Upregulation of c-mesenchymal epithelial transition expression and *RAS* mutations are associated with late lung metastasis and poor prognosis in colorectal carcinoma

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Received September 28, 2017; Accepted January 26, 2018

DOI: 10.3892/etm.2018.5966

Abstract. The present study aimed to investigate whether c-mesenchymal epithelial transition factor (C-MET) overexpression combined with *RAS* (including *KRAS, NRAS* and *HRAS*) or *BRAF* mutations were associated with late distant metastases and the prognosis of patients with colorectal cancer (CRC). A total of 374 patients with stage III CRC were classified into 4 groups based on *RAS*/*BRAF* and C-MET status for comprehensive analysis. Mutations in *RAS*/*BRAF* were determined using Sanger sequencing and C-MET expression was examined using immunohistochemistry. The associations between *RAS*/*BRAF* mutations in combination with C-MET overexpression and clinicopathological variables including survival were evaluated. In addition, their predictive value for late distant metastases were statistically analyzed via logistic regression and receiver operating characteristic analysis. Among 374 patients, mutations in *KRAS, NRAS, HRAS, BRAF* and C-MET overexpression were observed in 43.9, 2.4, 0.3, 5.9 and 71.9% of cases, respectively. Considering *RAS*/*BRAF* mutations and C-MET overexpression, vascular invasion (P=0.001), high carcino-embryonic antigen level (P=0.031) and late distant metastases (P<0.001) were more likely to occur in patients of group 4. Furthermore, survival analyses revealed *RAS*/*BRAF* mutations may have a more powerful impact on survival than C-MET overexpression, although they were both predictive factors for adverse prognosis. Further logistic regression suggested that *RAS*/*BRAF* mutations and C-MET overexpression may predict late distant metastases. In conclusion, *RAS*/*BRAF* mutations and C-MET overexpression may serve as predictive indicators for metastatic behavior and poor prognosis of CRC.

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Key words: colorectal cancer, RAS, c-mesenchymal epithelial transition factor, prognosis, metastasis

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth most frequent cause of cancer-associated mortality worldwide (1). It has recently been indicated that late distant metastases are common in CRC, particularly liver and lung metastases, which accounted for ~40% of all advanced patients (2). Although notable advances have been made in comprehensive therapy, the prognosis of metastatic CRC remains unfavorable (3). As the understanding of molecular mechanisms underlying tumorigenesis and progression of CRC develops, targeted therapy has already become a popular alternative to other, currently used treatments, representing a significant landmark in devising individualized treatment regimens.

It is known that epidermal growth factor receptor (EGFR) is an important molecular target in metastatic CRC (mCRC) (4). Furthermore, the success of cetuximab or panitumumab, agents that target EGFR, created a new milestone in precision medicine for mCRC (5). However, mutations of *RAS* genes (including *KRAS*, *NRAS* and *HRAS*) or *BRAF* may induce constitutive activation of downstream signaling pathways, independent of *EGFR* inhibition, which is associated with tumor proliferation and diffusion. Recent data (4) has demonstrated that *KRAS* exons 2, 3 and 4; *NRAS* exons 2 and 3; *HRAS* exon 2; and *BRAF* exon 15 occurs in ~50% of CRC patients, and exhibits facilitated neoplastic transformation *in vitro* of colorectal cells as well as resistance to anti-*EGFR* therapy (6). Therefore, screening of gene mutation profiling is important for appropriate therapeutic options and regular surveillance. Notably, the predictive and prognostic significance of *RAS*/*BRAF* mutations in CRC remains controversial. A recent retrospective study (7) indicated that distant metastasis was more likely to occur in patients with *KRAS* or *BRAF* mutation. In addition, Morris *et al* (8), previously demonstrated a trend toward lung metastasis and low survival for *RAS*/*BRAF*-mutant CRC. Conversely, certain studies have not demonstrated that

mutations in *RAS*/*BRAF* were independent prognostic factors for CRC (9,10). Therefore, the association of *RAS*/*BRAF* status with late distant metastases and prognosis of CRC requires further investigation.

The c-mesenchymal epithelial transition factor (C-MET), a tyrosine kinase receptor for hepatocyte growth factor, is associated with diverse biological functions ranging from embryogenesis to wound healing (11). However, aberrant C-MET expression is closely correlated with tumor progression and metastasis via regulating cell proliferation, scattering and apoptosis (12). It is well known that *C-MET* gene is upregulated in a variety of human malignancies, including CRC (11). Recently, Lorenzon *et al* (13), reported that in *KRAS* wild-type patients with CRC, high C-MET expression appeared as a negative predictor for disease-specific survival and may interfere with anti-*EGFR* strategies, although the patient cohort analyzed in the research was small.

Currently, use of a combination of biomarkers as a better predictor of metastasis and prognosis in patients with CRC has attracted more attention due to the potential of identifying distinct tumor subtypes bearing different prognoses. However, the clinicopathological relevance of *RAS*/*BRAF* mutations combined with high C-MET expression in CRC is yet to be fully elucidated. The majority of studies focused on western populations (8,11-13) and, with few deriving data from Chinese patients (10). To improve the current knowledge, the present study comprehensively characterized *RAS*/*BRAF* mutations and C-MET overexpression in stage III CRC, alone and in combination, to provide an insight into the association between gene abnormalities and patient survival in Chinese populations.

Materials and methods

Patients and follow-up. The observational model was developed in 374 stage III CRC samples (204 males and 170 females; age range, 23-92 years old) and corresponding non-cancerous tissues from patients who had undergone surgical resection at the department of gastrointestinal surgery of Guangdong General Hospital (Guangzhou, China) between January 2010 and October 2015. The inclusion criteria were as follows: All patients had to have undergone complete lesion removal, without having received any prior anticancer therapy. Patients were also required to have normal renal and hepatic function test results. Patients were excluded from the present study if they exhibited inflammatory bowel disease. All patients were classified into 4 groups: Group 1, *RAS*/*BRAF*-wild without C-MET overexpression; group 2, *RAS*/*BRAF*-wild with C-MET overexpression; group 3, *RAS*/*BRAF*-mutant without C-MET overexpression; and group 4, *RAS*/*BRAF*-mutant with C-MET overexpression. Genetic testing was performed as a part of integrated care and information on clinicopathological data were obtained from medical archives. Tumor grading was based on the American Joint Committee on Cancer TNM classification and pathological classification was in line with the World Health Organization criteria (14,15). Overall survival (OS) or disease-free survival (DFS) was calculated from the surgery of the primary CRC until death/censoring or local recurrence/late distant metastasis/censoring, respectively. Late distant metastasis was defined as metastasis that occurred during follow-up. Of the 374 participants, 272 (72.7%) received 5-fluorouracil (5-FU)-based postoperative adjuvant chemotherapy. An outpatient follow-up was conducted every 3 months in accordance with Response Evaluation Criteria in Solid Tumors 1.1 (16) during the initial 2 years following clinical treatments and subsequently every 6 months, until the end of a 3 year follow-up or mortality. Written, informed consent was obtained from all individual participants and the protocol was approved by the Ethics Committee of Guangdong General Hospital.

