extracted from primary tumor through surgical resection or endoscopic biopsy were formalin-fixed, paraffin-embedded, and histologically confirmed. Five sections (10 μm thick) that were cut from paraffin-embedded tumor tissue blocks were used per analysis. To obtain maximal tumor DNA, we chose tumor-rich paraffin block specimens whose tumor components were greater than at least 30%. DNA in the collected tissue samples was extracted using the QIAamp DNA FFPE Tissue Kit (Cat No. 56404, Qiagen) following the

![Flow diagram of the patient selection process.](image-url)
manufacturer’s protocol. DNA from each sample was eluted in 50 μL of ATE buffer (included in the kit).

Total DNA was quantified by using the Qubit dsDNA HS Assay kit (Invitrogen), libraries were constructed using the KAPA Hyper library preparation kit (KAPA, KX8504) following Illumina (San Diego, CA) protocols. Sequencing was performed to examine the mutation in KRAS (all exons), NRAS (all exons), BRAF (all exons) through a NovaSeq 6000 sequencing system (Illumina).

**Statistical Analysis**

Statistical analyses were performed with SPSS software (version 22 of SPSS, Chicago, IL, USA). The relationship between gene mutations and clinical characteristics was compared by Pearson’s Chi-squared (χ²) test or Fisher’s exact test. Statistical tests were two-sided, and P<0.05 was considered significant.

**Results**

**Frequency of Gene Mutations in Primary Lesions**

Within the study period, we retrospectively collected 200 therapy-naïve patients with mCRC at first diagnosis. Primary colorectal samples were analyzed for KRAS, NRAS and BRAF gene mutations. Among these samples, 43% (86/200) of carcinomas were all wild type. KRAS mutation occurred in 41% (82/200) of colorectal carcinomas. NRAS mutation was observed in 4.0% (8/200) of colorectal carcinomas. BRAF mutation was demonstrated in 11.5% (23/200) of colorectal carcinomas. Particularly, there was one sample that harbored both KRAS and BRAF mutations (KRAS p.A146T+ BRAF p. D594G), and in another 5 patients, double KRAS mutation existed (p.G12V+p.D33E, 2 of p.G12A+p.G12S, 2 of p.G12D+p.G12S); these cases were excluded from the analysis because they were too rare to analyze. Detailed distribution of mutation subtypes is summed up in Table 1, the percentage of each mutation subtype is shown in Figure 2.

**Patients’ Characteristics**

Table 2 summarizes the clinicopathological characteristics of study subjects. A total of 194 patients with metastatic colorectal carcinoma who had sufficient clinical data were evaluated. At diagnosis, the median age was 59 years (range 26–83 years); 121 (62.4%) of patients were male, the other 73 (37.6%) were female. Of these patients, 61 (31.4%) had right-sided colon tumors, 80 (41.2%) had left-sided colon tumors, and 53 (27.3%) patients’ tumors were located in rectum. For T staging, there were 3 T1, 5 T2, 106 T3, and 55 T4 stage cases, and for N staging, there were 41 N0, 62 N1, and 66 N2 stage cases. For gross type of tumor, 27 (13.9%) were swell type, 131 (67.5%) were ulcer type, and 11 (5.7%) were invasion type. For 25 cases, no surgery was carried out because the patients did not reach the indication of surgery, so their pathologic stage of T and N, tumor gross type was unknown. 105 patients had well/moderate differentiated tumors and 89 patients had poorly differentiated tumors. Regarding the histological type of tumors, 79.4% were classic adenocarcinoma and 20.6% were mucinous/rare histological type.

### Table 1 Mutation Frequency and Subtype Distribution of RAS and BRAF Genes

| Genes                     | Codon | Mutation | Cases (% of 200) |
|---------------------------|-------|----------|------------------|
| Total cases of KRAS mutation | 12    | p.G12A   | 82 (41.0%)       |
|                           | 12    | p.G12C   | 3 (1.5%)         |
|                           | 12    | p.G12D   | 2 (1.0%)         |
|                           | 12    | p.G12R   | 23 (11.5%)       |
|                           | 12    | p.G12S   | 1 (0.5%)         |
|                           | 12    | p.G12V   | 5 (2.5%)         |
|                           | 13    | p.G13A   | 18 (9.0%)        |
|                           | 13    | p.G13C   | 2 (1.0%)         |
|                           | 13    | p.G13D   | 1 (0.5%)         |
|                           | 59    | p.A59E   | 11 (5.5%)        |
|                           | 59    | p.A59T   | 1 (0.5%)         |
|                           | 61    | p.Q61H   | 1 (0.5%)         |
|                           | 61    | p.Q61L   | 1 (0.5%)         |
|                           | 117   | p.K117N  | 1 (0.5%)         |
|                           | 146   | p.A146T  | 2 (1.0%)         |
|                           |       | bi-mutation | 4 (2.0%)    |
|                           |       | p.G12A+p.G12S | 2 (1.0%)  |
|                           |       | p.G12D+p.G12S | 1 (0.5%)  |
|                           |       | p.G12V+p.D33E | 1 (0.5%)  |
| Total cases of NRAS mutation | 12    | p.G12D   | 8 (4.0%)         |
|                           | 60    | p.G60E   | 3 (1.5%)         |
|                           | 61    | p.Q61K   | 1 (0.5%)         |
|                           | 61    | p.Q61R   | 1 (0.5%)         |
| Total cases of BRAF mutation | 466   | p.G466V  | 23 (11.5%)       |
|                           | 600   | p.V600E  | 1 (0.5%)         |
|                           | 601   | p.K601E  | 2 (10.5%)        |
| Total cases of both KRAS and BRAF mutation | 12    | p.A146T+p.D594G | 1 (0.5%)  |

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**Reference:**

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**Figure 2:**

The percentage of each mutation subtype is shown in Figure 2.
Regarding metastasis, 132 (68%) had liver metastasis, 49 (25.3%) had lung metastasis, 26 (13.4%) had peritoneal metastasis, 34 (17.5%) had abdominal and pelvic implant metastasis, 42 (21.6%) had distant lymph node involvement and 17 (8.8%) had other locations’ involvement including spleen (3), bone (4), brain (1), ovary (6) and adrenal gland (3). 116 patients had only one organ involved in metastasis; liver-only metastasis, lung-only metastasis, peritoneum-only metastasis, abdominal and pelvic implant-only metastasis, distant lymph node-only metastasis were respectively observed in 75 (38.7%), 11 (5.7%), 4 (2.1%), 10 (5.2%), 10 (5.2%) of these patients. There were 6 remaining patients, including 3 with bone-only metastasis, 2 with ovary-only metastasis and 1 with spleen-only metastasis, who were excluded due to the low incidence in our study. 78 patients had more than one metastases. Of patients with dual-site metastases, 31 (16%) had liver-lung metastases, 14 (7.2%) had liver-peritoneum metastases and 4 (2.1%) had lung-peritoneum metastases (P=0.002), and was more likely located in right-sided colon (P=0.007) than all wild-type tumors. There was no statistically significant association between any gene mutations and other clinicopathological features like gender, age, differentiation degree, etc. When compared with all wild-type tumor, mutant KRAS tumor had a higher rate in right-sided colon (P=0.008) and had a significant relevance with peritoneum metastasis (P=0.017) and multi-organ metastases (P=0.008). When compared with all wild type, differences between NRAS mutation and clinical features did not reach statistical significance, BRAF mutation showed significant association with mucinous/rare histological subtypes, distant lymph node metastasis and multi-organ metastases in comparison with all wild type (P=0.026 and P=0.029, P=0.003, respectively). Moreover, mutant BRAF carcinomas also tended to be located in right-sided colon (P=0.052) and had peritoneal metastasis (P=0.052) compared to all wild-type carcinomas, although it did not reach statistical significance.

**Gene Mutation and Clinical Characteristics**

Table 3 summarizes the connection between clinicopathological characteristics and gene mutations. Tumor with any gene mutations was significantly correlated with mucinous/rare histological subtypes (P=0.016), peritoneal metastasis (P=0.019) and multi-organ metastases (P=0.002), and was more likely located in right-sided colon (P=0.007) than all wild-type tumors. There was no statistically significant association between any gene mutations and other clinicopathological features like gender, age, differentiation degree, etc. When compared with all wild-type tumor, mutant KRAS tumor had a higher rate in right-sided colon (P=0.008) and had a significant relevance with peritoneum metastasis (P=0.017) and multi-organ metastases (P=0.008). When compared with all wild type, differences between NRAS mutation and clinical features did not reach statistical significance, BRAF mutation showed significant association with mucinous/rare histological subtypes, distant lymph node metastasis and multi-organ metastases in comparison with all wild type (P=0.026 and P=0.029, P=0.003, respectively). Moreover, mutant BRAF carcinomas also tended to be located in right-sided colon (P=0.052) and had peritoneal metastasis (P=0.052) compared to all wild-type carcinomas, although it did not reach statistical significance.

**Differences Among Specific KRAS Mutations in mCRC**

For specific KRAS mutations, detailed information is shown in Table 4. Tumor with KRAS codon 12 mutation was more likely to present in right-sided colon (P=0.026) and present with peritoneal metastasis (P=0.014) and multi-organ metastases (P=0.001) than all wild-type tumors. After stratification, patients with peritoneal
metastasis had a tendency to carry mutant KRAS G12D (P=0.052). KRAS G12V was more frequent in right-sided colon (P=0.022) than all wild type. Both KRAS G12D and G12V mutated carcinomas were significantly linked to multi-organ metastases compared to all wild-type carcinomas (P=0.01 and P=0.002, respectively). There were no significant differences found in patients with mutant KRAS codon 13 and G13D.

Table 2 (Continued).

| Characteristics                      | Cases (% of 194) |
|--------------------------------------|------------------|
| Single-site metastasis               |                  |
| Liver-only metastasis                | 75(38.7%)        |
| Lung-only metastasis                 | 11(5.7%)         |
| Peritoneum-only metastasis           | 42(2.1%)         |
| Distant lymph node-only metastasis   | 10(5.2%)         |
| Abdominal and pelvic implant-only metastasis | 10(5.2%) |

Note: "25 patients without surgery were excluded here.

Single-Site and Dual-Site Metastases

For single-site metastasis, all wild type tumors were significantly correlated with distant lymph node metastasis compared to any gene mutations, KRAS mutation and KRAS codon 12 mutation cases (P=0.006, P=0.003 and P=0.014, respectively). For dual-site metastases, patients with any mutations, KRAS mutation, BRAF mutation, KRAS codon 12 and KRAS G12D mutations were statistically significantly more likely to carry liver-peritoneum metastases than those with all wild type (P=0.004, P=0.003, P=0.029, P=0.002, and P=0.007, respectively).

Discussion

As one of the most devastating diseases in the world, colorectal carcinoma is a pathologically and clinically heterogeneous malignancy. With the changing global lifestyle, the number of people suffering from CRC continues to rise. China is also facing a similar situation, and even worse. Early detection of CRC is often missed because of its unclear symptoms, while chemotherapy at advanced stages is generally unsatisfying. The appearance of monoclonal antibodies (MoAbs), like cetuximab and panitumumab, has significantly improved the outcome of mCRC patients. Since the efficacy of anti-EGFR therapy is bound up with KRAS status in CRC, many studies have estimated the role of
Table 3 Associations Between Gene Mutations and Clinicopathological Characteristics of Patients

| Characteristics | All Wild Type | Any Gene Mutations | P-value | KRAS Mutation | P-value | NRAS Mutation | P-value | BRAF Mutation | P-value |
|-----------------|--------------|--------------------|---------|---------------|---------|---------------|---------|---------------|---------|
| Gender          | Male         | 56/86 (65.1%)      | 48/77 (62.3%) | 0.481 | 6/8 (75.0%) | 0.712 | 6/8 (75.0%) | 0.712a | 11/23 (47.8%) | 0.130 |
|                 | Female       | 30/86 (34.9%)      | 29/77 (37.7%) |        | 2/8 (25.0%) |       | 2/8 (25.0%) |       | 12/23 (52.2%) |       |
| Age             | <60          | 46/86 (53.5%)      | 37/77 (48.1%) | 0.460 | 4/8 (50.0%) | 0.488 | 4/8 (50.0%) | 1*     | 11/23 (47.8%) | 0.629 |
|                 | ≥60          | 40/86 (46.5%)      | 40/77 (51.9%) |        | 4/8 (50.0%) |       | 4/8 (50.0%) |       | 12/23 (52.2%) |       |
| Primary Location| Right-sided colon | 20/86 (23.3%) | 30/77 (39.0%) | 0.007 | 1/8 (12.5%) | 0.008 | 1/8 (12.5%) | 0.780 | 10/23 (43.5%) | 0.052 |
|                 | Left-sided colon | 46/86 (53.5%) | 23/77 (29.9%) |        | 5/8 (62.5%) |       | 5/8 (62.5%) |       | 6/23 (26.1%) |       |
|                 | Rectum       | 20/86 (23.3%)      | 24/77 (31.2%) |        | 2/8 (25.0%) |       | 2/8 (25.0%) |       | 7/23 (30.4%) |       |
| Tumor infiltration | T1          | 2/80 (2.5%)        | 1/63 (1.6%) | 0.644 | 3/63 (4.8%) | 0.583 | 0/8 (0.0%) | 0.460 | 1/8 (12.5%) | 0.756 |
|                 | T2           | 2/80 (2.5%)        | 3/63 (4.8%) |        | 7/8 (87.5%) |       | 0/8 (0.0%) |       | 10/18 (55.6%) |       |
|                 | T3           | 4/80 (58.8%)       | 42/63 (66.7%) |        | 1/8 (12.5%) |       | 8/18 (44.4%) |       |       |       |
|                 | T4           | 29/80 (36.3%)      | 17/63 (27.0%) |        |       |       |       |       |       |       |
| Nodal Status    | N0           | 21/80 (26.3%)      | 15/63 (23.8%) | 0.407 | 3/8 (37.5%) | 0.765 | 3/8 (37.5%) | 0.309 | 2/18 (11.1%) | 0.178 |
|                 | N1           | 32/80 (40.0%)      | 23/63 (36.5%) |        | 1/8 (12.5%) |       | 6/18 (33.3%) |       | 10/18 (55.6%) |       |
|                 | N2           | 27/80 (33.8%)      | 25/63 (39.7%) |        | 4/8 (50.0%) |       | 10/18 (55.6%) |       |       |       |
| Gross type      | Swell type   | 13/80 (16.3%)      | 13/63 (20.6%) | 0.753 | 0/8 (0.0%) | 0.271 | 0/8 (0.0%) | 0.349 | 1/18 (5.6%) | 0.284 |
|                 | Ulcer type   | 63/80 (78.8%)      | 43/63 (68.3%) |        | 8/18 (44.4%) |       | 0/8 (0.0%) |       | 17/18 (94.4%) |       |
|                 | Invasion type| 4/80 (5.0%)        | 7/63 (11.1%) |        | 0/8 (0.0%) |       | 0/8 (0.0%) |       | 0/18 (0.0%) |       |
| Pathological type| Adenocarcinoma | 75/86 (87.2%) | 60/77 (77.9%) | 0.016 | 5/8 (62.5%) | 0.094a | 5/8 (62.5%) | 0.094a | 15/23 (65.2%) | 0.026a |
|                 | Mucinous/rare adenocarcinoma | 11/86 (12.8%) | 17/77 (22.1%) | 0.117 | 3/8 (37.5%) |       | 8/23 (34.8%) |       |       |       |
| Differentiation | Well-moderate | 45/86 (52.3%) | 47/77 (61.0%) | 0.654 | 4/8 (50.0%) | 0.263 | 4/8 (50.0%) | 1*     | 9/23 (39.1%) | 0.261 |
|                 | Poor         | 41/86 (47.7%)      | 30/77 (39.0%) |        | 4/8 (50.0%) |       | 4/8 (50.0%) |       | 14/23 (60.9%) |       |
| Location of metastasis | Liver     | 55/86 (64.0%) | 55/77 (71.4%) | 0.276 | 6/8 (75.0%) | 0.309 | 6/8 (75.0%) | 0.708a | 16/23 (69.6%) | 0.616 |
|                 | Lung         | 22/86 (25.6%)      | 20/77 (26.0%) | 0.926 | 3/8 (37.5%) | 0.954 | 3/8 (37.5%) | 0.435a | 4/23 (17.4%) | 0.413 |
|                 | Peritoneum   | 6/86 (7.0%)        | 15/77 (19.5%) | 0.019 | 0/8 (0.0%) | 0.017 | 0/8 (0.0%) | 1*     | 5/23 (21.7%) | 0.052a |
|                 | Distant lymph node | 21/86 (24.4%) | 10/77 (13.0%) | 0.403 | 0/8 (0.0%) | 0.063 | 0/8 (0.0%) | 0.192a | 11/23 (47.8%) | 0.029 |
|                 | Abdominal and pelvic implant | 11/86 (12.8%) | 17/77 (22.1%) | 0.122 | 1/8 (12.5%) | 0.117 | 1/8 (12.5%) | 1*     | 5/23 (21.7%) | 0.322a |
| Single-site metastasis | Liver-only metastasis | 39/86 (45.3%) | 27/77 (35.1%) | 0.088 | 3/8 (37.5%) | 0.182 | 3/8 (37.5%) | 0.728a | 6/23 (26.1%) | 0.096 |
|                 | Lung-only metastasis | 4/86 (4.7%) | 7/77 (6.5%) | 0.758a |       |       |       |       |       |       |
|                 | Peritoneum-only metastasis | 2/86 (2.3%) | 1/77 (1.3%) | 1*     |       |       |       |       |       |       |
|                 | Distant lymph node-only metastasis | 9/86 (10.5%) | 0/77 (0.0%) | 0.006a |       |       |       |       |       |       |
|                 | Abdominal and pelvic implant-only metastasis | 5/86 (5.8%) | 5/77 (6.5%) | 0.753a |       |       |       |       |       |       |

(Continued)
Table 3 (Continued).

|                  | All Wild Type | Any Gene Mutations | P-value | KRAS Mutation | P-value | NRAS Mutation | P-value | BRAF Mutation | P-value |
|------------------|---------------|--------------------|---------|---------------|---------|---------------|---------|---------------|---------|
| Dual-site metastases |               |                    |         |               |         |               |         |               |         |
| Liver-lung metastases | 14/86 (16.3%) | 17/108 (15.7%)     | 0.919   | 13/77 (16.9%) | 0.918   | 2/8 (25%)     | 0.620   | 2/23 (8.7%)   | 0.515   |
| Liver-peritoneum metastases | 1/86 (1.2%) | 13/108 (12.0%)     | 0.004   | 10/77 (13.0%) | 0.003   | 0/8 (0.0%)    | 1*      | 3/23 (13.0%)  | 0.029   |
| Lung-peritoneum metastases | 1/86 (1.2%) | 3/108 (2.8%)       | 0.631*  | 2/77 (2.6%)  | 0.603*  | 0/8 (0.0%)    | 1*      | 1/23 (4.3%)   | 0.379*  |
| Number of metastases |               |                    |         |               |         |               |         |               |         |
| 1                | 62/86 (72.1%) | 54/108 (50.0%)     | 0.002   | 40/77 (51.9%) | 0.008   | 5/8 (62.5%)   | 0.685   | 9/23 (39.1%)  | 0.003   |
| ≥1               | 24/86 (27.9%) | 54/108 (50.0%)     |         | 37/77 (48.1%) |         | 3/8 (37.5%)   |         | 14/23 (60.9%) |         |

Notes: *25 patients without surgery were excluded here. **Two-sided Fischer’s exact test, others are two-sided χ² test.

KRAS status in CRC. Some studies indicated that anti-EGFR therapy shows a response to mCRC people with wild-type KRAS. 21,22 However, even in KRAS wild-type cohorts, more than 65% of patients were still resistant to anti-EGFR MoAbs. 23 In further studies, Yuan et al concluded that patients with BRAF mutation are unlikely to gain benefit from anti-EGFR therapy. 24,25 For mutant NRAS, a similar conclusion was also reported by De Roock et al. 16 Furthermore, the latest ASCO guidelines also suggest that response to anti-EGFR treatment is confined to patients with all wild type. In addition, Foltran et al illustrated that patients with any mutation of the oncogenes have poorer survival compared to those with all wild type. 26 Despite the fact that the relation between gene mutation and survival of mCRC patients has been fully examined, there is ambiguous understanding of the link between mutant genes and clinicopathological features in mCRC patients in China, especially for distant metastasis.

To our best knowledge, this is the first study to discuss the association between gene mutations and clinicopathological features, especially for metastatic patterns, in first diagnosed mCRC of Chinese population compared with all wild type. We are the first to demonstrate that among patients with any gene mutations, those with KRAS mutation and BRAF mutation are more likely to carry peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases compared to all wild-type patients. After stratification, KRAS codon 12 mutation, but not codon 13 mutation, was remarkably associated with peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases compared to all wild-type patients. After stratification, KRAS codon 12 mutation, but not codon 13 mutation, was remarkably associated with peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases. We are also the first to demonstrate that distant lymph node-only metastasis is visibly linked to all wild-type tumors. In addition, we also found that tumors with any mutations are statistically located in right-sided colon, having mucinous or signet-ring cell component compared to all wild type, which is consistent with a previous study. 27

It is well known that there is a strong association between survival outcome and site of metastasis. Previous studies found that the survival of patients with peritoneal metastasis was poorer than those with metastases in other sites, 28,29 which could partly be blamed on local complications like ascites formation. However, the molecular mechanism is still controversial and not well-established. In addition, patients with any gene mutations had a shorter survival time than those with all wild type. Further, mutations in KRAS or BRAF, which also suffer inferior prognosis compared with all wild-type counterparts, 30 were reported by Liu et al. In our study, we observed that among tumors with any gene mutations, KRAS or BRAF mutation more frequently metastasized to peritoneum and liver-peritoneum compared to those with all wild type, which might be an explanation of previous studies.

The frequency of mutant KRAS in our study was 41%, which was similar to other studies. 12,13 Previous studies have examined the distribution of KRAS mutation from western populations, which reported that the most frequent subtype in codon 12 was G12D, followed by G12V, G12C, G12S and G12A and G12R. In codon 13 mutations, the majority was KRAS G13D, followed by G13C, G13R. 31,32 However, in the present study, the corresponding order was G12D, G12V, G12S, G12A, G12C and G12R in codon 12; in codon 13, the corresponding order was KRAS G13D and G13A. Moreover, we also found some rare mutations, including A59E, A59T in KRAS codon 59 and Q61H, Q61L in codon 61, but they were too rare in our study to analyze. Surprisingly, we found five cases
|                                      | All Wild Type | KRAS Codon 12 Mutation | P-value | KRAS Codon 13 Mutation | P-value | KRAS G12D Mutation | P-value | KRAS G12V Mutation | P-value | KRAS G13D Mutation | P-value |
|--------------------------------------|---------------|------------------------|---------|------------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|
| **Gender**                           |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| Male                                 | 56/86 (65.1%) | 34/52 (65.4%)          | 0.974   | 8/14 (57.1%)           | 0.564   | 13/23 (56.5%)     | 0.447   | 13/18 (72.2%)     | 0.562   | 6/11 (54.5%)      | 0.519*  |
| Female                               | 30/86 (34.9%) | 18/52 (34.6%)          |         | 6/14 (42.9%)           |         | 10/23 (43.5%)     |         | 5/18 (27.8%)      |         | 5/11 (45.5%)      |         |
| **Age**                              |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| <60                                  | 46/86 (53.5%) | 26/52 (50.0%)          | 0.691   | 6/14 (42.9%)           | 0.460   | 14/23 (60.9%)     | 0.527   | 6/18 (33.3%)      | 0.120   | 6/11 (54.5%)      | 0.947   |
| ≥60                                  | 40/86 (46.5%) | 26/52 (50.0%)          |         | 8/14 (57.1%)           |         | 9/23 (39.1%)      |         | 12/18 (66.7%)     |         | 5/11 (45.5%)      |         |
| **Primary location**                 |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| Right-sided colon                    | 20/86 (23.3%) | 21/52 (40.4%)          | 0.026   | 4/14 (28.6%)           | 0.181   | 9/23 (39.1%)      | 0.063   | 10/18 (55.6%)     | 0.022   | 3/11 (27.3%)      | 0.196   |
| Left-sided colon                     | 46/86 (53.5%) | 16/52 (30.8%)          |         | 4/14 (28.6%)           |         | 6/23 (26.1%)      |         | 6/18 (33.3%)      |         | 2/18 (11.1%)      |         |
| Rectum                               | 21/86 (23.3%) | 15/52 (28.8%)          |         | 6/14 (42.9%)           |         | 8/23 (34.8%)      |         |                   |         |                   |         |
| **Tumor infiltration**               |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| T1                                   | 2/80 (2.5%)   | 1/11 (9.1%)            | 0.319   | 0/21 (0.0%)            | 0.622   | 0/15 (0.0%)       | 0.237   | 1/8 (12.5%)       | 1/8 (12.5%) | 4/8 (50.0%) | 2/8 (25.0%) |
| T2                                   | 2/80 (2.5%)   | 1/11 (9.1%)            |         | 0/21 (0.0%)            |         | 15/21 (71.4%)     |         | 9/15 (60.0%)      |         |                   |         |
| T3                                   | 2/80 (2.5%)   | 7/11 (63.6%)           |         | 0/21 (0.0%)            |         | 6/21 (28.6%)      |         | 4/15 (26.7%)      |         |                   |         |
| T4                                   | 47/80 (58.8%) | 28/44 (63.6%)          |         | 0/21 (0.0%)            |         | 10/21 (47.6%)     |         | 5/15 (33.3%)      |         |                   |         |
| **Nodal status**                     |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| N0                                   | 21/80 (26.3%) | 10/44 (22.7%)          | 0.432   | 3/11 (27.3%)           | 0.906   | 3/21 (14.3%)      | 0.389   | 5/15 (33.3%)      | 0.617   | 2/8 (25.0%)       | 0.839   |
| N1                                   | 32/80 (40.0%) | 14/44 (31.8%)          |         | 5/11 (45.5%)           |         | 8/21 (38.1%)      |         | 4/15 (26.7%)      |         | 4/8 (50.0%)       |         |
| N2                                   | 27/80 (33.8%) | 20/44 (45.5%)          |         | 3/11 (27.3%)           |         | 10/21 (47.6%)     |         | 6/15 (40.0%)      |         | 2/8 (25.0%)       |         |
| **Gross type**                       |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| Swell type                           | 13/80 (16.3%) | 10/44 (22.7%)          | 0.587   | 3/11 (27.3%)           | 0.535   | 4/21 (19.0%)      | 0.954   | 4/15 (26.7%)      | 0.254   | 3/8 (37.5%)       | 0.293   |
| Ulcer type                           | 63/80 (78.8%) | 31/44 (70.5%)          |         | 7/11 (63.6%)           |         | 16/21 (76.2%)     |         | 9/15 (60.0%)      |         | 5/8 (62.5%)       |         |
| Invasion type                        | 4/80 (5.0%)   | 3/44 (6.8%)            |         | 1/11 (9.1%)            |         | 1/21 (4.8%)       |         | 2/15 (13.3%)      |         | 0/8 (0.0%)        |         |
| **Pathological type**                |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| Adenocarcinoma                       | 75/86 (87.2%) | 39/52 (75.0%)          | 0.067   | 13/14 (92.9%)          | 1*      | 17/23 (73.9%)     | 0.191*  | 13/18 (72.2%)     | 0.147*  | 10/11 (90.9%)     | 1*      |
| Mucinous/rare adenocarcinoma         | 11/86 (12.8%) | 13/52 (25.0%)          |         | 1/14 (7.1%)            |         | 6/23 (26.1%)      |         | 5/18 (27.8%)      |         | 1/11 (9.1%)       |         |
| **Differentiation**                  |               |                        |         |                        |         |                   |         |                   |         |                   |         |
| Well-moderate                        | 45/86 (52.3%) | 31/52 (59.6%)          | 0.404   | 10/14 (71.4%)          | 0.183   | 14/23 (60.9%)     | 0.465   | 13/18 (72.2%)     | 0.122   | 8/11 (72.7%)      | 0.335*  |
| Poor                                 | 41/86 (47.7%) | 21/52 (40.4%)          |         | 4/14 (28.6%)           |         | 9/23 (39.1%)      |         | 5/18 (27.8%)      |         | 3/11 (27.3%)      |         |

(Continued)
Table 4 (Continued).

| Location of metastasis                      | All Wild Type | KRAS Codon 12 Mutation | P-value | KRAS Codon 13 Mutation | P-value | KRAS G12D Mutation | P-value | KRAS G12V Mutation | P-value | KRAS G13D Mutation | P-value |
|---------------------------------------------|---------------|------------------------|---------|------------------------|---------|--------------------|---------|--------------------|---------|--------------------|---------|
| Liver                                       | 55/86 (64.0%) | 41/52 (78.8%)          | 0.065   | 7/14 (50.0%)           | 0.319   | 19/23 (82.6%)      | 0.089   | 13/18 (72.2%)      | 0.503   | 6/11 (54.5%)       | 0.530*  |
| Lung                                        | 22/86 (25.6%) | 15/52 (28.8%)          | 0.675   | 4/14 (28.6%)           | 0.754*  | 7/23 (30.4%)       | 0.640   | 6/18 (33.3%)       | 0.562*  | 3/11 (27.3%)       | 1*      |
| Peritoneum                                  | 6/86 (7.0%)   | 11/52 (21.2%)          | 0.014   | 1/14 (7.1%)            | 1*      | 5/23 (21.7%)       | 0.052*  | 4/18 (22.2%)       | 0.068*  | 1/11 (9.1%)        | 0.582*  |
| Distant lymph node                          | 21/86 (24.4%) | 6/52 (11.5%)           | 0.065   | 2/14 (14.3%)           | 0.512*  | 3/23 (13.0%)       | 0.242   | 3/18 (16.7%)       | 0.759*  | 2/11 (18.2%)       | 1*      |
| Abdominal and pelvic implant                | 11/86 (12.8%) | 11/52 (21.2%)          | 0.193   | 2/14 (14.3%)           | 1*      | 3/23 (13.0%)       | 1*      | 4/18 (22.2%)       | 0.288*  | 2/11 (18.2%)       | 0.639*  |

Single-site metastasis

| Liver-only metastasis                       | 39/86 (45.3%) | 17/52 (32.7%)          | 0.142   | 6/14 (42.9%)           | 0.862   | 8/23 (34.8%)       | 0.363   | 4/18 (22.2%)       | 0.070   | 5/11 (45.5%)       | 1*      |
| Lung-only metastasis                        | 4/86 (4.7%)   | 2/52 (3.8%)            | 1*      | 3/14 (21.4%)           | 0.055*  | 2/23 (8.7%)        | 0.604*  | 0/18 (0.0%)        | 1*      | 2/11 (18.2%)       | 0.137*  |
| Peritoneum-only metastasis                  | 2/86 (2.3%)   | 1/52 (1.9%)            | 1*      | 0/14 (0.0%)            | 0.352*  | 0/23 (0.0%)        | 0.200*  | 0/18 (0.0%)        | 0.353*  | 0/11 (0.0%)        | 0.592*  |
| Distant lymph node-only metastasis          | 9/86 (10.5%)  | 0/52 (0.0%)            | 0.014*  | 0/14 (0.0%)            | 0.582*  | 1/18 (5.6%)        | 2/11 (18.2%)        | 0.179*  |
| Abdominal and pelvic implant-only metastasis| 5/86 (5.8%)   | 3/52 (5.8%)            | 1*      | 2/14 (14.3%)           | 0.253*  | 1/18 (5.6%)        | 2/11 (18.2%)        | 0.179*  |

Dual-site metastases

| Liver-Lung metastases                       | 14/86 (16.3%) | 12/52 (23.1%)          | 0.322   | 1/14 (7.1%)            | 0.687*  | 5/23 (21.7%)       | 0.544*  | 5/18 (27.8%)       | 0.313*  | 1/11 (9.1%)        | 1*      |
| Liver-peritoneum metastases                 | 1/86 (1.2%)   | 8/52 (15.4%)           | 0.002*  | 1/14 (7.1%)            | 0.262*  | 4/23 (17.4%)       | 0.007*  | 2/18 (11.1%)       | 0.077*  | 1/11 (9.1%)        | 0.215*  |
| Lung-peritoneum metastases                  | 1/86 (1.2%)   | 1/52 (1.9%)            | 1*      | 1/14 (7.1%)            | 0.262*  | 0/23 (0.0%)        | 0.379*  | 0/18 (0.0%)        | 1*      | 1/11 (9.1%)        | 0.215*  |

Number of metastases

| 1                                           | 62/86 (72.1%) | 23/52 (44.2%)          | 0.001   | 12/14 (87.5%)          | 0.346   | 10/23 (43.5%)      | 0.010   | 6/18 (33.3%)       | 0.002   | 9/11 (81.8%)       | 0.722*  |
| ≥1                                          | 24/86 (27.9%) | 29/52 (55.8%)          | 0.001   | 2/14 (14.3%)           | 0.346   | 13/23 (56.5%)      | 0.010   | 12/18 (66.7%)      | 2/11 (18.2%)        | 0.002   | 9/11 (81.8%)       | 0.722*  |

Notes: *25 patients without surgery were excluded here. **Two-sided Fisher’s exact test, others are two-sided χ² test.
with double KRAS mutations (2 of G12D+G12S, 2 of G12A+G12S, 1 of G12V+D33E). In addition, NRAS mutation was detected in 3% of mCRC patients and similar prevalence was obtained in other studies.\textsuperscript{14,15} The majority of mutant subtypes in NRAS were G12D in codon 12 and Q61R in codon 61, followed by Q61K in codon 61, G60E in codon 60. According to previous Western and Chinese studies, BRAF mutation could be detected in 8%-12% of all patients who suffered from CRC.\textsuperscript{28,33-37} The most common subtype of BRAF mutation was V600E, which accounted for approximately 90% of mutant BRAF.\textsuperscript{38} In addition, non-V600 BRAF mutations were considered as a special and uncommon category (they occurred in 2% of mCRC patients).\textsuperscript{14} Certain differences between patients with V600 and those with non-V600 BRAF mutations were reported in other studies,\textsuperscript{39} which was not illustrated specifically in our report. In our study, the incidence of BRAF mutation was 11.5%, which was consistent with previous research.\textsuperscript{18,19} V600E was the most frequent subtype of BRAF mutation. For non-V600BRAF Mutation, BRAF G466V and K601E were found. Previous studies suggested that KRAS and BRAF mutations were mutually exclusive in mCRC.\textsuperscript{40,41} However, in this study, we found one case harbored both KRAS and BRAF mutations, which demonstrated that KRAS and BRAF mutations were not mutually exclusive. The result was in line with that of Mao et al.\textsuperscript{42}

Furthermore, we confirmed the strong relationship between KRAS-mutated carcinomas and right-sided colon, which was also confirmed by several pieces of research.\textsuperscript{33-45} However, we could not find obvious significance among mucinous carcinoma, lung metastasis\textsuperscript{2,15} and KRAS mutation, which might be due to small sample size in our study. In concordance with previous studies,\textsuperscript{55,46,47} our study showed that BRAF-mutated colorectal cancers were more commonly located in right colon; histologically, the mucinous/rare type was more frequently associated with BRAF mutation; and in terms of distant metastases, tumors with mutated BRAF were more likely to metastasize to peritoneum and distant lymph nodes. Previous research\textsuperscript{15} found that mucinous histology was less frequent in NRAS mutated tumors compared to all wild type, which was in line with our result.

For specific KRAS mutation, its connection with clinical features is still controversial. Li et al reported that both KRAS codon 12 and 13 mutated carcinomas were more likely to be found in right-sided colon, and were more frequently mucinous histology type when compared with KRAS/BRAF wild-type carcinomas.\textsuperscript{48} However, other studies\textsuperscript{49,50} observed that KRAS codon 12 mutation was closely related to mucinous differentiation and right-sided colon; KRAS codon 13 mutations were more frequently located in right-sided colon, but there was no statistical linkage with mucinous differentiation. Our conclusion was similar to the latter.

Previous laboratory studies\textsuperscript{50-52} suggested that the presence of mutation in KRAS codon 12 confers substantially greater oncogenic potential as compared with codon 13 mutation. Regulation of RAS involves binding of GTP, which activates the protein. Activation of RAS enables high affinity interactions with downstream effectors such as RAF-MAPK and phosphoinositide 3-kinase. Subsequently, slow intrinsic GTPase activity leads to RAS functional in activation. This on and off switch regulation is tightly controlled by ARHGAP (Rho-GTPase activating proteins) and RAPGEF (Rap guanine-nucleotide exchange factors). Interestingly, RAS mutants are resistant to ARHGAP-mediated GTPase activation, leading to elevated cellular levels of RAS-GTP.\textsuperscript{52} Guerrero et al\textsuperscript{50} found that KRAS codon 12 mutation, by altering the threshold for induction of apoptosis, confers a more aggressive tumor phenotype than codon 13 mutation. This suggests that codon 12 mutation results in greater resistance to ARHGAP-mediated GTPase activation than codon 13 mutation. Several research\textsuperscript{53,54} has also confirmed that KRAS mutation in codon 12, rather than in codon 13, is a negative factor of survival outcome, when compared with all wild type. In a word, these experimental and clinical data are consistent with our observations that KRAS codon 12 mutation may be associated with more aggressive tumor behavior to metastasize to peritoneum and liver-peritoneum, which more frequently present with multi-organ metastases.

Despite some positive findings observed in the present study, our study still has some limitations. First of all, owing to the nature of retrospective research, there is unavoidable selection bias in our outcomes. Secondly, based on a relatively small sample size, the amount of samples was not enough to examine other less common mutations, like KRAS mutation in codon 59, 61, 117 and 146. Finally, survival analysis was not performed due to the short follow-up for patients. In further study, we will continuously collect the survival data and therapy regimens for further investigations.

**Conclusion**

Our study suggests that clinicopathological characteristics (specifically for metastasis) are related to KRAS/NRAS/
BRAF mutations in therapy-naïve synchronous mCRC population in China. We demonstrated that distant lymph node-only metastasis is visibly linked with all wild-type tumors. We found that patients with any gene mutations, KRAS mutation are more likely to carry peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases than those with all wild type. After stratification, KRAS codon 12 mutation, but not codon 13 mutation, was remarkably associated with peritoneal metastasis, liver-peritoneum metastases and multi-organ metastases compared to all wild type. These results may be useful for aiding in the prediction of prognosis and choosing the appropriate regimens for therapy.

Disclosure

The authors report no conflicts of interest for this work.

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