ORIGINAL ARTICLE
Oncology & Hematology

JKMS

https://doi.org/10.3346/jkms.2017.32.1.38 • J Korean Med Sci 2017; 32: 38-46

BRAF-Mutated Colorectal Cancer Exhibits Distinct Clinicopathological Features from Wild-Type *BRAF*-Expressing Cancer Independent of the Microsatellite Instability Status

Min Hye Jang,[1*†] Sehun Kim,[1*] Dae Yong Hwang,[2] Wook Youn Kim,[1] So Dug Lim,[1] Wan Seop Kim,[1] Tea Sook Hwang,[1] and Hye Seung Han[1]

[1]Department of Pathology, Konkuk University School of Medicine, Seoul, Korea; [2]Department of Surgery, Konkuk University School of Medicine, Seoul, Korea

*Sehun Kim and Min Hye Jang contributed equally to this work.
†Current address: Department of Pathology, Yeungnam University Hospital, Daegu, Korea.

Received: 24 May 2016
Accepted: 15 September 2016

Address for Correspondence:
Hye Seung Han, MD
Department of Pathology, Konkuk University School of Medicine, 120-1, Neungdong-ro, Gwangjin-gu, Seoul 05030, Korea
E-mail: 20040002@kuh.ac.kr

In patients with colorectal cancer (CRC), the *BRAF* V600E mutation has been reported to be associated with several clinicopathological features and poor survival. However, the prognostic implications of *BRAF* V600E mutation and the associated clinicopathological characteristics in CRCs remain controversial. Therefore, we reviewed various clinicopathological features, including *BRAF* status, in 349 primary CRCs and analyzed the relationship between *BRAF* status and various clinicopathological factors, including overall survival. Similar to previous studies conducted in Eastern countries, the incidence of the *BRAF* V600E mutation in the current study was relatively low (5.7%). *BRAF*-mutated CRC exhibits distinct clinicopathological features from wild-type *BRAF*-expressing cancer independent of the microsatellite instability (MSI) status. This mutation was significantly associated with a proximal tumor location ($P = 0.002$); mucinous, signet ring cell, and serrated tumor components ($P < 0.001$, $P = 0.003$, and $P = 0.008$, respectively); lymphovascular invasion ($P = 0.004$); a peritumoral lymphoid reaction ($P = 0.009$); tumor budding ($P = 0.046$); and peritoneal seeding ($P = 0.012$). In conclusion, the incidence of the *BRAF* V600E mutation was relatively low in this study. *BRAF*-mutated CRCs exhibited some clinicopathological features which were also frequently observed in MSI-H CRCs, such as a proximal location; mucinous, signet ring cell, and serrated components; and marked peritumoral lymphoid reactions.

Keywords: *BRAF* V600E; Colorectal Cancer; Clinicopathological Feature; Microsatellite Instability

INTRODUCTION

Colorectal cancer (CRC) is among the leading causes of cancer-related death worldwide (1) and is also the fourth-leading cause of death among Korean cancer patients, based on a 2013 report from the Korean National Cancer Center (2). The propagation of an early endoscopic detection program and changes in environmental factors, such as an increase in the adoption of western dietary patterns, have led to continued increase of the CRC incidence in Korea.

The mutation status of *BRAF*, a gene that encodes a major component of the ERK/MAP kinase signaling pathway, is a molecular classification criterion for CRC. The most common type of *BRAF* mutation is a CTG → CAG transversion at residue 1799, leading to a substitution from glutamic acid to valine at codon 600 (V600E); this substitution occurs in CRCs, as well as in other malignancies (3). *KRAS* mutation is generally associated with tumors attributed to the chromosomal instability (CIN) pathway, whereas *BRAF* mutation is known to be associated with microsatellite instability (MSI), the CpG-island methylator phenotype (CIMP), and the serrated pathway (4). Mutations in *BRAF* and *KRAS* are mutually exclusive, and activating *BRAF* mutations occur almost exclusively in microsatellite-unstable and CIMP-high CRCs (5).

In contrast to other oncogenes such as *KRAS*, the incidence of *BRAF* mutation is fairly low among CRCs. This incidence ranges from 5% to 15% according to previous studies. However, the *BRAF* V600E mutation has been highlighted as a new predictive biomarker of anti-*EGFR* monoclonal antibody efficacy in CRCs (6-8). In addition, this mutation is considered a new candidate for targeted therapy. A few researchers have attempted to use the *BRAF* V600E mutation as a new therapeutic target in advanced and metastatic CRCs (9,10).

However, the prognostic implication of the *BRAF* V600E mutation remains controversial. Several studies have shown that the *BRAF* V600E mutation is associated with poor survival in advanced CRCs, and particularly in microsatellite stable (MSS) CRCs (11). Other studies have failed to prove a relationship between *BRAF* status and prognosis (12,13). Furthermore, characterization of the clinicopathological features of *BRAF*-mutated CRCs remains unclear. Therefore, we aimed to investigate the association between the *BRAF* V600E mutation status and

pISSN 1011-8934
eISSN 1598-6357

clinicopathological features of CRC and to validate the clinical significance of this mutation with regard to survival prognosis.

MATERIALS AND METHODS

Study population and clinicopathological review

Three hundred sixty-five CRC cases registered between 2011 and 2014 were collected from the Konkuk University Medical Center pathologic archives. Fourteen patients who had undergone neoadjuvant chemotherapy and 2 patients who had undergone surgery for recurrent tumors were excluded from the study. Finally, a total of 349 primary CRC cases were evaluated. Two hundred eighteen patients underwent conventional adjuvant chemotherapy comprising FOLFOX (folinic acid/5-fluorouracil/oxaliplatin), FOLFORI, or XELOX (capecitabine/oxaliplatin). Among them, 2 patients were treated with cetuximab, an anti-*EGFR* monoclonal antibody.

