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Coexistence of MSI with KRAS mutation is associated with worse prognosis in colorectal cancer

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Abstract

Kristen rat sarcoma viral oncogene homolog (KRAS) and microsatellite instability (MSI) are prognostic markers of colorectal cancer (CRC). However, the clinical value is still not fully understood, when giving the consideration to both the molecular makers. Five hundred fifty-one patients with CRC were retrospectively assessed by determining their clinicopathological features. KRAS mutations were detected by polymerase chain reaction. MSI, a defect in the mismatch repair (MMR) system, was detected by immunohistochemistry. The prognostic value of KRAS in combination with MSI was studied. Among 551 CRC patients, mutations in KRAS codon 12 and KRAS codon 13 were detected in 34.5% and 10.5% of patients, respectively. Four hundred one tumors were randomly selected to detect for MMR proteins expression. In this analysis, 30 (7.5%) tumors that had at least 1 MMR protein loss were defined as MMR protein-deficient (MMR-D), and the remaining tumors were classed as MMR protein-intact (MMR-I). According to KRAS mutation and MSI status, CRC was classified into 4 groups: Group 1, KRAS-mutated and MMR-I; Group 2, KRAS-mutated and MMR-D; Group 3, KRAS wild and MMR-I; and Group 4, KRAS wild and MMR-D. We found that patients in Group4 had the best prognosis. In conclusion, combination status of KRAS and MSI status may be used as a prognostic biomarker for CRC patient, if validated by larger studies.

Abbreviations: CRC = colorectal cancer; KRAS = Kristen rat sarcoma viral oncogene homolog; MMR = mismatch repair gene; MSI = microsatellite instability; OS = overall survival; PFS = progression-free survival.

Keywords: clinicopathological features, colorectal cancer, CRC, KRAS, MSI, prognosis

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide.[1] Although the incidence of CRC has historically been lower in China than in Western countries, it has increased rapidly in recent years.[2] Consequently, due to the increasing incidence of CRC, it is important to identify the factors contributing to CRC susceptibility and progression in China.

In metastatic colorectal cancer (mCRC), most therapeutic regimens are based on 5-fluorouracil. Moreover, the emergence of anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (mAbs) has brought great achievement to mCRC treatment.[3,4] The Kristen rat sarcoma viral oncogene homolog (KRAS) mutation is the most common somatic mutation in CRC and is predictive of resistance to anti-EGFR antibodies in mCRC. However, the status of KRAS in CRC as a prognostic factor remains controversial. Hutchins et al[5] showed that tumor recurrence was high likely to occur in stage II CRC patients with mutant-KRAS MT. Moreover, chemotherapy could reduce the risk of recurrence and metastasis. A similar study has been previously published with regard to Chinese patients.[6] It was showed that there were no differences in survival between stage II and III KRAS mutant-type (KRAS MT) and KRAS wild-type (KRAS WT) CRC patients who received postoperative adjuvant chemotherapy. However, for those did not receive chemotherapy, Mutant KRAS had a negative impact on overall survival (OS).[6] In addition, several studies showed that KRAS status was not an independent prognostic factor for CRC.[7–10] Thus, the association of KRAS with CRC prognosis needs to be further investigated.

Microsatellite instability (MSI), a mismatch repair (MMR) system defect, accounts for approximately 15% of CRC cases, of which 12% are sporadic colorectal cancer cases, and the other 3% are hereditary nonpolyposis CRC.[11] According to MMR protein expression, tumors are classified as MMR protein-deficient (MMR-D) and MMR protein-intact (MMR-I).[12] It was previously reported that MMR-D tumors tended to occur in

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females, proximal location, and were poorly differentiated of the mucinous phenotype with marked peritumoral and intratumoral lymphocytic infiltration.[13] The association of KRAS and MSI with prognosis has been previously studied in CRC, and it was demonstrated that KRAS WT and microsatellite stable were negative predictors for disease-specific survival.[14,15]

However, development of a predictor using a combination of biomarker has the potential to identify distinct tumor subtypes with varying prognosis. Most studies focusing on KRAS and MSI were performed in Western countries. Although some studies were conducted in the Chinese population, little is known about the clinical value of KRAS in combination with MSI.

The aim of this study is to determine the clinical relevance of KRAS MT and positive MMR proteins, alone or in combination, in 551 patients in the Chinese population.

2. Materials

2.1. Patients and tissues

We obtained 551 CRC tissue samples by surgical resection from patients in the Affiliated Drum Tower Hospital of Nanjing University Medical School between 2013 and 2015. This study was approved by the Medical Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. Patients were staged based on the pTNM staging system of the 7th edition American Joint Committee on Cancer staging. All patients in this study received surgery. Patients with high-risk stage II and III CRC tumors received adjuvant chemotherapy, and those with rectal cancer received radiotherapy after surgery. High-risk stage II was defined as T4 lesion, poorly or mucinous-differentiated histology, bowel obstruction, or lymphovascular invasion. All CRC patients with stage IV received chemotherapy. There were 134 patients with stage IV tumors at diagnosis or had underwent recurrence and/or metastasis during their follow-up period, 34 of whom with wild-type KRAS received EGFR inhibitor, including cetuximab and nimotuzumab.

Principal inclusion criteria were colon or rectal cancer with surgical procedures including radical resection or a palliative surgical conducted at our hospital; no preoperative therapy; pathologically confirmed malignances; and with available clinical information. Criteria for exclusion were tumors located in the appendix and anal canal; genital tumor; second primary cancers out of colorectal; pathologically confirmed squamous cell carcinoma, melanoma, and gastrointestinal stromal tumor; in situ carcinoma (high-grade intraepithelial neoplasia); and patients who did not received normative treatment after operation.

OS was defined as the interval between surgery and death date. Progression-free survival (PFS) was defined as the interval between the surgery and date of tumor progression, including tumor increased >30%, reoccurrence, new metastasis, or patient death. The postoperative period was measured from the surgery date to the time of the last follow-up or death. Patients were followed up postoperatively every 6 months for 2 years, and then annually for 3 to 5 years. At the end of the study period (January 2016), the median follow-up time for all patients was 14 months, ranging from 1 to 35 months. Follow-up data were retrieved from medical records and confirmed by direct interviews with patients' physicians or family members. Follow-up investigations included clinical examination, routine blood chemistry, serum carcinoembryonic antigen screening, annual colonoscopy, chest radiography, and abdominopelvic and chest computed tomography.

2.2. KRAS mutation analysis

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue of all patients. Five to ten sections of 4 μm in thickness were cut from FFPE tissue. Then FFPE tissue was used to extract DNA with a "Recover All" total nucleic acid isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. A negative control (without sample) was performed to exclude the possibility of contamination during extraction. The mutations in KRAS in the tumor samples were evaluated by a multiplex allele-specific polymerase chain reaction-based assay (ACCB, Beijing, China), together with the Stratagene Mx3000P (Agilent Technologies Inc, Santa Clara, CA), which assesses 7 different potential mutations in KRAS codons 12 and 13 (i.e., G12A, G12C, G12D, G12S, G12V, G13A, G13C, and G13D).

2.3. Tumor MMR protein expression detected by IHC

Immunohistochemical analysis of 4 MMR (i.e., MSH2, MSH6, PMS2, and MLH1) proteins was carried out on FFPE tumor samples. After the tumor area adjacent to normal mucosa and/or lymphocytic infiltration was marked, 4 mm paraffinized tissue was removed and the multiple tissue blocks were prepared. Four-micron-thick sections were obtained for immunohistochemistry (IHC). Immunostaining was done with the use of standard protocols. The mouse mAbs used were anti-MSH2, anti-MSH6, anti-MLH1, and anti-PMS2 (BD, New Jersey, USA). Adjacent normal tissues from each sample served as positive controls. MMR protein loss was defined as an absence of nuclear staining in tumor cells but positive nuclear staining in normal colonic epithelial cells and lymphocytes. The tumor was defined as MMR-D when any one of the MMR protein was negatively expressed.

2.4. Subtype classifications

Tumor subtypes were defined according to the KRAS mutation and MSI status as follows: Group 1, KRAS-mutant and MMR-I;

Table 1

Clinicopathological information of the studied patients.

Clinicopathological features	N	%
No. of patients	551	
Gender		
Male	341	61.9
Female	210	38.1
Age, median (range)	64 (22–91)	
≤60	221	40.1
>60	330	59.9
Histology		
Mucinous	54	9.8
Nonmucinous	497	90.2
Grade		
G1	29	5.3
G2	405	73.5
G3 + G4	117	21.2
Primary tumor site		
Proximal colon	173	31.4
Distal colon	158	28.7
Rectum	220	39.9
TNM stage		
I	48	8.7
II	211	38.3
III	247	44.8
IV	45	8.2

Group 2, KRAS-mutant and MMR-D; Group 3, KRAS-wild and MMR-I; and Group 4, KRAS-wild and MMR-D.

2.5. Statistical analysis

All statistical calculations were performed using SPSS software version 21.0. Differences in the clinical pathological characters among different tumor subtypes were performed by Fisher exact test or Pearson χ^2 test. PFS and OS were compared among different tumor subtypes using the Kaplan–Meier method with a log-rank test. Univariate Cox-proportional hazard models were used to explore associations of patient characteristics and biomarkers with PFS and OS. Thereafter, multivariate Cox-regression models were utilized and unless otherwise specified, all models were adjusted for KRAS status, MMR status, tumor grade, and TNM stage. In addition, 95% confidence intervals [CIs] are provided for all hazard ratios (HRs). Differences were taken as significant when a 2-tailed $P < 0.05$ was achieved.

3. Results

3.1. Clinical characteristics of the participants

This study recruited 551 CRC patients, among which, 341 cases (61.9%) were male. The median age of the included patients was 64.0 years (22–91 years). Of all the patients, there were 173 cases (31.4%) with tumors located in proximal colon cancer, 158 cases (28.7%) with tumors located in distal colon cancer, and 220 (39.9%) rectal cancer patients. Additionally, 259 cases (47.0%) were staged as I and II, and 292 cases (53.0%) were staged as III and IV. Clinicopathological features are shown in Table 1.

3.2. Frequencies of KRAS mutations and MMR status in CRC, and its association with the clinicopathological features of CRC

Mutations in KRAS codon 12 or KRAS codon 13 were detected in 190 (34.5%) and 58 (10.5%) of the patients, respectively. Four

Table 2

Association of KRAS and MMR status with clinicopathological features.

Clinicopathological features	KRAS MT	KRAS WT	*P*	MMR-D	MMR-I	*P*
Tumor location						
Proximal colon	83	90	0.798	18	114	0.017
Distal colon	72	86		6	107	
Rectum	93	127		6	150	
Gender						
Male	141	200	0.010	16	226	0.442
Female	107	103		14	145	
Age	64 (22–91)	63 (26–87)		55 (33–86)	64 (22–91)	
≤60	101	120	0.794	18	139	0.019
>60	147	183		12	232	
Histological type						
Mucinous	30	24	0.114	6	28	0.032
Nonmucinous	218	279		24	343	
Tumor size, cm						
≤4.5	133	173	0.439	13	216	0.127
>4.5	115	130		17	155	
Tumor grade						
G1	17	12	0.270	3	18	0.354
G2	169	236		22	277	
G3 + G4	62	55		5	76	
T stage						
T1	10	3	0.230	1	8	0.863
T2	21	36		2	42	
T3	183	238		25	278	
T4	34	26		2	43	
N stage						
N0	115	148	0.710	23	175	0.003
N1	91	104		5	139	
N2	42	51		2	57	
Synchronous metastasis						
No	227	279	0.816	29	337	0. 227
Yes	21	24		1	34	
TNM stage						
I	20	28	0.353	3	34	0.005
II	91	120		20	137	
III	116	131		6	166	
IV	21	24		1	34	
Nerve invasion						
Negatively	126	168	0.303	18	199	0.570
Positively	122	135		12	172	

KRAS = Kristen rat sarcoma viral oncogene homolog, KRAS MT = KRAS mutant-type, KRAS WT = KRAS wild-type, MMR = mismatch repair gene, MMR-D = MMR protein-deficient, MMR-I = MMR protein-intact.

Table 3

Association of combinational status of KRAS and MMR status with clinicopathological features.

Clinicopathological features	Group 1 (KRAS MT, MMR-I)	Group 2 (KRAS MT, MMR-D)	Group 3 (KRAS WT, MMR-I)	Group 4 (KRAS WT, MMR-D)	*P*
Gender					
Male	100 (54.9%)	3 (50%)	126 (66.7%)	13 (54.2%)	0.110
Female	82 (45.1%)	3 (50%)	63 (33.3%)	11 (45.8%)	
Age	53 (35–86)	65 (22–91)	55 (33–81)	63 (26–87)	
≤60	65 (35.7%)	4 (66.7%)	74 (39.2%)	14 (58.3%)	0.089
>60	117 (64.3%)	2 (33.3%)	115 (60.8%)	10 (41.7%)	
Histological type					
Mucinous	16 (8.8%)	2 (33.3%)	12 (6.3%)	4 (16.7%)	0.046
Nonmucinous	166 (91.2%)	4 (67.7%)	177 (93.7%)	20 (83.3%)	
Tumor size, cm					
≤4.5	103 (56.6%)	3 (50.0%)	113 (59.8%)	10 (41.7%)	0.386
>4.5	79 (43.4%)	3 (50.0%)	76 (40.2%)	14 (58.3%)	
Tumor grade					
G1	11 (6.0%)	1 (16.7%)	7 (3.7%)	2 (8.3%)	0.765
G2	130 (71.4%)	4 (66.7%)	147 (77.8%)	18 (75.0%)	
G3 + G3	41 (22.6%)	1 (16.7%)	35 (18.5%)	4 (16.7%)	
T stage					
T1 + T2	25 (13.7%)	1 (16.7%)	25 (13.2%)	2 (8.3%)	0.896
T3 + T4	157 (86.3%)	5 (83.3%)	164 (86.8%)	22 (91.7%)	
Lymph node metastasis					
Negatively	85 (46.7%)	4 (66.7%)	90 (47.6%)	19 (79.2%)	0.019
Positively	97 (53.3%)	2 (33.3%)	99 (52.4%)	5 (20.8%)	
Synchronous metastasis					
No	165 (90.7%)	6 (100%)	172 (91.0%)	23 (95.8%)	0.738
Yes	17 (9.3%)	0 (0%)	17 (9.0%)	1 (4.2%)	
TNM stage					
I + II	81 (44.5%)	4 (67.7%)	90 (47.6%)	19 (79.2%)	0.004
III + IV	101 (54.5%)	2 (33.3%)	99 (52.4%)	5 (20.8%)	
Nerve invasion					
Negatively	94 (51.6%)	4 (66.7%)	105 (55.6%)	14 (58.3%)	0.763
Positively	88 (48.4%)	2 (33.3%)	84 (44.4%)	10 (41.7%)	

KRAS = Kristen rat sarcoma viral oncogene homolog, KRAS MT = KRAS mutant-type, KRAS WT = KRAS wild-type, MMR = mismatch repair gene, MMR-D = MMR protein-deficient, MMR-I = MMR protein-intact.

hundred and one tumors were randomly detected for MMR proteins expression using IHC. Of these tumors, 30 (7.5%) had at least 1 MMR protein lost. We analyzed the correlations of KRAS and MMR status, alone, or in combination, with the clinicopathological features of CRC. There were no significant differences in the frequency of KRAS status among the proximal colon, distal colon, and rectal cancer. In addition, MMR-D type tumors tended to locate in the proximal colon, in comparison with the MMR-I type tumor. Compared with wild-type KRAS, mutant KRAS status was more commonly detected in female. No significant association was observed between KRAS status and age, histology type, tumor size, tumor grade, T stage, lymph node metastases, synchronous metastasis, TNM stage, and nerve invasion (Table 2). Moreover, we found that compared with MMR-I, MMR-D was more likely to occur in young patients, mucinous type tumors, patients with less lymph nodes invasion,

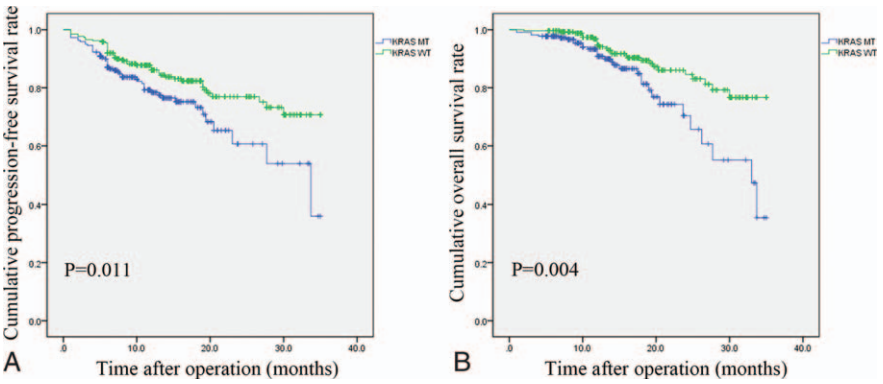

Figure 1. Progression-free survival (A) and overall survival (B) based on KRAS status in patients with colorectal cancer. KRAS = Kristen rat sarcoma viral oncogene homolog.

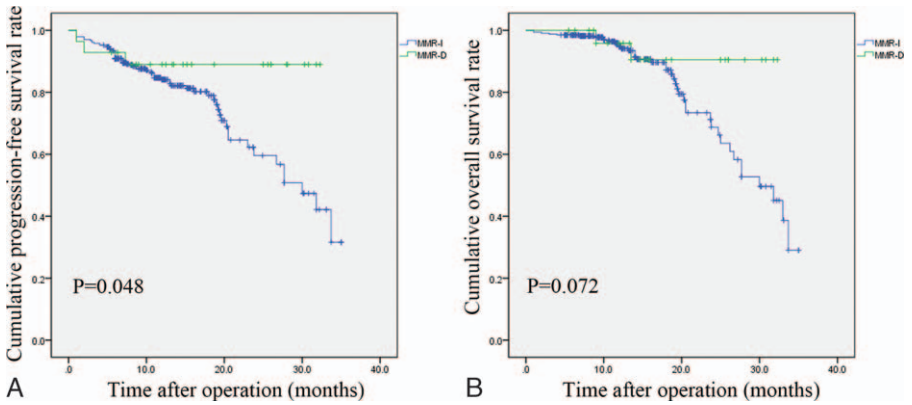

Figure 2. Progression-free survival (A) and overall survival (B) based on MMR proteins expression in patients with colorectal cancer. MMR = mismatch repair gene.

and early stage tumors (Table 2). There were also significant differences in the clinical characteristics distribution among the patients giving consideration to both MMR and KRAS status (Table 3).

3.3. Survival analysis

We found that there were 58 deaths (10.5%) in this study. As compared with KRAS WT cancers, mutations in KRAS were associated with a worse PFS and OS. The mean PFS of KRAS mutant type and KRAS wild-type CRC patients were 25.3 and 29.0 months, respectively (log-rank test, $P=0.011$). The mean OS of KRAS mutant type and KRAS wild-type CRC patients were 27.6 and 31.4 months, respectively (log-rank test, $P=0.004$; Fig. 1). Patients with MMR-D had longer PFS than those with MMR-I (log-rank test, $P=0.048$), though the difference did not achieve a statistical significant in OS (Fig. 2).

In multivariate analysis, KRAS wild-type patients had significantly lower risk of tumor recurrence (HR = 0.753, 95% CI: 0.607–0.935; $P=0.010$) and death (HR = 0.639, 95% CI: 0.482–0.847; $P=0.002$) as compared with KRAS WT tumors patients, after adjustment for the prognostic influence of tumor grade, the depth of the tumor, and TNM stage (Table 4).

The combination of KRAS and MSI status provided a classification of the patients into 4 different groups, Group 4 KRAS wild-type and MMR-D patients had the longest OS, while Group 2 KRAS mutant and MMR-D patients had the shortest OS. For PFS, there was no statistically significant among the 4 groups, which may be caused by the low frequency of MSI in CRC and the short follow-up time (Fig. 3).

4. Discussion

The incidence and mortality of CRC in China is increasing, whereas in Western countries, the trend for this condition is decreasing. The pathogenic mechanism of CRC differs between China and Western counties. Western studies have previously shown that KRAS and MSI were prognosis biomarkers for CRC.[6,8–10,16,17] Many studies also showed that there was a significant association of KRAS or MSI with the clinicopathological features in Chinese CRC patients. However, few Chinese studied had paid close attention on the prognostic relevance of the 2 markers either alone or in combination.

Overall, our study showed that 45.0% CRC patients had mutations in KRAS, which were similar to reports in other cohorts of Chinese patients,[18–20] but the mutant frequency in present study was higher than found in Western countries.[21,22] For MSI status, only 7.5% of the tumors were MMR-D, which was lower than the proportion in other Chinese studies.[23,24] Testing techniques used in previous studies might have contributed to this difference. Our findings suggest that compared with KRAS WT tumors, KRAS MT tumors are more likely to be found in male subjects ($P=0.010$), observations which are in consistent with previously published studies.[25,26]

To our best knowledge, this is the first study that has assessed the clinical relevance of combinational status of KRAS and MSI in Chinese CRC patients. There was no significant difference in the histological type between KRAS MT and KRAS WT patients. However, KRAS WT CRC patients with MMR-I phenotype had the lowest incidence of mucinous tumors ($P=0.046$). MMR-I tumors tend to have higher lymph node metastasis and a later TNM stage ($P=0.019$ and $P=0.012$). Moreover, in line with

Table 4

Multivariate analysis of PFS and OS.

Characteristics	PFS			OS		
	HR	95% CI	P	HR	95% CI	P
KRAS, KRAS MT vs KRAS WT	0.753	0.607–0.935	0.010	0.639	0.482–0.847	0.002
MMR, MMR-I vs MMR-D	1.049	0.840–1.310	0.672	0.817	0.610–1.094	0.176
Grade, G1 vs G2 vs G3	1.518	1.017–2.266	0.041	1.428	0.827–2.469	0.201
T stage, T1 vs T2 vs T3 vs T4	2.460	1.579–3.832	<0.001	1.487	0.814–2.717	0.197
N stage, N0 vs N1 vs N2	1.340	0.928–1.935	0.118	1.263	0.793–2.010	0.326
TNM stage, I vs II vs III vs IV	1.919	1.252–2.941	0.003	2.013	1.220–3.322	0.006

CI = confidence interval, HR = hazard ratio, KRAS = Kristen rat sarcoma viral oncogene homolog, KRAS MT = KRAS mutant-type, KRAS WT = KRAS wild-type, MMR = mismatch repair gene, MMR-D = MMR protein-deficient, MMR-I = MMR protein-intact, OS = overall survival, PFS = progression-free survival.

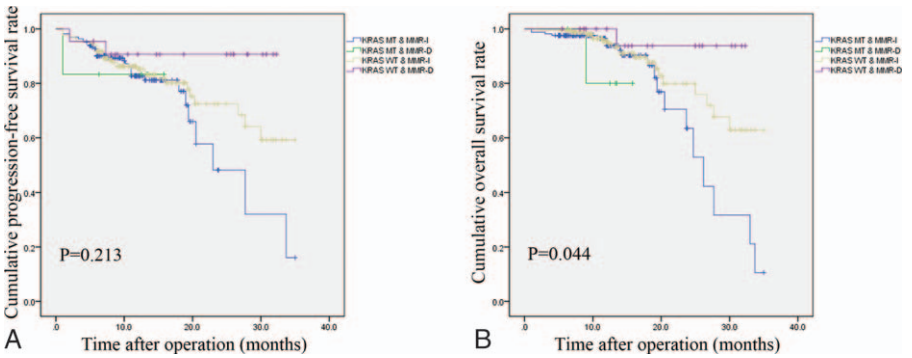

Figure 3. Progression-free survival (A) and overall survival (B) based on the combinational status of KRAS and MSI. KRAS = Kristen rat sarcoma viral oncogene homolog, MSI = microsatellite instability.

previous studies, mutant KRAS is a negative predictor for PFS and OS.[7,27]

The mechanism responsible for the results might be that KRAS is an oncogene that could drive metabolic reprogramming in CRC,[28] and KRAS mutation is associated with a risk of thromboembolism in metastatic CRC.[29] On the other hand, MMR-D was reported as a favorable prognostic marker when compared with MMR-I tumors.[16,30] Thus, theoretically, according to these evidences, it is likely that patients with KRAS WT and MMR-D tumors would have the longest OS and those with KRAS MT and MMR-I would have the shortest OS. If fact, our results showed that Group 4 (KRAS WT and MMR-D) had the best OS, whereas Group 2 (KRAS MT and MMR-D) had the worst OS. Moreover, combinational status of KRAS and MSI did not have the predictive ability for PFS. The major reason for our results might due to the short follow-up time. Alternatively, it is possible that there was a lower frequency of MMR-D, which led to bias of this study. In present study, there were 134 patients in stage IV tumors at diagnosis or had underwent recurrence and/or metastasis during their follow-up period, 34 of whom with wild-type KRAS received chemotherapy in combination with EGFR inhibitor, including 28 cases in Group 3 and 6 cases in Group 4. However, although anti-EGFR treatment prolonged the PFS and OS when compared with chemotherapy alone in Group 3 and Group 4 (data not shown), there was no significant difference in PFS and OS between patients who received EGFR inhibitor in Group 3 and Group 4, indicating that MSI status was not a predictor for treatment efficiency of EGFR inhibitor.

The main limitation of the present study was its retrospective design in nature and the short follow-up time, both of which may lead to bias. In addition, all CRC patients in this study were recruited from a single hospital, which may not be representative of the general population.

In summary, our data indicate the utility of KRAS MT in combination with MSI as prognostic factors in CRC Chinese patients. CRC patients with KRAS WT and MMR-D tumors in Chinese individuals might have a good prognosis. Larger studies are needed to validate our findings.

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