Tissue sampling and mutation assessment. Comprehensive genomic profiling was analyzed in 374 resected CRC tissue samples, which were fixed with 10% formalin overnight at room temperature and embedded in paraffin wax. Tissues were then sliced longitudinally to a thickness of 4 μm. Genomic DNA was isolated from each FFPE specimen using a QIAamp DNA FFPE Tissue Kit 56404 (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. In addition, cancer cell-rich regions were identified prior to sample DNA isolation via application of hematoxylin and eosin (HE) staining to ascertain that all cases exhibited enrichment of ≥70% malignant cells. HE staining was performed according to manufacturers' instructions. Following washing with xylene and dehydration with ethanol, the sections were rehydrated in distilled water and then stained with the alum haematoxylin (Shanghai XIBAO Biology Co., Ltd., Shanghai, China) for 13 min at room temperature. After rinsing under running tap water, slides were differentiated with 0.3% acid alcohol for 5 min and washed in running tap water for 10 sec. Next, the tissue sections were stained with eosin (Shanghai XIBAO Biology Co., Ltd.) for 1 min at room temperature, dehydrated and mounted in crystal mount. Staining was analyzed by two independent observers under an optical microscope (magnification, x400; CX31; Olympus Corporation, Tokyo, Japan). Ultimately, extracted DNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

Each tumor specimen was examined for *KRAS* exon 2, 3 and 4; *NRAS* exon 2 and 3; *HRAS* exon 2; and *BRAF* exon 15 (codon 600). AmpliSeq Designer v.1.2.6 software (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to design primer pairs for PCR amplification of each gene region of interest (17). DNA was amplified using GoTaq Hot Start Polymerase (Promega Corporation, Madison, WI, USA) and 0.2 μM each primer on the GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cycling conditions were as previously described (18). Amplicons were finally Sanger sequenced bidirectionally on an ABI 3730XL genetic analyzer (Invitrogen; Thermo Fisher Scientific, Inc.). Primers and procedures were the same as previously reported (19).

Immunohistochemical (IHC) analysis of C-MET protein expression. Immunohistochemistry was performed as described previously (11). Briefly, slides were dewaxed, rehydrated and antigens were retrieved with EDTA (pH 8) by microwave heating at 95˚C. Following the inhibition of

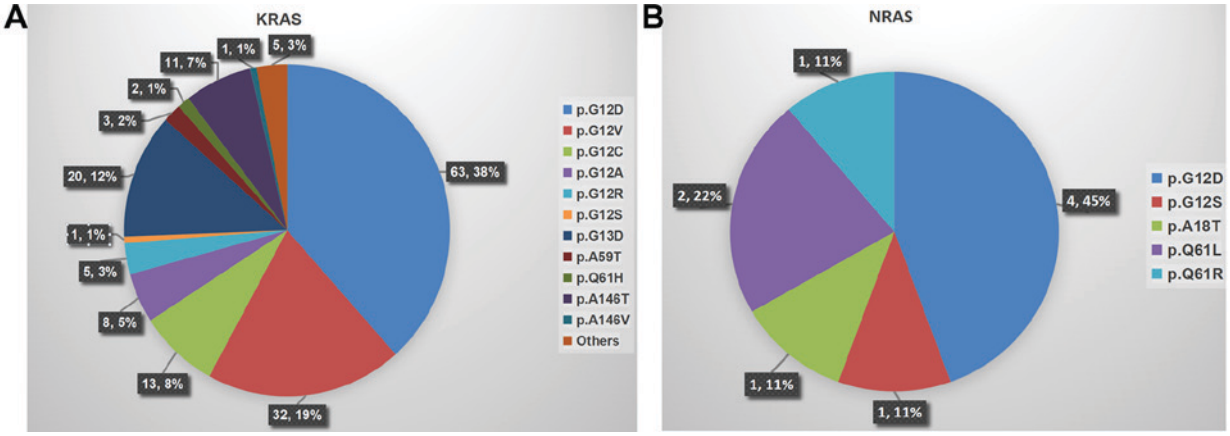

Figure 1. Mutation subtypes frequency distribution of (A) *KRAS* and (B) *NRAS*.

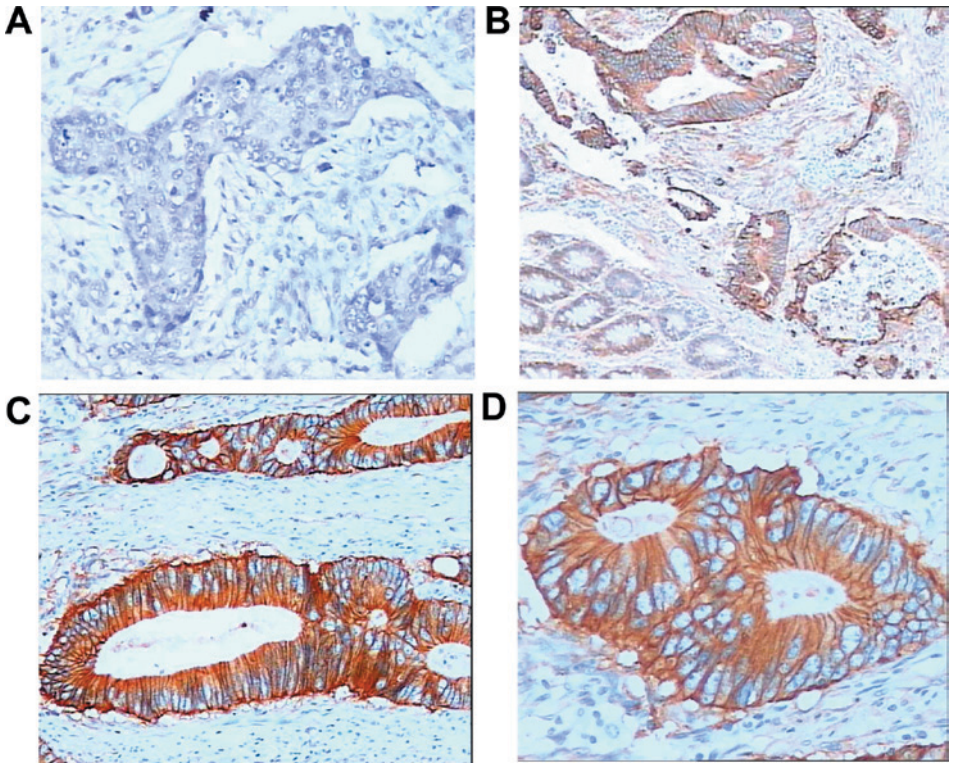

Figure 2. Analysis of C-MET expression by immunohistochemistry in colorectal carcinomas. C-MET expression was localized in the membrane and its expression was observed predominantly in cancer cells. (A) Negative C-MET staining in a cancerous tissue sample (magnification, x100). (B) Positive C-MET staining in tumor cells (upper), with negative or weak staining in adjacent epithelial cells (lower) (magnification, x100). (C) Strong C-MET staining in tumor nests (magnification, x100). (D) Positive membrane staining, as observed in the majority of tumor cells (magnification, x200). C-MET, c-mesenchymal epithelial transition factor.

endogenous peroxidase activity and blocking non-specific antibody binding, sections were incubated with lyophilized primary antibody against C-MET (1:100; EP1454Y; BD Biosciences, Franklin Lakes, NJ) overnight at 4˚C. Following a 30-min incubation at room temperature with secondary antibodies (cat. no. sc-3699; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), immunoreaction was visualized using the streptavidin-biotin peroxidase complex method. Subsequently, slides were examined under an optical microscope (magnification, x400, CX31; Olympus Corporation). C-MET staining was assessed according to Hercep Test guidelines (20) as follows: 0, no membrane staining or membrane staining in <10% of tumor cells; 1+, faint membrane staining; 2+, moderate and smooth membrane staining; 3+, strong and granular membrane staining in ≥10% of tumor cells. C-MET overexpression was defined as IHC 2+/3+. The results were judged by two independent pathologists.

Statistical analysis. Data analysis was performed using SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). Pearson's Chi-square (χ^2) test was used to compare the correlation between RAS/BRAF mutations and clinicopathological variables. Kruskal-Wallis test or Mann Whitney U test were performed to compare treatment response. Survival curves

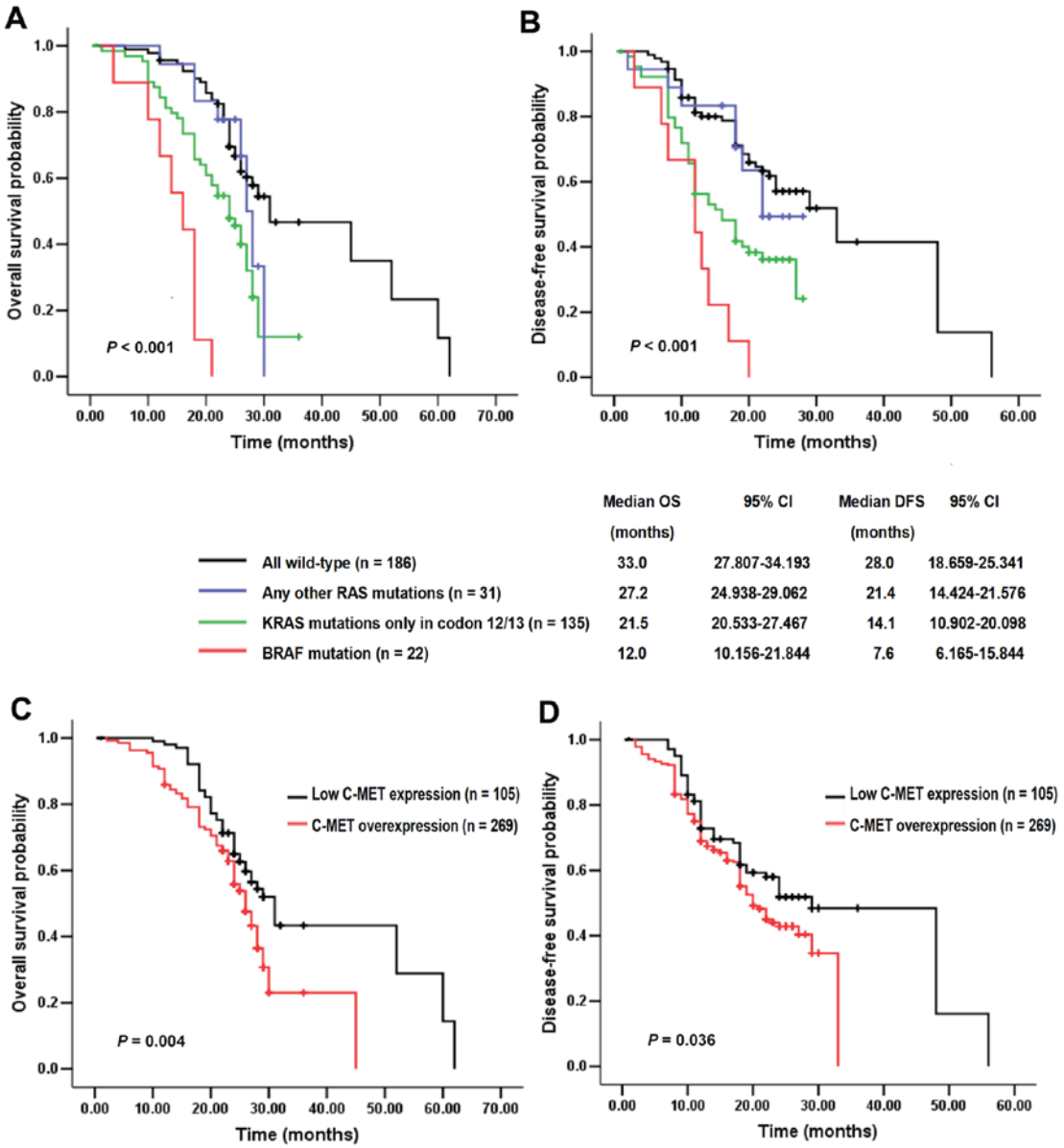

Figure 3. Kaplan-Meier survival curves of patients with stage III colorectal carcinoma. (A) OS and (B) DFS in all wild-type vs. *RAS*/*BRAF* mutations. (C) OS and (D) DFS in low C-MET expression vs. C-MET overexpression of entire study population. OS, overall survival; DFS, disease-free survival; C-MET, c-mesenchymal epithelial transition factor; CI, confidence interval.

of OS and DFS were plotted via Kaplan-Meier analysis with significance assessed using log-rank test. Univariate and multivariate proportional Cox models were performed to assess independent prognostic factors. Logistic regression using a backward stepwise method and receiver operating characteristic (ROC) analysis were performed to evaluate synchronous liver metastasis of patients with CRC. P<0.05 was considered to indicate a statistically significant difference.

Results

Frequencies of gene mutations and C-MET status in stage III CRC patients. Mutations in *KRAS*, *NRAS* and *HRAS* were observed in 43.9% (164/374), 2.4% (9/374) and 0.3% (1/374) of patients, respectively. In addition, as another vital component of the *EGFR* pathway, *BRAF* mutations were observed in 5.9% (22/374) cases. Mapping correlations between molecular biomarkers demonstrated that 4 patients carried concurrent *KRAS* and *NRAS* mutations (combinations were p.G12D/p.G12D, p.G12D/p.A18T and p.A146T/p.Q61L), and in another 4 patients, *KRAS* and *BRAF* mutations (combinations were all p.G12D/p.V600E) were concomitantly observed. However, no co-mutations of *NRAS* with *BRAF* were observed in the present study. Notably, the most prevalent mutation occurred in exon 2 (codons 12 and 13) of *KRAS* (38.0%, 142/374). The detailed distribution of *KRAS* and *NRAS* mutation subtypes is presented in Fig. 1A and B.

In addition, the status of C-MET protein in all stage III CRC biopsies were investigated via IHC assay (Fig. 2). It was observed that 269 (71.9%) cases exhibited C-MET overexpression (Fig. 2B-D). In paired non-tumorous specimens, C-MET staining was either absent or present in the membrane of only a few cells (Fig. 2A).

Table I. Correlation between mutation profile and clinicopathological features in 374 patients with stage III colorectal cancer.

		KRAS status			BRAF status			NRAS status					
Clinicopathological features	Patients, n	Wild-type (n=210)	Mutation (n=164)	P-value	Wild-type (n=352)	Mutation (n=22)	P-value	Wild-type (n=365)	Mutation (n=9)	P-value	All wild-type (n=186)	Any mutation (n=188)	P-value
Sex				0.470			<0.001			0.951			0.076
Male	204	118 (57.8)	86 (42.2)		200 (98.0)	4 (2.0)		199 (97.5)	5 (2.5)		110 (53.9)	94 (46.1)	
Female	170	92 (54.1)	78 (45.9)		152 (89.4)	18 (10.6)		166 (97.6)	4 (2.4)		76 (44.7)	94 (55.3)	
Age, years				0.355			0.074			0.089			0.079
<65	188	110 (58.5)	78 (41.5)		181 (96.3)	7 (3.7)		186 (98.9)	2 (1.1)		102 (54.3)	86 (45.7)	
≥65	186	100 (53.8)	86 (46.2)		171 (91.9)	15 (8.1)		179 (96.2)	7 (3.8)		84 (45.2)	102 (54.8)	
Tumor location				0.360			0.002			0.622			0.300
Left colon	166	100 (60.2)	66 (39.8)		152 (91.6)	14 (8.4)		162 (97.6)	4 (2.4)		84 (50.6)	82 (49.4)	
Right colon	46	24 (52.2)	22 (47.8)		40 (87.0)	6 (13.0)		44 (95.7)	2 (4.3)		18 (39.1)	28 (60.9)	
Rectum	162	86 (53.1)	76 (46.9)		160 (98.8)	2 (1.2)		159 (98.1)	3 (1.9)		84 (51.9)	78 (48.1)	
Differentiation				0.609			0.068			0.372			0.611
Well/Moderate	238	136 (57.1)	102 (42.9)		220 (92.4)	18 (7.6)		231 (97.1)	7 (2.9)		116 (48.7)	122 (51.3)	
Poor	136	74 (54.4)	62 (45.6)		132 (97.1)	4 (2.9)		134 (98.5)	2 (1.5)		70 (51.5)	66 (48.5)	
Depth of invasion				0.406			0.712			0.404			0.360
T1	2	0 (0.0)	2 (100.0)		2 (100.0)	0 (0.0)		2 (100.0)	0 (0.0)		0 (0.0)	2 (100.0)	
T2	24	14 (58.3)	10 (41.7)		22 (91.7)	2 (8.3)		24 (100.0)	0 (0.0)		12 (50.0)	12 (50.0)	
T3	284	158 (55.6)	126 (44.4)		266 (93.7)	18 (6.3)		275 (96.8)	9 (3.2)		138 (48.6)	146 (51.4)	
T4	64	38 (59.4)	26 (40.6)		62 (96.9)	2 (3.1)		64 (100.0)	0 (0.0)		36 (56.3)	28 (43.8)	
Nodal stage				0.299			0.941			0.489			0.126
N1	260	148(56.9)	112 (43.1)		244(93.8)	16 (6.2)		255 (98.1)	5 (1.9)		132 (50.8)	128 (49.2)	
N2a	74	44 (59.5)	30 (40.5)		70 (94.6)	4 (5.4)		72 (97.3)	2 (2.7)		40 (54.1)	34 (45.9)	
N2b	40	18 (45.0)	22 (55.0)		38 (95.0)	2 (5.0)		38 (95.0)	2 (5.0)		14 (35.0)	26 (65.0)	
Vascular invasion				<0.001			0.222			0.160			0.001
No	308	186 (60.4)	122 (39.6)		292 (94.8)	16 (5.2)		299 (97.1)	9 (2.9)		166 (53.9)	142 (46.1)	
Yes	66	24 (36.4)	42 (63.6)		60 (90.9)	6 (9.1)		66 (100.0)	0 (0.0)		20 (30.3)	46 (69.7)	
Initial CEA, ng/ml				0.613			0.350			0.757			0.950
<20	100	54 (54.0)	46 (46.0)		96 (96.0)	4 (4.0)		98 (98.0)	2 (2.0)		50 (50.0)	50 (50.0)	
≥20	274	156 (56.9)	118 (43.1)		256 (93.4)	18 (6.6)		267 (97.4)	7 (2.6)		136 (49.6)	138 (50.4)	

Table I. Continued.

Clinicopathological features	Patients, n	KRAS status			BRAF status			NRAS status					
		Wild-type (n=210)	Mutation (n=164)	P-value	Wild-type (n=352)	Mutation (n=22)	P-value	Wild-type (n=365)	Mutation (n=9)	P-value	All wild-type (n=186)	Any mutation (n=188)	P-value
Late distant metastases				0.001			0.628			0.574			0.001
No	46	36 (78.3)	10 (21.7)		44 (95.7)	2 (4.3)		46 (100.0)	0 (0.0)		34 (73.9)	12 (26.1)	
Liver	126	76 (60.3)	50 (39.7)		116 (92.1)	10 (7.9)		121 (96.0)	5 (4.0)		61 (48.4)	65 (51.6)	
Lung	68	29 (42.6)	39 (57.4)		63 (92.6)	5 (7.4)		67 (98.5)	1 (1.5)		24 (35.3)	44 (64.7)	
Abdomen	72	42 (58.3)	30 (41.7)		69 (95.8)	3 (4.2)		70 (97.2)	2 (2.8)		41 (56.9)	31 (43.1)	
Others	62	27 (43.5)	35 (56.5)		60 (96.8)	2 (3.2)		61 (98.4)	1 (1.6)		26 (41.9)	36 (58.1)	
COX-2 expression				0.080			0.180			<0.001			0.126
Negative/Weak	32	24 (75.0)	8 (25.0)		31 (96.9)	1 (3.1)		28 (87.5)	4 (12.5)		21 (65.6)	11 (34.4)	
Moderate	66	36 (54.5)	30 (45.5)		59 (89.4)	7 (10.6)		66 (100.0)	0 (0.0)		29 (43.9)	37 (56.1)	
Strong	276	150 (54.3)	126 (45.7)		262 (94.9)	14 (5.1)		271 (98.2)	5 (1.8)		136 (49.3)	140 (50.7)	
MSI				0.466			0.111			0.448			0.979
MSI-H	22	14 (63.6)	8 (36.4)		19 (86.4)	3 (13.6)		22 (100.0)	0 (0.0)		11 (50.0)	11 (50.0)	
MSI-L/MSS	352	196 (55.7)	156 (44.3)		333 (94.6)	19 (5.4)		343 (97.4)	9 (2.6)		175 (49.7)	177 (50.3)	

Data are presented as n (%), unless otherwise stated. COX-2, cyclooxygenase-2; CEA, carcinoembryonic antigen; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, stable MSI.

Table II. Correlation between C-MET overexpression and clinicopathological features in 374 patients with stage III colorectal cancer.

Clinicopathological features	Patients, n	C-MET overexpression		P-value
		No (n=105)	Yes (n=269)	
Gender				0.690
Male	204	59 (28.9)	145 (71.1)	
Female	170	46 (27.1)	124 (72.9)	
Age, years				0.610
<65	188	55 (29.3)	133 (70.7)	
≥65	186	50 (26.9)	136 (73.1)	
Tumor location				0.699
Left colon	166	50 (30.1)	116 (69.9)	
Right colon	46	13 (28.3)	33 (71.7)	
Rectum	162	42 (25.9)	120 (74.1)	
Differentiation				0.103
Well/Moderate	238	60 (25.2)	178 (74.8)	
Poor	136	45 (33.1)	91 (66.9)	
Depth of invasion				0.251
T1	2	0 (0.0)	2 (100.0)	
T2	24	6 (25.0)	18 (75.0)	
T3	284	75 (26.4)	209 (73.6)	
T4	64	24 (37.5)	40 (62.5)	
Nodal stage				0.019
N1	260	84 (32.3)	176 (67.7)	
N2a	74	15 (20.3)	59 (79.7)	
N2b	40	6 (15.0)	34 (85.0)	
Vascular invasion				0.023
No	308	94 (30.5)	214 (69.5)	
Yes	66	11 (16.7)	55 (83.3)	
Initial CEA, ng/ml				0.072
<20	100	35 (35.0)	65 (65.0)	
≥20	274	70 (25.5)	204 (74.5)	
Late distant metastases				<0.001
No	46	23 (50.0)	23 (50.0)	
Liver	126	27 (21.4)	99 (78.6)	
Lung	68	7 (10.3)	61 (89.7)	
Abdomen	72	26 (36.1)	46 (63.9)	
Others	62	22 (35.5)	40 (64.5)	
COX-2 expression				0.490
Negative/Weak	32	10 (31.2)	22 (68.8)	
Moderate	66	22 (33.3)	44 (66.7)	
Strong	276	73 (26.4)	203 (73.6)	
MSI				0.167
MSI-H	22	9 (40.9)	13 (59.1)	
MSI-L/MSS	352	96 (27.3)	256 (72.7)	

Data are presented as n (%), unless otherwise stated. C-MET, c-mesenchymal epithelial transition factor; COX-2, cyclooxygenase-2; CEA, carcinoembryonic antigen; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, stable MSI.

Associations between RAS/BRAF mutations and C-MET overexpression with clinicopathological features. The present study evaluated the correlations of *RAS/BRAF* and C-MET status, alone or in combination, with the clinicopathological characteristics in patients with stage III CRC. Briefly, *KRAS* mutations were significantly correlated with

Table III. Association of combinational status of *RAS*/*BRAF* genes and C-MET protein with clinicopathological features.

Clinicopathological features	Patients, n	Group 1 (n=62)	Group 2 (n=124)	Group 3 (n=43)	Group 4 (n=145)	P-value
Gender						0.053
Male	204	32 (51.6)	77 (62.1)	27 (62.8)	68 (46.9)	
Female	170	30 (48.4)	47 (37.9)	16 (37.2)	77 (53.1)	
Age, years						0.068
<65	188	39 (62.9)	63 (50.8)	16 (32.6)	70 (50.3)	
≥65	186	23 (37.1)	61 (49.2)	27 (67.4)	75 (49.7)	
Tumor location						0.190
Right colon	46	8 (12.9)	10 (8.1)	5 (11.6)	23 (15.9)	
Left colon/Rectum	328	54 (87.1)	114 (91.9)	38 (88.4)	112 (84.1)	
Differentiation						0.293
Well/Moderate	238	33 (53.2)	83 (66.9)	27 (62.8)	95 (65.5)	
Poor	136	29 (46.8)	41 (33.1)	16 (37.2)	50 (34.5)	
Depth of invasion						0.310
T1+T2	26	2 (3.2)	12 (9.7)	4 (9.3)	8 (5.5)	
T3+T4	348	60 (96.8)	112 (90.3)	39 (90.7)	137 (94.5)	
Nodal stage						0.054
N1	260	50 (80.6)	82 (66.1)	34 (79.1)	94 (64.8)	
N2	114	12 (19.4)	42 (33.9)	9 (20.9)	51 (35.2)	
Vascular invasion						0.001
No	308	57 (91.9)	109 (87.9)	37 (86.0)	105 (72.4)	
Yes	66	5 (8.1)	15 (12.1)	6 (14.0)	40 (27.6)	
Initial CEA (ng/ml)						0.031
<20	100	16 (25.8)	34 (27.4)	19 (44.2)	31 (21.4)	
≥20	274	46 (74.2)	90 (72.6)	24 (55.8)	114 (78.6)	
Late distant metastases						<0.001
No	46	18 (29.0)	16 (12.9)	5 (11.6)	7 (4.8)	
Yes	328	44 (71.0)	108 (87.1)	38 (88.4)	138 (95.2)	
COX-2 expression						0.657
Negative/Weak	32	4 (6.5)	12 (9.7)	2 (4.7)	14 (9.7)	
Moderate/Strong	342	58 (93.5)	112 (90.3)	41 (95.3)	131 (90.3)	
MSI						0.523
MSI-H	22	5 (8.1)	5 (4.0)	4 (9.3)	8 (5.5)	
MSI-L/MSS	352	57 (91.9)	119 (96.0)	39 (90.7)	137 (94.5)	

Group 1, *RAS*/*BRAF*-wild without C-MET overexpression; group 2, *RAS*/*BRAF*-wild with C-MET overexpression; group 3, *RAS*/*BRAF*-mutant without C-MET overexpression; and group 4, *RAS*/*BRAF*-mutant with C-MET overexpression. Data are presented as n (%), unless otherwise stated. C-MET, c-mesenchymal epithelial transition factor; COX-2, cyclooxygenase-2; CEA, carcinoembryonic antigen; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, stable MSI.

vascular invasion (P<0.001) and late distant metastasis, particularly lung metastases (P=0.001). *NRAS* mutations were more likely to exhibit low COX-2 expression (P=0.001). Furthermore, *BRAF* exhibited a higher mutation rate in female patients than males (P<0.001) and right colon than other tumor locations (P=0.002; Table I). The present study demonstrated that, compared with low C-MET expression, C-MET overexpression was more likely to occur in cases with late nodal stage (P=0.019), vascular invasion (P=0.023) and late distant metastases, particularly lung and liver metastases (P<0.001; Table II). Considering both *RAS*/*BRAF* mutations and C-MET status, there were significant differences in the clinicopathological features distribution among different groups. For patients in group 4, vascular invasion (P=0.001), high carcino-embryonic antigen level (P=0.031) and late distant metastases (P<0.001) were observed at significantly higher levels than in the other groups (Table III).

Survival analysis. By May 1, 2017, the end of follow-up period, 68.4% (256/374) of patients had succumbed.

Table IV. Univariate and multivariate analyses of OS and DFS for 374 patients.

		OS univariate analysis		OS multivariate analysis		DFS univariate analysis		DFS multivariate analysis	
Parameter	Variables	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Gender	Male vs. female	1.041 (0.701-1.545)	0.843			1.061 (0.714-1.576)	0.771		
Age, years	<65 vs. ≥65	1.258 (0.845-1.874)	0.258			1.048 (0.706-1.554)	0.817		
Tumor location	Left/right colon vs. rectum	0.911 (0.623-1.377)	0.658			1.076 (0.871-1.330)	0.496		
Differentiation	Well/moderate vs. poor	1.062 (0.702-1.605)	0.776			1.061 (0.085-1.000)	0.771		
Depth of invasion	T1+T2 vs. T3+T4	1.011 (0.818-1.250)	0.916			1.140 (0.765-1.700)	0.520		
Nodal stage	N0+N1 vs. N2a+N2b	1.042 (0.806-1.347)	0.752			1.123 (0.868-1.453)	0.377		
Vascular invasion	No vs. yes	0.982 (0.782-1.234)	0.879			0.968 (0.772-1.214)	0.779		
Initial CEA, ng/ml	<20 vs. ≥20	1.154 (0.890-1.497)	0.281			1.186 (0.916-1.536)	0.195		
Late distant metastases	No vs. yes	3.334 (2.139-5.197)	<0.001	2.678 (1.655-4.334)	<0.001	3.291 (2.092-5.178)	<0.001	2.782 (1.678-4.435)	<0.001
COX-2 expression	Negative/weak vs. moderate/strong	0.991 (0.758-1.294)	0.946			0.991 (0.759-1.293)	0.946		
MSI	MSI-H vs. MSI-L/MSS	0.713 (0.345-1.471)	0.360			0.619 (0.300-1.277)	0.194		
C-MET overexpression	No vs. yes	3.032 (1.323-6.948)	0.009	2.837 (1.103-6.053)	0.031	2.642 (1.154-6.045)	0.021	2.382 (0.892-4.753)	0.083
RAS/BRAF mutations	No vs. yes	2.459 (1.617-3.739)	<0.001	2.045 (1.276-3.279)	0.003	2.222 (1.460-3.382)	<0.001	1.976 (1.230-3.175)	0.005
Anti-EGFR therapy	No vs. yes	0.497 (0.229-1.080)	0.077			0.396 (0.182-0.864)	0.020	1.055 (0.411-2.710)	0.911

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; COX-2, cyclooxygenase-2; CEA, carcinoembryonic antigen; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stability; C-MET, c-mesenchymal epithelial transition factor; EGFR, epidermal growth factor receptor.

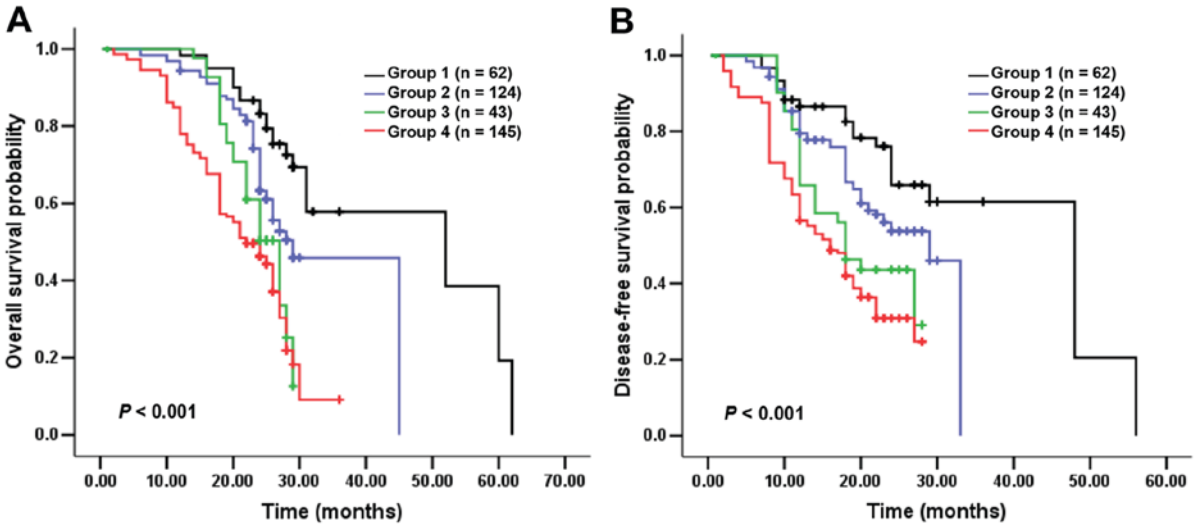

Figure 4. Kaplan-Meier survival curves of patients with colorectal carcinoma classified according to *RAS/BRAF* mutations and C-MET status. (A) OS and (B) DFS based on the combinational status of *RAS/BRAF* and C-MET. OS, overall survival; DFS, disease-free survival; C-MET, c-mesenchymal epithelial transition factor.

The median follow-up duration was 32.0 months (range, 0.6-76.3 months) and 19 (5.1%) patients were lost to follow-up. The potential influence of *RAS/BRAF* mutations and C-MET status on survival was analyzed. In the entire study cohort, it was concluded that OS and DFS for *RAS/BRAF* mutant patients, particularly those exhibiting *BRAF* mutation, were significantly reduced compared with those of cases with all wild-type. The any-other-*KRAS/NRAS*-mutated group exhibited longer median OS and DFS (27.2 and 21.4 months, respectively) than the other two mutational groups (Fig. 3A and B). As compared with C-MET low expression cancers (median OS and DFS, 38.7 and 32.3 months, respectively), C-MET overexpression cases (median OS and DFS, 26.4 and 21.2 months, respectively) were correlated with worse OS (P=0.004) and DFS (P=0.036; Fig. 3C and D). Notably, patients in Group 2 exhibited a more favorable survival than those in Group 3, indicating that tumors which harbor single *RAS/BRAF* mutations demonstrate higher malignant potential in comparison with cases carrying a single C-MET overexpression. Therefore *RAS/BRAF* mutations may have a more powerful impact on OS and DFS than elevated C-MET (Fig. 4A and B).

Furthermore, the Cox proportional hazards model was applied to estimate prognostic factors. As confirmed by multivariate analyses, *RAS/BRAF* mutations emerged as independent risk factors for OS [hazard ratio (HR), 2.045; 95% confidence interval (CI), 1.276-3.279; P=0.003)] and DFS (HR, 1.976; 95% CI, 1.230-3.175; P=0.005), whereas C-MET overexpression only exerted a significant prognostic effect on OS (HR, 2.837; 95% CI, 1.103-6.053; P=0.031; Table IV).

Predictive value of RAS/BRAF mutations and C-MET overexpression to late metastasis in patients with CRC. As distant metastasis was significantly associated with malignant progression and poor survival in patients with CRC, the potential predictors for late metastasis were investigated using unconditional logistic regression and ROC curves. Items that were verified to be statistically significant were regarded as independent variables. It was observed that *RAS/BRAF* mutations [yes=1, no=0; odds ratio (OR), 2.544; P=0.002], C-MET overexpression (yes=1, no=0; OR, 3.408; P=0.003) and depth of invasion (T3+T4=1, T1+T2=0; OR, 3.363; P<0.001) were all significantly correlated with the occurrence of late distant metastases (Table V).

The number of cases included the whole study population. With ROC curve analysis, the sensitivity and specificity of *RAS/BRAF* mutations alone, C-MET overexpression alone, depth of invasion alone, or their combination for predicting late distant metastasis among patients with CRC were evaluated. The predictive findings presented in Fig. 5, demonstrated that the combination of *RAS/BRAF* mutations, C-MET overexpression and depth of invasion [area under curve (AUC), 0.734; 95% CI, 0.672-0.797; P<0.001] exhibited a better predictive value compared with single *RAS/BRAF* mutations (AUC, 0.618; 95% CI, 0.545-0.691; P=0.003), C-MET overexpression (AUC, 0.600; 95% CI, 0.531-0.670; P=0.011) or depth of invasion (AUC, 0.628; 95% CI, 0.553-0.702; P=0.001).

Efficacy of anti-EGFR therapies. In the present study, 342 patients suffered from late distant metastasis and/or recurrence during the follow-up period, 46 of whom received cetuximab combined with first-line FOLFIRI (irinotecan/5-Fu/leucovorin) or FOLFOX6 (oxaliplatin/5-Fu/leucovorin) chemotherapy, including 1 patient in group 1, 41 in group 2 and 4 in group 4. No instances of patient complete response (CR) were observed; 1 case in group 1 and 7 cases in group 2 exhibited partial response (PR); 24 cases in group 2 exhibited stable disease (SD), whereas 4 cases in group 4 exhibited all progressive disease (PD) for the first response evaluation at 3 months. The disease control rate (including CR, PR and SD) was 69.6% (32/46). Therefore, the efficacy of anti-*EGFR* therapy in *RAS/BRAF* wild-type patients were better than that in mutant counterparts, although no statistical significance

Table V. Logistic regression analysis of factors associated with late distant metastases in patients with colorectal cancer.

Characteristics	OR	95% CI	P-value
Depth of invasion: T3+T4 vs. T1+T2	3.363	1.911-5.916	<0.001
RAS/BRAF mutations: Yes vs. no	2.544	1.402-4.613	0.002
C-MET overexpression: Yes vs. no	3.408	1.527-7.604	0.003
Constant	0.001		

CI, confidence interval; OR, odds ratio; C-MET, c-mesenchymal epithelial transition factor.

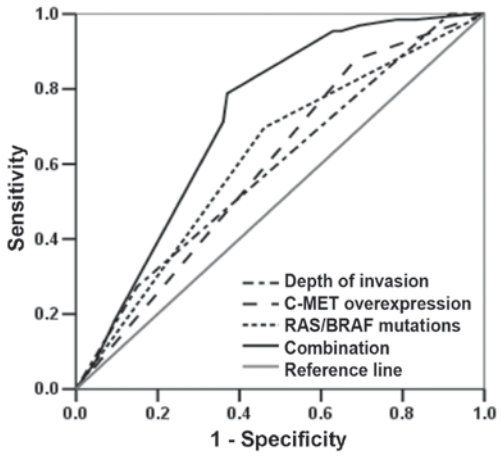

Figure 5. ROC curves for the predictive ability of *RAS*/*BRAF* mutations and C-MET overexpression to late distant metastasis. ROC, receiver operating characteristic curve; C-MET, c-mesenchymal epithelial transition factor.

was observed. However, the influence of C-MET status on anti-*EGFR* therapies were not assessed due to the low number of suitable cases.

Discussion

CRC is a clinically and pathologically heterogeneous malignancy, presenting high incidence of metastasis and a consequent poor clinical outcome on account of its invasive nature (1). Despite the complexity of carcinogenesis, a number of molecular studies have been performed in search of more specific and feasible markers with predictive and prognostic significance. As a result, multiple genes, such as vascular endothelial growth factor, cyclooxygenase-2, *PIK3CA*, protein kinase B and *ERBB2* (7,21), have been considered as biomarkers of the aggressiveness of CRC. In recent years, increasing attention has been given to extended *RAS* and C-MET status, whose abnormalities have been demonstrated to contribute to uncontrolled cell growth and malignant transformation in CRC (18,22). To the best of our knowledge, this is the first study where a combined analysis of *RAS*/*BRAF* mutations plus C-MET overexpression was performed, which clarified their clinical value in a large cohort of Chinese patients with stage III CRC.

According to the present data, mutations in *KRAS*, *NRAS*, *HRAS*, *BRAF* and C-MET overexpression were observed in 43.9% (164/374), 2.4% (9/374), 0.3% (1/374), 5.9% (22/374) and 71.9% (269/374) of cases, respectively. The prevalence of genetic abnormalities was in accordance with previous publications (7,23-26). Different from intra-tumoral heterogeneity of *KRAS* mutations and rare *NRAS* or *HRAS* mutations, *BRAF* aberrance exhibited relative intra-tumoral homogeneity. In addition, the present study also demonstrated that mutations in *RAS*/*BRAF* oncogenes were not mutually exclusive, although the findings conflicted with several reports from other populations (27-29). One likely explanation for this may be the disparity of sample sources (Chinese vs. European population). Notably, emerging studies (30,31) have observed a high concordance of *RAS*/*BRAF* mutations between primary CRCs and corresponding metastases, indicating that these genetic changes existed early in tumorigenesis, and maintained their status during development (21). However, the level of concordance for C-MET expression was controversial (22,32). Shoji *et al* (31), previously indicated that c-MET protein was more highly expressed in liver metastases than in paired primary tumors. In contrast, another study (33) revealed that C-MET expression in late metastases tended to be decreased, which supported the outcome of the present study. Therefore, more studies in ethnically-diverse populations are required.

In the present study, the association between combinational status of *RAS*/*BRAF* plus C-MET and clinicopathological features were investigated. Briefly, it was indicated that *KRAS* mutations and C-MET overexpression, or their combination, may be important indicators to identify subsets of CRC with vascular invasion and late distant metastases. Particularly, 35% of patients in the present study developed liver metastases during their disease course and >50% of cases exhibited metastases in other sites, including lung metastases. Of the cases with liver metastases, 39.7% had *KRAS* mutations and 78.6% exhibited high C-MET expression. By contrast, genetic abnormalities were more closely associated with lung metastases. In addition, *NRAS* mutations were correlated with low *COX-2* expression, suggesting the reduced aggression of tumors carrying *NRAS* mutations compared with those with other *RAS*/*BRAF* mutations. This is in accordance with previous studies (10,23). Recently, a retrospective study (34) reported that *BRAF* mutations were observed more frequently in right colon and female patients, which supported the conclusions of the present study. Numerous experimental model systems have confirmed *RAS*/*BRAF* mutations and upregulated C-MET collaboration, or their interactions, contributed to cell proliferation and the invasion-metastasis cascade, which may yield tumor aggressiveness and distant organ involvement (6,35). Furthermore, Bradley *et al* (22), recently illustrated that small interfering RNA-mediated knockdown of c-MET inhibited the migration and invasion potential of CRC cells, thereby suppressing tumor progression and metastasis *in vivo*. These outcomes indicated that genetic abnormalities are important in promoting CRC malignancy.

The initiation and development of CRC is a complex, multi-step process that is accompanied by the accumulation of diverse gene alterations (3,6). *RAS*/*BRAF* mutations are

typically the most frequent driver mutations in CRC (36), C-MET overexpression is regarded as adjuvant pro-metastatic marker, both of which represent the principle aspect of somatic genetic changes (37,38). Another focus of the present study was further exploring the predictive value of *RAS*/*BRAF* mutations and C-MET status. In one prior study (39), *KRAS* exon 2-mutated CRC patients exhibited a marked propensity for lung metastases. Similar results have also been described by Morris *et al* (8), in which all *RAS*/*BRAF* mutant cases harbored the trend towards distant metastases. The present data highlighted that *RAS*/*BRAF* mutations combined with C-MET overexpression were significant predictors for higher risk of late distant metastasis, suggesting their importance in distinguishing CRCs with highly aggressive behavior from low metastatic lesions. The results also demonstrated that these mutations provide powerful insights into the complexity of tumor foci genotype and provide a rationale for the combination therapeutic strategies. Previous studies have proposed that the block of C-MET, the HDAC inhibitor and CDK1 inhibition may markedly attenuate CRC development (40-42).

Previously, *KRAS* mutation was regarded as an adverse prognostic indicator in 1990 (43). Only in the last several years has the prognostic value of extended *RAS* mutations in CRC received more attention. Conversely, high C-MET expression has been documented to be associated with lower survival in diverse human tumors (12,32). A previous study (31) has demonstrated that C-MET overexpression indicated a poor outcome in terms of the risk of recurrence and mortality in patients with mCRC following metastasectomy. Similarly, the present data also revealed that C-MET overexpression and *RAS*/*BRAF* mutations, particularly *BRAF* mutation, were significantly associated with shorter OS and DFS in the entire study population. Notably, compared with C-MET overexpression, *RAS*/*BRAF* mutations appeared to be more powerful prognostic markers of a short interval to low survival and late metastasis following surgery. Furthermore, as the National Comprehensive Cancer Network recommends patients with mCRC and *RAS*/*BRAF* wild-type for anti-*EGFR* treatment (44), the present results also illustrated wild-type cases may gain survival benefits from cetuximab. Regarding C-MET status, Inno *et al* (32) previously proposed that C-MET overexpression was significantly associated with a worse outcome and anti-EGFR resistance; whereas in the present study, too small sample size in low C-MET expression patients treated with cetuximab prevented the elucidation of potential therapeutic importance of C-MET. A focus on this issue is required in future studies.

In view of the retrospective nature of the current methodology, there has been an inevitable selection bias in the present outcomes. Firstly, certain participants and their medical record documentation may have been lost to follow-up, particularly for those who were not hospitalized following first-line chemotherapy. Secondly, the patients were heterogeneous and selected according to the availability of genetic detection, which limited data analyses. Therefore, further prospective studies are required to confirm the present conclusions.

In conclusion, the status of *RAS*/*BRAF* and C-MET may serve as significant predictors for metastatic behavior and refining prognosis in CRC. Accordingly, radiological diagnosis in combination with *RAS*/*BRAF* and C-MET detection may help in the prognostic evaluation for postoperative stage III CRC cases, as well as devised appropriate individualized medicine in the future.

Acknowledgements

Not applicable.

Funding

The present work was supported by a grant from the Program of Health and Family Planning Commission Foundation of Guangzhou City, Guangdong Province, China (grant no. A2017418); and a grant from the Program of Science and Technology Commission Foundation of Guangzhou City, Guangdong Province, China (grant no. 20140705).

Availability of data and materials

The data in the present study are available from Guangdong General Hospital (Guangzhou, China).

Authors' contributions

JL designed the study, analyzed the data, wrote the present manuscript and gave final approval of the manuscript to be published. CH analyzed data, WZ conducted the follow-up, JW performed IHC and Sanger sequencing, LX performed survival analysis and DM designed the experiments.

Ethics approval and consent to participate

Written, informed consent was obtained from all individual participants and the protocol was approved by the Ethics Committee of Guangdong General Hospital.

Consent for publication

Written informed consent was obtained from each participant.

Competing interests

The authors declare that they have no competing interests.

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