Evaluation of pathologic features

Various demographic features were obtained from the Konkuk University Medical Center electric medical records, including each patient's age, sex, tumor size, tumor location, treatment methodology, and follow-up history. We also reviewed slides containing hematoxylin and eosin-stained tumor sections. One pathologist (JMH) evaluated the pathological features of the 349 CRCs, including the pathologic (p) T/N stages, tumor differentiation, lymphovascular invasion, perineural invasion, tumor border, tumor budding, Crohn-like lymphoid reaction, peritumoral lymphoid reaction, and proportions of mucinous, signet ring cell, medullary, serrated, and cribriform-comedo components (Fig. 1).

Tumor differentiation was graded according to the 2010 World

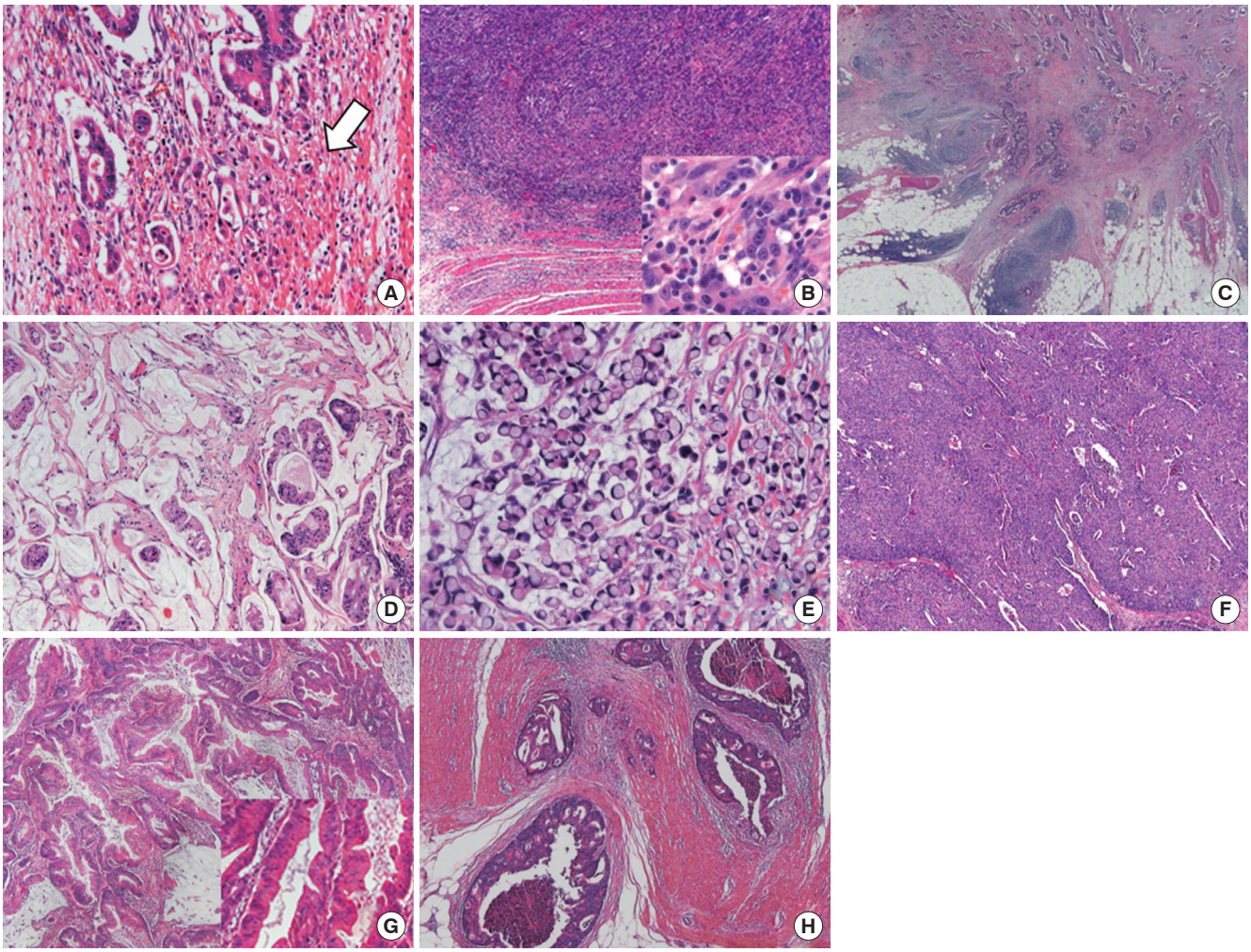

Fig. 1. Histopathological features of CRCs. (**A**) CRC with positive tumor budding (arrow, ≥ 5 buds). (**B**) CRC with a marked peritumoral lymphoid reaction and tumor cell destruction by infiltrating lymphocytes. (**C**) CRC with an active Crohn-like lymphoid reaction (≥ 1-mm-sized lymphoid aggregate). (**D**) Mucinous component. (**E**) Signet ring cell component. (**F**) Medullary component. (**G**) Serrated component. (**H**) Cribriform-comedo component. Stain, hematoxylin-eosin; original magnification: 1.25× (**C**), 40× (**B**, **F**, **H**), 100× (**A**, **D**, **G**), 400× (**E**, inset of **B**, inset of **G**).
CRC = colorectal cancer.

Health Organization (WHO) classification. The proportion of each component in a tumor was assessed using the following 3 categories based on a previous study by Kim et al. (14): absent, < 50%, and ≥ 50%. The histologic features of each component were determined based on the 2010 WHO classification. Medullary features were defined as a syncytial growth pattern of large tumor cells with a high nuclear/cytoplasmic ratio and prominent nucleoli. Serrated features were defined as glandular serration with abundant eosinophilic cytoplasm, intra- and extracellular mucin, and a lack of tumor necrosis. Cribriform-comedo features were defined as cribriform glandular architecture with central comedo-like necrosis.

Tumor budding was evaluated according to a previous study by Ueno et al. (15). This characteristic was defined as a single tumor cell or cluster of < 5 tumor cells at the invasive front. A tumor with ≥ 5 buds visible in a 200 × magnification field was classified as a budding-positive tumor. Peritumoral lymphoid reactions were assessed according to Klintrup's scoring system (16), as follows: G0, no increase of lymphoid cells at the invasive margin; G1, mild increase of lymphoid cells without tumor cell destruction caused by lymphoid infiltration; G2, band-like infiltration of lymphoid cells at the invasive margin with focal tumor cell destruction by lymphocytes; or G3, cup-like intense lymphoid infiltration at the invasive margin with frequent tumor cell destruction by lymphocytes. An active Crohn-like lymphoid reaction was defined as the presence of a ≥ 1-mm peritumoral lymphoid aggregate, based on the criteria presented by Ueno et al. (15).

Immunochemistry and interpretation of *EGFR* and p53

An automatic Ventana ES immunohistochemistry staining device (Ventana Medical Systems, Tucson, AZ, USA) was used for all immunohistochemistry (IHC) procedures. Four-micrometer tissue sections were cut, dried, deparaffinized, and rehydrated according to standard procedures. Primary antibodies specific for *EGFR* (clone E30, dilution 1:100; Dako, Glostrup, Denmark) and p53 (clone D07, dilution 1:500; Thermo Fisher Scientific, Fremont, CA, USA) were used for this study. Internal controls were used in all IHC evaluations to ensure the quality of staining.

EGFR staining was graded using a 4-tiered system (0, 1+, 2+, 3+) according to the membranous staining intensity and frequency in the cancer cells, as follows: 0, negative/no membranous staining; 1+, faint/partial membrane staining; 2+, weak/complete membrane staining in > 10% of total invasive cancer cells; and 3+, intense/complete membrane staining in > 10% of total invasive cancer cells.

p53 positivity was defined according to the distribution patterns of stained nuclei. Only cases involving widespread positivity among tumor cell nuclei in either the entire tissue section or segments of the tumor sample were considered positive. Tumors with a scattered distribution of positive nuclei were considered negative even if > 10% of the tumor cells exhibited nuclear staining (17).

MSI and *BRAF* V600E mutation tests

DNA extraction was performed in our study as previously described (18). The MSI status was analyzed via polymerase chain reaction (PCR) amplification with fluorescent dye-labeled primers comprising mononucleotide (BAT25 and BAT26) and dinucleotide markers (D2S123, D5S346, and D17S250) specific for microsatellite sites. MSI was defined as a single-band shift in either of 2 alleles or the appearance of a variably sized band in the cancer sample. Cases were stratified as a high incidence of MSI (MSI-H) when instability was identified in > 30% of markers and as a low incidence of MSI (MSI-L) when instability was identified in < 30% of markers. A MSS status was defined as a lack of definite evidence of MSI.

The *BRAF* V600E mutation was identified using pyrosequencing as previously described (19). The following PCR and sequencing primers were used: 5'-biotin-TTCATAATGCTTGCTCTGATAGG-3´ (PCR, F), 5´-GGCCAAAAATTTAATCAGTGGAA-3´ (PCR, R), and 5´-CCACTCCATCGAGATT-3´ (Sequencing R). The PCR conditions comprised 50 repeats of a 30-minute incubation at 55°C. Pyrosequencing TM was executed using a single-nucleotide polymorphism (SNP) reagent kit (Biotage, Uppsala, Sweden).

Statistical analysis

The χ^2 test was used to analyze differences in various clinicopathological factors between the *BRAF* V600E mutant and wild-type CRC groups. Student's *t*-test was used to compare the mean age and mean tumor size between these two groups. The Kaplan-Meier method was used to produce overall survival curves. The log-rank test was used to analyze differences in survival probability. A Cox regression model was applied to univariate and multivariate analyses regarding survival and used to generate hazard ratios (HRs) and confidence intervals (CIs). All statistical parameters were considered significant at a *P* value < 0.05.

Ethics statement

This study was approved by the Institutional Review Board of Konkuk University Medical Center (IRB No.: KUH1210031). The requirement for informed consent was waived by the board.

RESULTS

Demographic features of the patients studied

A total of 349 patients with primary CRC were evaluated in this study. Table 1 presents the various clinicopathological features of the baseline study population. More than half of the tumors were located in the distal colon (proximal, 30.4% vs. distal, 69.6%). More than 90% of cases exhibited moderate differentiation (94.0%).

 https://doi.org/10.3346/jkms.2017.32.1.38

Table 1. Clinicopathological features of 349 primary CRCs and relationships with MSI status

Pathological characteristics	Total patients (n = 349)	MSI status		*P* value
		MSI-H (n = 35)	MSS/MSI-L (n = 314)	
Age, yr				
Mean ± SD	63.1 ± 11.2	59.2 ± 141.0	63.5 ± 10.8	0.031
Sex				
Male	208 (59.6)	17 (48.6)	191 (60.8)	0.161
Female	141 (40.4)	18 (51.4)	123 (39.2)	
Peritoneal carcinomatosis				
Absent	319 (91.4)	35 (100.0)	284 (90.4)	0.057
Present	30 (8.6)	0 (0.0)	30 (9.6)	
Tumor location				
Proximal	106 (30.4)	25 (71.4)	81 (25.8)	< 0.001
Distal	243 (69.6)	10 (28.6)	233 (74.2)	
Tumor size, cm				
Mean ± SD	4.7 ± 2.2	5.2 ± 2.7	4.6 ± 2.1	0.255
Tumor multiplicity				
Solitary	335 (96.0)	33 (94.3)	302 (96.2)	0.640
Multiple	14 (4.0)	2 (5.7)	12 (3.8)	
Tumor border*				
Expanding	72 (20.8)	11 (31.4)	61 (19.6)	0.103
Infiltrative	274 (79.2)	24 (68.6)	250 (80.4)	
pT stage				
pTis/pT1	35 (10.0)	3 (8.6)	32 (10.2)	0.047
pT2	44 (12.6)	10 (28.6)	34 (10.8)	
pT3	234 (67.0)	19 (54.3)	215 (68.5)	
pT4	69 (10.3)	3 (8.6)	33 (10.5)	
pN stage				
pN0	196 (56.2)	26 (74.3)	170 (54.1)	0.040
pN1	84 (24.0)	7 (20.0)	77 (24.5)	
pN2	69 (19.8)	2 (5.7)	67 (21.3)	
LVI				
Absent	253 (72.5)	29 (82.9)	224 (71.3)	0.148
Present	96 (27.5)	6 (17.1)	90 (28.7)	
Perineural invasion				
Absent	287 (82.2)	33 (94.3)	254 (80.9)	0.049
Present	62 (17.8)	2 (5.7)	60 (19.1)	
Peritumoral lymphoid reaction*				
Absent (G0)	66 (19.1)	2 (5.7)	64 (20.4)	< 0.001
Mild (G1)	177 (51.2)	9 (25.7)	168 (53.5)	
Moderate (G2)	84 (24.3)	17 (48.6)	67 (21.3)	
Marked (G3)	22 (6.4)	7 (20.0)	15 (4.8)	
Crohn-like lymphoid reaction*				
Inactive (largest LA size < 1 mm)	235 (67.9)	17 (48.6)	218 (70.1)	0.010
Active (largest LA size ≥ 1 mm)	111 (32.1)	18 (51.4)	93 (29.9)	
Tumor differentiation				
WD	8 (2.3)	0 (0.0)	8 (2.5)	0.011
MD	328 (94.0)	30 (85.8)	298 (94.9)	
PD	13 (3.7)	5 (14.3)	8 (2.5)	
Tumor budding*				
Negative (< 5 bud)	100 (28.9)	10 (28.6)	90 (28.9)	0.964
Positive (≥ 5 bud)	246 (71.1)	25 (71.4)	221 (71.1)	
Mucinous component*				
Absent	245 (70.8)	12 (34.3)	233 (74.9)	< 0.001
< 50%	91 (26.3)	22 (62.9)	69 (22.2)	
≥ 50%	10 (2.9)	1 (2.8)	9 (2.9)	
Signet ring cell component*				
Absent	329 (95.1)	32 (91.4)	297 (95.5)	0.293
< 50%	16 (4.6)	3 (8.6)	13 (4.2)	
≥ 50%	1 (0.3)	0 (0.0)	1 (0.3)	
Medullary component*				
Absent	332 (96.0)	33 (94.3)	299 (96.1)	0.642
< 50%	14 (4.0)	2 (5.7)	12 (3.86)	
≥ 50%	0 (0.0)	0 (0.0)	0 (0.0)	
Serrated component*				
Absent	312 (90.2)	28 (80.0)	284 (91.3)	0.064
< 50%	34 (9.8)	7 (20.0)	27 (8.7)	
≥ 50%	0 (0.0)	0 (0.0)	0 (0.0)	
Cribriform comedo component*				
Absent	335 (96.8)	35 (100.0)	300 (96.5)	0.611
< 50%	11 (3.2)	0 (0.0)	11 (3.5)	
≥ 50%	0 (0.0)	0 (0.0)	0 (0.0)	
EGFR expression				
Negative	97 (27.8)	7 (20.0)	90 (28.7)	0.703
1+	97 (27.8)	10 (28.6)	87 (27.7)	
2+	119 (34.1)	14 (40.0)	105 (33.4)	
3+	36 (10.3)	4 (11.4)	32 (10.2)	
P53 overexpression				
Positive	162 (46.4)	31 (88.6)	156 (49.7)	< 0.001
Negative	187 (53.6)	4 (11.4)	158 (50.3)	

CRC = colorectal cancer, MSI = microsatellite instability, MSI-H = microsatellite instable-high, MSS = microsatellite stable, MSI-L = microsatellite instable-low, SD = standard deviation, WD = well differentiated, MD = moderately differentiated, PD = poorly differentiated, LVI = lymphovascular invasion, LA = lymphoid aggregate, *EGFR* = epidermal growth factor receptor.

*Indicated characteristics were evaluable in 346 cases. Three of 349 CRC cases were excluded from the evaluation because of a lack of appropriate amounts of tumor sample resulting from previous endoscopic polypectomy or submucosal dissection.

Most cases involved locally advanced tumors. pT3 or pT4 cancers accounted for 77.3% of the total study cases, and lymph node metastasis was also identified in 43.8% of cases (n = 153). Sixty-three (18.1%) of the 349 cancers had already generated distant metastases at the time of diagnosis. Peritoneal carcinomatosis was identified in 30 cases. Of the 349 patients with CRC, 214 had received standard adjuvant chemotherapy after surgery.

Associations between MSI status and clinicopathological characteristics

Among the 349 patients, 35 (10.0%) had MSI-H CRC. Accordingly, we assessed differences in clinicopathological characteristics between MSI-H and MSS/MSI-L CRCs (Table 1). Patients with MSI-H CRC were statistically younger than those with MSS/MSI-L CRC ($P = 0.031$). Most (25/35, 71.4%) MSI-H CRCs were located in the proximal colon. The MSI-H subgroup included fewer pT4-stage tumors and cases of lymph node metastasis

than did the MSS/MSI-L subgroup. Furthermore, CRCs with a MSI-H status were less likely to be associated with distant metastasis ($P = 0.002$). Histologically, poorly differentiated tumors were more frequently observed among MSI-H CRCs ($P = 0.011$). However, MSI-H CRC was associated with a lower rate of perineural invasion ($P = 0.049$). MSI-H CRC was found to more frequently associate with Crohn-like lymphoid reactions and high-grade peritumoral lymphoid reactions ($P = 0.010$ and $P = 0.011$, respectively). MSI-H CRCs had a significantly larger mucinous component than did MSS/MSI-L CRCs ($P < 0.001$). In addition, MSI-H CRCs were more likely to exhibit serrated features, although this difference was not statistically significant ($P = 0.064$).

Incidence of *BRAF* V600E mutation and its association with clinicopathological characteristics

BRAF V600E mutation was detected in 20 of 349 (5.7%) cases. We evaluated the relationship between *BRAF* V600E mutation and clinicopathological characteristics in the investigated tumors (Table 2). CRCs harboring the *BRAF* V600E mutation were mostly located in the proximal colon (70.0%; $P < 0.001$). These tumors also more frequently presented with mucinous, signet ring cell, and serrated components than did wild-type CRCs ($P < 0.001$, $P = 0.002$, and $P = 0.008$, respectively). In addition, *BRAF* V600E-mutated CRCs more frequently exhibited peritumoral lymphoid reactions than did *BRAF* wild-type CRCs ($P < 0.001$). These findings were similar to the features of MSI-H CRCs. Several factors associated with a worse prognosis were also found to correlate with the *BRAF* V600E mutant phenotype. Tumors harboring the *BRAF* V600E mutation more frequently exhibited poorly differentiated histology, infiltrative borders, and tumor budding, compared to tumors harboring wild-type *BRAF* ($P = 0.007$, $P = 0.019$, and $P = 0.015$, respectively). Lymphovascular invasion was also more frequent among tumors with the *BRAF* V600E mutation. Specifically, 12 (65.0%) of 20 *BRAF*-mutated cases exhibited lymphovascular invasion ($P < 0.001$). Peritoneal carcinomatosis was also significantly more frequently identified in *BRAF*-mutated cancers ($P = 0.021$).

Cancers harboring the *BRAF* V600E mutation tended to have a larger tumor size and more advanced pathologic T stage, although these differences were not statistically significance ($P = 0.051$ and $P = 0.094$, respectively). However, *EGFR* and p53 immunoreactivity were observed significantly more frequently in *BRAF* V600E mutant CRCs than in wild-type CRCs ($P < 0.001$ and $P = 0.015$, respectively). Mean age, sex, pN stage, and perineural invasion were not found to differ significantly according to *BRAF* status. In addition, no correlation was observed between the *BRAF* status and MSI ($P = 0.128$), although the latter is well known to be associated with *BRAF* mutation in CRC.

Table 2. Associations between *BRAF* and variable clinicopathological characteristics

Characteristics	*BRAF* V600E (n = 20)	Wild type (n = 329)	*P* value
Age (mean ± SD)	63.1 ± 11.7	63.1 ± 11.2	0.990
Sex (male:female)	12:8	196:133	0.970
cM stage (M0:M1)	14:6	272:57	0.131
Peritoneal carcinomatosis (absent:present)	15:5	304:25	0.021
Location (proximal:distal)	14:6	92:237	< 0.001
Tumor size (mean ± SD)	5.6 ± 2.1	4.6 ± 2.2	0.051
Tumor multiplicity (solitary:multiple)	20:0	315:14	1.000
Tumor border* (expanding:infiltrative)	0:20	72:254	0.019
pT stage (pTis/T1:T2:T3:T4)	0:2:13:5	35:42:221:31	0.094
pN stage (pN0:N1:N2)	9:5:6	187:79:63	0.399
LVI (absent:present)	7:13	246:83	< 0.001
Perineural invasion (absent:present)	15:5	272:57	0.271
Peritumoral lymphoid reaction* (grade 0:1:2:3)	7:4:3:6	59:173:81:16	< 0.001
Crohn-like lymphoid reaction* (inactive:active)	11:9	224:102	0.202
Tumor differentiation (WD:MD:PD)	0:16:4	8:312:9	0.007
Tumor budding* (< 5 bud: ≥ 5 bud)	1:19	99:227	0.015
Mucinous component* (0%: < 50%: ≥ 50%)	6:13:1	239:78:9	< 0.001
Signet ring cell component* (0%: < 50%: ≥ 50%)	15:5:0	314:11:1	0.002
Medullary component* (0%: < 50%: ≥ 50%)	18:2:0	314:12:0	0.190
Serrated component* (0%: < 50%: ≥ 50%)	14:6:0	298:28:0	0.008
Cribriform-comedo component* (0%: < 50%: ≥ 50%)	20:0	315:11	1.000
MSI (MSI-H:MSS/MSI-L)	4:16	31:298	0.128
EGFR expression (0:1+:2+:3+)	1:4:6:9	96:93:113:27	< 0.001
P53 overexpression (negative:positive)	16:4	171:158	0.015

SD = standard deviation, LVI = lymphovascular invasion, WD = well differentiated, MD = moderately differentiated, PD = poorly differentiated, MSI = microsatellite instability, MSI-H = microsatellite instable-high, MSS = microsatellite stable, MSI-L = microsatellite instable-low, *EGFR* = epidermal growth factor receptor.
*Indicated characteristics were evaluable in 346 cases. Three of 349 CRCs cases were excluded from the evaluation because of an inappropriate amount of tumor for evaluation resulting from previous endoscopic polypectomy or submucosal dissection.

https://doi.org/10.3346/jkms.2017.32.1.38

We also evaluated the clinicopathological characteristics of CRCs with respect to *BRAF* V600E mutation status in the MSS/MSI-L subgroup (Supplementary Table 1). In this subgroup, CRCs harboring the *BRAF* mutation more frequently exhibited peritoneal carcinomatosis, a proximal location, advanced pT stage, larger tumor size, infiltrative border, tumor budding, and mucinous, signet ring cell, and serrated components when compared with *BRAF* wild-type CRCs. The former also more frequently exhibited marked peritumoral lymphoid reactions.

Univariate and multivariate survival analyses of various clinicopathological characteristics of CRCs

We evaluated the prognostic value of various clinicopathological parameters, including the *BRAF* V600E mutation and MSI statuses (Table 3). To ensure a precise statistical analysis, we excluded 22 cases with follow-up durations of < 6 months. Accordingly, 327 cases were subjected to overall survival analysis. The median follow-up duration was 23.1 months (range, 2.1–43.0 months). Fourteen (4.3%) cancer-related deaths were recorded.

In the univariate analysis, advanced pT and pN stages were associated with poorer overall survival ($P = 0.040$ and $P < 0.001$, respectively; Table 3). Distant metastases at diagnosis and/or peritoneal carcinomatosis were also associated with reduced overall survival ($P < 0.001$ for both). Regarding tumor features, histologic differentiation, lymphovascular invasion, and perineural invasion correlated significantly with overall survival ($P = 0.028$, $P = 0.001$, and $P = 0.018$, respectively), as did the presence of a signet ring cell component ($P = 0.042$). Patients whose CRCs exhibited marked peritumoral lymphoid reaction (G3) demonstrated poorer overall survival ($P = 0.060$). However, *BRAF* mutation and MSI status were not found to be associated with overall survival in the current study ($P = 0.773$ and $P = 0.397$, respectively; Fig. 2).

A multivariate analysis was conducted to include peritumoral lymphoid reaction and all other factors that exhibited significant associations with overall survival in the univariate analysis. However, only pT stage (HR = 4.212; 95% CI, 1.290–13.752; $P = 0.017$) remained as an independent prognostic factor of overall survival (Table 3).

We further assessed the prognostic value of *BRAF* mutation status in stage-stratified subgroups using a Kaplan-Meier survival analysis and log-rank test. However, we found no difference in overall survival between patients with *BRAF* V600E-mutated CRC and wild-type CRC in each subgroup (stage II/III CRC, $P = 0.631$; stage IV CRC, $P = 0.934$). We also evaluated overall survival in several different subgroups through a Kaplan-Meier anal-

Table 3. Univariate and multivariate analyses of overall survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age at diagnosis (≥ 60 vs. < 60)	0.396 (0.132–1.184)	0.097	-	-
Sex (male vs. female)	0.817 (0.306–2.543)	0.882	-	-
Adjuvant chemotherapy (done vs. not done)	5.724 (0.747–43.883)	0.093	-	-
Distant metastasis at diagnosis	15.040 (4.680–44.332)	< 0.001	3.793 (0.923–15.597)	0.065
Peritoneal carcinomatosis (present vs. absent)	8.995 (3.116–25.961)	< 0.001	-	-
Location (distal vs. proximal)	0.516 (0.179–1.490)	0.222	-	-
Tumor size (≥ 4.5 cm vs. < 4.5 cm)	2.481 (0.777–7.917)	0.125	-	-
Tumor multiplicity (multiple vs. solitary)	0.046 (0.000–3,958.568)	0.596	-	-
Tumor border (infiltrative vs. expanding)	29.935 (0.138–6,503.835)	0.216	-	-
pT stage (T4 vs. Tis/T1/T2/T3)	15.495 (5.267–45.583)	< 0.001	4.212 (1.290–13.752)	0.017
pN stage (pN1/2 vs. pN0)	19.754 (2.582–151.143)	0.004	4.528 (0.444–46.187)	0.202
LVI (present vs. absent)	9.515 (2.654–34.117)	0.001	2.296 (0.532–90.918)	0.266
Perineural invasion (present vs. absent)	3.604 (1.250–10.396)	0.018	1.027 (0.306–3.449)	0.966
Peritumoral lymphoid reaction (G0 vs. G1/2/3)	0.349 (0.116–1.046)	0.060	0.859 (0.273–2.709)	0.796
Crohn-like lymphoid reaction (active vs. inactive)	0.531 (0.148–1.905)	0.331	-	-
Tumor differentiation (PD vs. MD/WD)	5.409 (1.201–24.370)	0.028	1.879 (0.371–9.506)	0.446
Tumor budding (≥ 5 bud vs. < 5 bud)	5.320 (0.696–40.678)	0.107	-	-
Mucinous component (≥ 50% vs. < 50%)	1.029 (0.322–3.285)	0.961	-	-
Signet ring cell component (≥ 50% vs. < 50%)	4.733 (1.057–21.193)	0.042	3.003 (0.533–16.922)	0.213
Medullary component (≥ 50% vs. < 50%)	2.353 (0.307–18.009)	0.410	-	-
Serrated component (≥ 50% vs. < 50%)	0.731 (0.096–5.592)	0.763	-	-
Cribriform comedo component (≥ 50% vs. < 50%)	2.475 (0.323–18.944)	0.383	-	-
MSI status (MSI-H vs. MSS/MSI-L)	0.042 (0.000–65.463)	0.397	-	-
BRAF (V600E mutated vs. wild type)	1.349 (0.176–10.324)	0.773	-	-
EGFR expression (1+/2+/3+ vs. 0)	0.475 (0.164–1.370)	0.168	-	-
P53 overexpression (positive vs. negative)	0.687 (0.230–2.051)	0.501	-	-

HR = hazard ratio, CI = confidence interval, LVI = lymphovascular invasion, PD = poorly differentiated, MD = moderately differentiated, WD = well differentiated, MSI = microsatellite instability, MSI-H = microsatellite instable-high, MSS = microsatellite stable, MSI-L = microsatellite instable-low, *EGFR* = epidermal growth factor receptor.

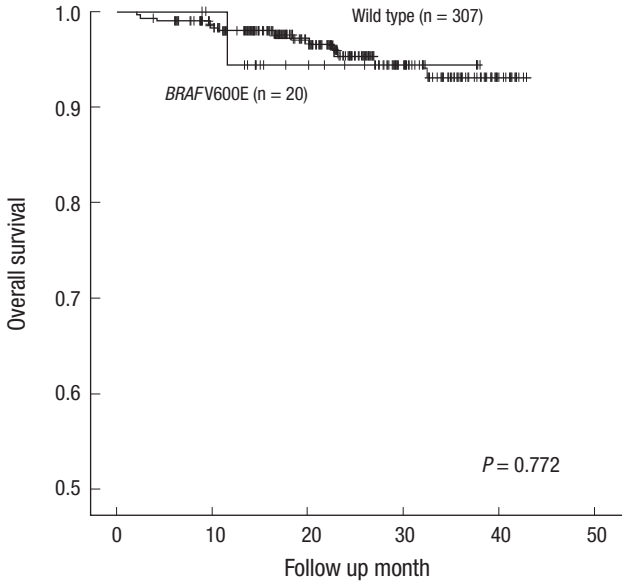

Fig. 2. Comparison of overall survival between patients with *BRAF* V600E-mutated CRC and those with wild-type CRC among 327 cases of primary CRC.
CRC = colorectal cancer.

ysis. However, we found no difference in overall survival between patients with *BRAF* V600E-mutated CRC and wild-type CRC in each subgroup (CRCs with adjuvant chemotherapy, $P = 0.670$; MSS/MSI-L CRC, $P = 0.759$; proximal-located CRC, $P = 0.308$).

DISCUSSION

In the current study, we evaluated the *BRAF* V600E mutation statuses of CRCs using pyrosequencing techniques. According to a previous review by Kim et al. (20), the prevalence of *BRAF*-mutated CRC is lower in Eastern Asian countries than in Western countries. *BRAF* mutations have been reported to account for 3.7%–20.6% of all CRCs in Western countries, but only 0.7%–11.4% of CRCs in Eastern Asian countries (e.g., China, Japan, and Korea). Similarly, the incidence of *BRAF* V600E mutation was quite low in the current study, as only 20 (5.7%) cases harbored this mutation. In addition, the incidence of MSI-H CRC was also quite low (10.0%) in this study, as in other studies of Korean patients (21). Various genetic and/or environmental factors appear to influence these ethnic differences; however, the cause of the ethnic molecular heterogeneity observed in CRCs is not yet fully understood.

To characterize *BRAF* V600E-mutated CRCs, we analyzed associations between the *BRAF* V600E mutation and other clinicopathological factors. We found that this mutation associated significantly with a proximal tumor location ($P < 0.001$), poor differentiation ($P = 0.007$), presence of a mucinous component ($P = 0.003$), and peritoneal carcinomatosis ($P = 0.021$). Many previous studies regarding the clinicopathological features of *BRAF*-mutated CRCs reported results consistent with those of our study. A recent meta-analysis of 25 major studies also revealed that *BRAF* mutation was significantly associated with poor differentiation, mucinous histology, and a proximal location (22). Generally, *BRAF*-mutated CRC has been associated with a MSI-H status (25-27). Although our study revealed that *BRAF* mutation was more frequently observed in the MSI-H subgroup (4/35; 11.4%) relative to the MSS/MSI-L subgroup (16/314; 5.1%), this difference was not statistically significant ($P = 0.128$).

In our study, the *BRAF* V600E mutation was found to be associated with other histopathological features of CRCs. Specifically, an infiltrative tumor border, lack of lymphovascular invasion, marked peritumoral lymphoid reaction, negative tumor budding status, and presence of a serrated or signet ring cell component were more frequently identified in *BRAF* V600E-mutated CRCs than in wild-type CRCs. Some of these features have also been identified in MSI-H CRCs (23). Therefore, we assessed the association between *BRAF* V600E mutation and clinicopathological findings in MSS/MSI-L CRCs. With the exception of tumor differentiation, all features that differed significantly between *BRAF*-mutated and wild-type CRCs retained this significant difference independently of the MSI status. In addition, pT stage ($P = 0.045$) and tumor size ($P = 0.025$) were found to be associated with *BRAF* mutation.

Tumor-associated inflammatory reactions are commonly observed in various types of cancers and are usually associated with prognostic behaviors. In CRCs, the immune microenvironment has been highlighted as a new marker of prognostic prediction. Tumor-infiltrating lymphocytes (TILs), especially CD8+ T cells, with anti-tumor effects and positive effects on survival have been discovered in several studies of CRC (24). In addition, some studies revealed that MSI might be associated with increased tumor-associated lymphocyte infiltration (25,26). Therefore, we evaluated peritumoral lymphoid reaction according to Klintrup's criteria in the current study. We found that a marked peritumoral lymphoid reaction was associated not only with the MSI-H status, but also with the *BRAF* V600E mutation independently of the MSI status. However, an active Crohn-like reaction was not found to be associated with the *BRAF* status. In addition, a peritumoral lymphoid reaction did not have a prognostic effect in the current study.

Whereas the peritumoral lymphocytic reaction is a well-known characteristic of MSI-H CRC, the direct relationship between *BRAF* mutation and tumor-associated lymphocytic inflammation remained unclear. In contrast to our findings, a previous study by Zlobec et al. (4) revealed that *BRAF* mutation was associated with the absence of a peritumoral lymphocytic reaction. These authors suggested that this absence might partly explain the poor prognosis of patients with *BRAF*-mutated CRCs. Yoon et al. (27) further demonstrated a lack of association between CD8+ T cell and FoxP3+ regulatory T cell infiltration and

 https://doi.org/10.3346/jkms.2017.32.1.38

BRAF mutation. Although we cannot explain the association of a marked peritumoral lymphoid reaction with *BRAF* mutation among MSS/MSI-L CRCs in our study or the clinical implication of this finding, the correlation between *BRAF* and tumor-associated lymphocytic reactions is clearly controversial, and related studies are limited. Accordingly, additional large population studies are needed.

In the overall survival analyses, only an advanced TNM stage, peritoneal carcinomatosis, lymphovascular invasion, and poor differentiation, all of which are well-known prognostic factors, were associated with poor overall survival. In contrast, although *BRAF* V600E mutation was associated with peritoneal seeding, lymphovascular invasion, and poor differentiation, it did not associate directly with survival even in the univariate analysis. Notably, this mutation was not related to overall survival even in the MSS/MSI-L subgroup. Furthermore, the MSI status, which is usually considered a prognostic factor for CRC, was not predictive of overall survival in our study.

However, several limitations of our study prevent us from concluding that the *BRAF* V600E mutation is not associated with prognosis in CRCs. First, the follow-up duration was too short to allow a survival analysis involving significant statistical outputs. In medical statistics, an average follow-up period of at least 5 years should be implemented. In contrast, the mean follow-up period in the current study was only 16 months, and the maximum follow-up duration was < 3 years for all cases. Second, the number of cancer-related deaths during the follow-up period was too small to achieve the minimum statistical power. Only 14 patients died from CRC. Therefore, an additional follow-up is needed to validate the prognostic significance of the *BRAF* mutation.

In conclusion, the incidence of the *BRAF* V600E mutation was relatively low in this study, in agreement with previous studies conducted in Eastern Asian countries. *BRAF*-mutated CRCs exhibited some clinicopathological features which were also frequently observed in MSI-H CRCs, such as a proximal location; mucinous, signet ring cell, and serrated components; and marked peritumoral lymphoid reactions. However, in this study even *BRAF* mutated CRCs without MSI-H status also show these findings. Thus they are distinct characteristics of *BRAF* mutated CRCs independently of the MSI status.

DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Research conception and design: Jang MH, Han HS. Data acquisition, analysis, and interpretation: Jang MH, Kim S. Statistical analysis: Jang MH. Drafting of the manuscript: Jang MH, Kim S. Critical revision of the manuscript: Hwang DY, Kim WY, Lim SD, Kim WS, Hwang TS, Han HS. Approval of final manuscript: all authors.

ORCID

Min Hye Jang http://orcid.org/0000-0002-4270-8897
Sehun Kim http://orcid.org/0000-0002-2637-9229
Dae Yong Hwang http://orcid.org/0000-0001-9082-8431
Wook Youn Kim http://orcid.org/0000-0003-4024-8791
So Dug Lim http://orcid.org/0000-0003-2036-0313
Wan Seop Kim http://orcid.org/0000-0001-7704-5942
Tea Sook Hwang http://orcid.org/0000-0002-3602-9257
Hye Seung Han http://orcid.org/0000-0002-3591-9995

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55: 74-108.
2. National Cancer Information Center (KR). Cancer Incidence trend analysis [Internet]. Available at http://www.cancer.go.kr/mbs/cancer/subview.jsp?id=cancer_040201000000 [accessed on 7 June 2015].
3. Ikenoue T, Hikiba Y, Kanai F, Tanaka Y, Imamura J, Imamura T, Ohta M, Ijichi H, Tateishi K, