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RESEARCH REPORT

Clinical meaning of *BRAF* mutation in Korean patients with advanced colorectal cancer

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Abstract

AIM: To evaluate the clinicopathological features of colorectal cancer (CRC) with a v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutation and its molecular interaction with microsatellite instability (MSI) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) in patients with advanced CRCs.

METHODS: From October 2009 to December 2011, 141 patients with stage Ⅲ (n = 51) or Ⅳ (n = 90) CRCs who were tested for the *BRAF* mutation at Severance Hospital were included. Among 141 patients, five were excluded due to follow-up loss. Therefore, 136 patients were included in the study. The clinicopathological data, MSI status, and *KRAS*/*BRAF* mutation status were reviewed retrospectively. In addition, to evaluating the value of *BRAF* mutation status, progression-free survival and overall survival in all patients were collected and compared between the *BRAF* wild-type group and *BRAF* mutation group.

RESULTS: Of 136 patients, 80 (58.8%) were male and the mean age was 59 years. *BRAF* and *KRAS* mutations were detected in 9.6% and 35.3% of patients, respectively. Only 4.3% of patients had MSI-high tumors and there were no MSI-high in tumors with a BRAF mutation. *BRAF* mutations tended to be more frequent in stage Ⅳ than in stage Ⅲ (11.76% *vs* 5.88%, P = 0.370). Patients with a *BRAF* mutation had a lower incidence of *KRAS* mutation than those without (7.69% *vs* 38.21%, P = 0.033). Overall survival was significantly shorter in the *BRAF* mutation group than in the *BRAF* wild-type group both by univariate analysis (P = 0.041) and multivariate analysis (HR = 2.195; 95%CI: 1.039-4.640; P = 0.039), while progression-free survival was not different according to *BRAF* mutation status.

CONCLUSION: CRCs with a *BRAF* mutation have distinct molecular features and resulted in a poor prognosis in Korean patients with advanced CRC.

Key words: *BRAF*; Colorectal cancer; Molecular features; Chemotherapy response; Prognosis

Core tip: This study identified the clinicopathological features of colorectal cancer (CRC) with *BRAF* mutation and its molecular interaction with microsatellite instability and *KRAS* targeting only to stage Ⅲ/Ⅳ CRCs. These molecular markers enable the classification of CRCs into meaningful subtypes for prognosis. Our data strongly support the prognostic role of *BRAF* mutation in Korean patients with advanced CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in Western countries. In Korea, CRC is the second most common cancer in males and the third in females, with an estimated 25782 new cases and 7645 deaths each year[1]. TNM staging of CRC has become a promising tool in determining treatment and prognosis, but it is evident that CRC has a significant clinical heterogeneity even within the same pathologic stage[2-4]. Recent advances in molecular genetics enable the classification of CRC using molecular markers, including microsatellite instability (MSI) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutations, to predict prognosis and treatment response[5-8].

BRAF is a part of the Ras/Raf/mitogen-activated protein kinase kinase (MAP2K)/mitogen-activated protein kinases (MAPK) signaling pathway. Many of the transcription factors activated by the Ras/Raf/MAP2K/MAPK pathway are involved in cell proliferation and differentiation and many growth factor genes have binding sites for transcription factors activated by the Ras–ERK pathway, located in their promoter regions. Thus, aberrant activation of this pathway may provoke self-sufficiency in proliferative signals and continuous stimulation of cell growth[9]. Mutations of *KRAS* or *BRAF* activate this pathway and are an established mechanism that drives colorectal carcinogenesis[10]. The most common mutation of *BRAF* is the classic GTG-GAG substitution at position 1799 of exon 15, which results in the V600E amino acid change and the subsequent constitutive activation of the EGFR signaling pathway. Generally, 10% to 20% of CRCs have *BRAF* mutations, and the incidence of *BRAF* mutation varies according to the status of the MSI. It has been reported that 34% to 70% of CRCs classified as MSI-high have *BRAF* mutations[11-13]. Compared to *KRAS* mutations, patients with CRC and *BRAF* mutations show some different clinical manifestations. It is well known that CRCs with *KRAS* mutations respond poorly to cetuximab[14]. However, the predictive role of *BRAF* mutations in response to cetuximab is not yet clear. As for prognostic markers, CRC with *BRAF* mutations have been shown to have significantly poor prognosis compared to those with *BRAF* wild-type[15-17], while *KRAS* mutations have no prognostic role[18].

Although CRCs have rapidly increased in Korea, the incidence of *BRAF* mutation and its clinical meaning have not yet been explored in Korean patients with CRC. The aim of this study was to evaluate the clinicopathological features of CRC with a *BRAF* mutation and its molecular interaction with MSI and *KRAS* mutation status in patients with stage Ⅲ or Ⅳ CRC.

MATERIALS AND METHODS

From October 2009 to December 2011, 141 patients in stage Ⅲ (n = 51) and Ⅳ (n = 90) CRC underwent molecular testing, including MSI analysis and determination of *KRAS* and *BRAF* mutation status in Severance Hospital. Among the 141 patients, five were excluded due to follow-up loss. In total, 136 patients were included in the present study. After an initial staging work-up including a CT scan, patients without metastasis or with resectable liver or lung metastasis received surgery and adjuvant chemotherapy with FOLFOX. Patients with unresectable metastatic disease received palliative chemotherapy with FOLFOX or FOLFIRI[19,20]. The clinicopathological data, including age, sex, family history of CRC, BMI (body mass index), Eastern Cooperative Oncology Group (ECOG) performance status tumor stage, tumor location, tumor grade, initial CEA and chemotherapeutic regimen were reviewed retrospectively. This study was approved by the Institutional Review Boards of Yonsei University College of Medicine.

DNA extract, MSI analysis, BRAF and KRAS sequencing

Before obtaining tissue samples, written informed consent was obtained from all patients. Tissue samples from the tumor and normal colonic mucosa were obtained from each patient after resection. DNA extracted from each tumor was amplified by a standard polymerase chain reaction using five Bethesda guidelines panel loci (BAT25, BAT26, MFD15, D2S123, and D5S346)[21]. In accordance with the consensus definitions of the National Cancer Institute, tumor samples were classified as displaying high-degree microsatellite instability (MSI-H, instability at 30% or more of the markers tested), low-degree microsatellite instability (MSI-L, instability at less than 30% of the markers tested), or microsatellite stability (MSS, stability at all of the markers tested). Due to the similar biological properties between MSI-L and MSS, these two molecular phenotypes were grouped together in all analyses.

KRAS and *BRAF* were charged at ISU ABXI (Seoul, South Korea). Genomic DNA was extracted from 10-μm-thick paraffin sections containing a portion of tumor tissue by the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Fifty nanograms of DNA were amplified in a 20 μL reaction solution containing 10 μL of 2 X concentrated HotStarTaq Master Mix (Qiagen, Hilden, Germany), including polymerase chain reaction (PCR) buffer with 3 mmol/L MgCl2, 400 μmol/L of each dNTP, and 0.3 μmol/L of each primer pair (KRAS F: 5'-ttatgtgtgacatgttctaat, R: 5'-agaatggtcctgcaccagtaa/BRAF, F: 5'-atgcttgctctgataggaaaatga, R: 5'-agcagcatctcagggcca). Amplifications were performed using a 15-min initial denaturation at 95 ℃, followed by 35 cycles of 30 s at 94 ℃, 30 s at 59 ℃, and 30 s at 72 ℃, and a 10-min final extension at 72 ℃. PCR

Table 1 Baseline characteristics of enrolled patients *n* (%)

	BRAF wild-type (*n* = 123)	*BRAF* Mutant (*n* = 13)	*P* value
Sex Male/Female	74 (60.2)/49 (39.8)	6 (46.2)/7 (53.8)	0.329
Age (mean ± SD, age)	58.54 ± 12.91	61.08 ± 7.87	0.488
Age (< 60 yr)	60 (48.8)	5 (38.5)	0.479
BMI (mean ± SD, kg/m^2)	22.77 ± 3.64	22.70 ± 3.31	0.950
Family history of colorectal cancer	25 (20.3)	1 (7.7)	0.462
ECOG performance status			0.421
0-1	116 (94.3)	13 (100.0)	
2-3	7 (5.7)	0 (0.0)	
Tumor type			0.871
Colon	97 (78.9)	10 (76.9)	
Rectum	26 (21.1)	3 (23.1)	
Tumor location			0.211
Proximal	35 (28.5)	6 (46.2)	
Distal	88 (71.5)	7 (53.8)	
Histology			0.111
WD and MD	113 (91.9)	10 (76.9)	
PD and UD	10 (8.1)	3 (23.1)	
AJCC tumor stage			0.370
Stage Ⅲ	48 (39.0)	3 (23.1)	
ⅢA	5 (4.1)	1 (7.7)	
ⅢB	30 (24.4)	1 (7.7)	
ⅢC	13 (10.6)	1 (7.7)	
Stage Ⅳ	75 (61.0)	10 (76.9)	
ⅣA	27 (22.0)	3 (23.1)	
ⅣB	48 (39.0)	7 (53.8)	
MSI			0.604
MSS and MS-low	110 (89.4)	12 (92.3)	
MSI-high	5 (4.1)	0	
Unchecked	8 (6.5)	1 (7.7)	
K-ras			0.033
Wild	76 (61.8)	12 (92.3)	
Mutant	47 (38.2)	1 (7.7)	
Initial CEA (mean ± SD, ng/mL)	251.41 ± 1520.96	14.29 ± 23.90	0.576

SD: Standard deviation; BMI: Body mass index; ECOG: Eastern Cooperative Oncology Group; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; UD: Undifferentiated; AJCC: American Joint Committee on Cancer; MSI: Microsatellite instability; MSS: Microsatellite stable; CEA: Carcinoembryonic antigen.

products separated in 2% gel were purified with a QIAgen gel extraction kit (Qiagen). DNA templates were processed for the DNA sequencing reaction using the ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, United States) with both forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were used in a 10 μL sequencing reaction solution containing 1 μL of BigDye Terminator v3.1 and 0.1 μmol/L of the same PCR primer. Sequencing reactions were performed using 25 cycles of 10 s at 96 ℃, 5 s at 50 ℃, and 4 min at 60 ℃. Sequence data were generated with the ABI PRISM 3730 DNA Analyzer (Applied Biosystems) and analyzed by Sequencing Analysis 5.1.1. software (Applied Biosystems) to compare variations.

Statistical analysis

The primary outcome was to compare overall survival (OS) and progression-free survival (PFS) of patients with *BRAF* mutation to those with *BRAF* wild-type. The secondary outcome was to evaluate the gene-gene interaction between *BRAF* mutation and *KRAS* mutation or MSI. Continuous variables were expressed as mean ± SD. Each patient's baseline characteristics were analyzed by descriptive statistics. OS was calculated from the time of diagnosis until death or the last follow-up visit, and PFS was calculated from the time of diagnosis until disease recurrence or progression. OS and PFS were analyzed using the Kaplan-Meier method, and survival curves were compared using the log-rank method. Cox proportional hazards modeling was used to control multiple risk factors that have been shown to influence CRC survival by computing 95% confidence intervals (CIs). A *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Patient characteristics

Of 136 patients, 107 (78.7%) patients were diagnosed with colon cancer and 29 (21.3%) with rectal cancer. Fifty-one (37.5%) patients were stage Ⅲ and 85 (62.5%) were stage Ⅳ. Thirteen patients (9.6%) had *BRAF* mutations, and 48 (35.3%) patients had *KRAS* mutations. The most frequent mutation at *KRAS* was G12D, which accounted for 29.2% of *KRAS* mutations (14/48). The second most frequent mutation was G13D (12/48), and the remainder occurred in the following order: G12V (9/48), G12C (6/48), G12S (4/48), G13C (2/48) and G12A (1/48). Five (4.3%) patients had MSI-high tumors. Molecular characteristics such as MSI status and *KRAS* and *BRAF* mutation status were not significantly different according to the tumor location.

The clinicopathological characteristics of patients according to *BRAF* mutation are summarized in Table 1. Tumors with *BRAF* wild-type tended to exhibit more differentiated histology (91.9% *vs* 76.9%, *P* = 0.211) and an earlier stage (stage Ⅲ, 39.0% *vs* 23.1%; *P* = 0.370) than tumors with *BRAF* mutation. There was no MSI-H in tumors with *BRAF* mutation. Tumors with *BRAF* wild-type had significantly more *KRAS* mutations than tumors with *BRAF* mutation (38.2% *vs* 7.7%, *P* = 0.033). Only one (7.7%) tumor with *BRAF* mutation had *KRAS* mutation. The other clinicopathological findings, such as sex, age, BMI, family history of CRC, ECOG performance status, tumor location and initial CEA level, were not different between tumors with *BRAF* wild-type and mutation. Treatment modalities in enrolled patients are summarized in Table 2. Ninety-three (75.6%) patients with *BRAF* wild-type and nine (69.2%) with *BRAF* mutation received surgery (*P* = 0.737). One hundred nineteen (96.7%) patients with *BRAF* wild-type and 11 (84.6%) with *BRAF* mutation received chemotherapy (*P* = 0.182). Chemotherapy regimens and targeted agents were not significantly different between the two groups. The mean ± SD follow-up duration in all patients was 21.5 ± 13.3 mo.

Table 2 Treatment modality of the patients *n* (%)

	BRAF wild-type (*n* = 123)	*BRAF* Mutant (*n* = 13)	*P* value
Surgery			0.737
Yes	93 (75.6)	9 (69.2)	
No	30 (24.4)	4 (30.8)	
Chemotherapy			
Yes	119 (96.7)	11 (84.6)	
No	4 (3.3)	2 (15.4)	
Chemotherapy regimen			0.441
FOLFOX	97 (82.2)	9 (81.8)	
FOLFIRI	5 (4.2)	0	
FL	7 (5.9)	2 (15.4)	
Xeloda	8 (6.8)	0	
SOX	1 (0.8)	0	
Target agent use			0.128
No	91 (74.0)	8 (61.5)	
Bevacizumab	19 (15.4)	5 (38.5)	
Cetuximab	13 (10.6)	0	

Tumor response and survival according to BRAF mutation status

To determine the value of *BRAF* mutation status as a prognostic marker, PFS and OS were compared between patients with *BRAF* wild-type and *BRAF* mutation (Figure 1). PFS was not statistically different between the two groups (*BRAF* wild type *vs* *BRAF* mutation, 10.1 ± 7.8 mo *vs* 7.2 ± 5.0 mo; $P = 0.135$). The OS was significantly shorter in patients with *BRAF* mutation than those with *BRAF* wild-type (*BRAF* wild type *vs* *BRAF* mutation, 22.2 ± 13.2 mo *vs* 18.8 ± 13.6 mo; $P = 0.041$). In contrast to *BRAF* mutation, *KRAS* mutation status was not associated with PFS or OS in patients with stage Ⅲ/Ⅳ CRCs.

Univariate and multivariate analyses were performed to validate the prognostic factors for survival in patients with stage Ⅲ/Ⅳ CRC. Univariate analysis revealed that histology, tumor stage, surgery and *BRAF* status were significant prognostic factors for survival ($P = 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.041$, respectively). Multivariate analysis showed that TNM stage Ⅳ (HR = 3.183; 95%CI: 1.517-6.679; $P = 0.002$), poor differentiation and lack of differentiation in histology (HR = 2.821; 95%CI: 1.378-5.776; $P = 0.005$), no surgery (HR = 3.694; 95%CI: 1.972-6.918; $P < 0.001$) and *BRAF* mutation (HR = 2.195; 95%CI: 1.039-4.640; $P = 0.039$) were significant poor prognostic factors for survival in patients with stage Ⅲ/Ⅳ CRC (Table 3).

Table 3 Prognostic factors in colorectal cancer patients in multivariate analysis

	HR (95%CI)	*P* value
Age (older than 60 yr *vs* younger)	1.164 (0.682-1.984)	0.578
Sex (male *vs* female)	0.927 (0.541-1.586)	0.781
Tumor type (rectum *vs* colon)	0.931 (0.511-1.697)	0.816
Initial stage (stage Ⅳ *vs* Ⅲ)	3.183 (1.517-6.679)	0.002
Histology (PD and UD *vs* WD and MD)	2.821 (1.378-5.776)	0.005
Surgical treatment (no *vs* yes)	3.694 (1.972-6.918)	< 0.001
BRAF mutation (mutant *vs* wild-type)	2.195 (1.039-4.640)	0.039
KRAS mutation (mutant *vs* wild-type)	1.305 (0.766-2.221)	0.327

CI: Confidence interval; PD: Poorly differentiated; UD: Undifferentiated; WD: Well differentiated; MD: Moderately differentiated; CEA: Carcinoembryonic antigen.

DISCUSSION

This retrospective study demonstrated that stage Ⅲ or Ⅳ CRC in Korean patients have distinct molecular characteristics, including exclusive mutations between *KRAS* and *BRAF* genes and a low incidence of MSI-H. *BRAF* mutant tumors showed significantly shorter survival than *BRAF* wild-type tumors, while the *KRAS* mutation had no prognostic impact.

It has been reported that 10% to 20% of CRCs have a *BRAF* mutation[3,22]. The *BRAF* mutation is associated with MSI-H through hMLH1 promoter hypermethylation, which is known to be associated with a high level of CpG island methylator phenotype (CIMP)[11]. Several reports have revealed a low incidence of *BRAF* mutations in CRC. Although it is not clear if ethnicity affects the status of the *BRAF* mutation, previous studies with small sample sizes of Koreans reported only 3.8%-7% of *BRAF* mutant CRCs in Korean patients[23,24]. A few studies showed that *BRAF* mutations were extremely uncommon in rectal cancer, with an incidence of 0%-2%[25,26]. However, the present study indicated that the incidence of *BRAF* mutation was not affected by ethnicity or tumor location. Although it is known that *BRAF* genes are exclusively mutated with *KRAS* genes, coincident mutations of *KRAS* and *BRAF* rarely occur in CRC with an incidence of 0.001%[27]. Herein, we found that one case had both *KRAS* (G12V) and *BRAF* mutations. This patient was a 49-year-old male and had rectosigmoid junction cancer of MSS type in MSI. It is not clear whether these tumors have a different biology and natural history than *KRAS* or *BRAF* only mutant tumors or which of the two mutations is the dominant oncogene driving tumor proliferation because coincident *KRAS* and *BRAF* mutation were infrequently observed[27].

The present study showed a small number of MSI-H tumors compared to previous studies[5,28] and thus failed to demonstrate the relationship between MSI status and *BRAF* mutation. This may have been due to the advanced stage of CRCs in the present study. Our previous study demonstrated that MSI-H tumors were strongly associated with early tumor stage, and only 6% of stage Ⅲ/Ⅳ CRC had MSI-H tumors[29]. Compared with colon cancer, rectal cancer has a low incidence of MSI-H tumors and this may have affected the results of the present study[29].

CRC has significant clinical heterogeneity based on several molecular markers such as MSI, *KRAS* and *BRAF*[2-4]. It has been well documented that MSI-H tumors have a better prognosis than MSS/MSI-L tumors[5,29]. In contrast, *BRAF* mutant tumors have a poor prognosis compared to *BRAF* wild-type tumors[6,7]. The

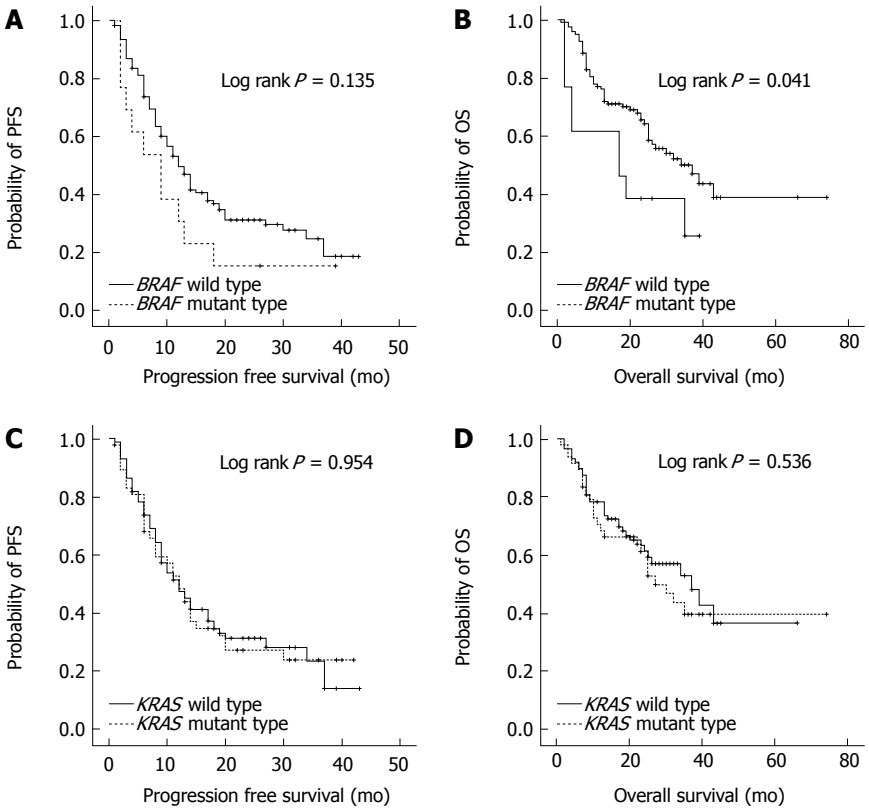

Figure 1 Progression-free survival and overall survival according to *BRAF* or *KRAS* mutation status. A: PFS was not significantly different by the *BRAF* mutation status (P = 0.135); B: OS was significantly poorer in the *BRAF* mutant group than in the *BRAF* wild-type group (P = 0.041); PFS (C) and OS (D) were not significantly different by the *KRAS* mutation status (P = 0.954 and P = 0.536, respectively). PFS: Progression-free survival; OS: Overall survival.

BRAF mutation has been used as a strong prognostic factor for overall survival in patients with CRCs, which was also confirmed in the present study. Thus, CRCs can be classified into four subtypes by these two distinct prognostic markers of MSI status and *BRAF* mutation[7]. MSS/*BRAF* mutant tumors are known to exhibit the worst prognosis, while MSI-H/*BRAF* wild-type tumors have the best prognosis. MSI-H/*BRAF* mutant or MSS/*BRAF* wild-type tumors have been suggested as intermediate subtypes[7]. However, several recent studies suggested that the association of *BRAF* mutation with poor prognosis is limited to MSS tumors[16,30]. Thus, further studies are necessary to adapt this molecular classification for clinical practice.

This study has several limitations. First, a retrospective study design has inherent limitations. Second, because of the small number of MSI-H tumors, the present study failed to evaluate the association of *BRAF* mutation with MSI status. Finally, even for advanced cancer, the follow-up period was relatively insufficient at a mean of 21 mo.

In conclusion, CRCs have distinct molecular features, including MSI status and mutations of *KRAS* and *BRAF*. These molecular markers enable the classification of CRCs into meaningful subtypes for prognosis. Our data strongly support the prognostic role of *BRAF* mutation in Korean patients with advanced CRC.

COMMENTS

Background

Approximately 10% of colorectal cancers (CRCs) have *BRAF* mutations and these CRCs have a worse prognosis than those with *BRAF* wild-type. In addition, the *BRAF* gene is thought to be closely associated with several molecular markers such as microsatellite instability (MSI) and *KRAS* in CRCs. However, although the prevalence of CRCs has rapidly increased in South Korea, the incidence of *BRAF* mutation and its clinical meaning are unknown in Korean CRC patients.

Research frontiers

Recent advances in molecular genetics enable the classification of CRC by molecular markers, including *MSI* and *KRAS* and *BRAF* mutations, to predict prognosis and treatment response. It is well known that CRCs with *KRAS* mutations respond poorly to cetuximab. However, the predictive role of *BRAF* mutations in cetuximab response is not clear. As for prognostic markers, MSI-high tumors have a better prognosis than MSS/MSI-low tumors. In contrast, *CRC* with *BRAF* mutations have a worse prognosis than those with *BRAF* wild-type, while *KRAS* mutations have no prognostic role. Future research should aim to uncover the role and purpose of molecular markers in CRC and to identify their potential usage clinically.

Innovations and breakthroughs

This study identified the clinicopathological features of CRC with a *BRAF* mutation and its molecular interaction with MSI and KRAS targeting for stage Ⅲ/Ⅳ CRC.

Applications

Molecular markers such as BRAF, KRAS, MSI may be used to classify cases of colorectal carcinoma into subtypes for prognosis.

Terminology

BRAF: *BRAF* is a human gene that makes a protein called B-Raf. The gene is also referred to as proto-oncogene B-Raf and v-Raf murine sarcoma viral onco-

gene homolog B1, while the protein is more formally known as serine/threonine-protein kinase B-Raf. The B-Raf protein is involved in sending signals inside cells, which are involved in directing cell growth. *KRAS*: *KRAS* is a human gene that makes a protein called KRAS. Like other members of the Ras family, the KRAS protein is a GTPase and is an early player in many signal transduction pathways. The protein product of the normal *KRAS* gene performs an essential function in normal tissue signaling, and the mutation of a *KRAS* gene is an essential step in the development of many cancers.

Peer review

The *BRAF* mutant tumors had a significantly shorter survival than that of *BRAF* wild-type tumors, while the *KRAS* mutation had no prognostic impact. These results are interesting and this finding allows these molecular markers to be used to classify cases of colorectal carcinoma into subtypes for prognosis.

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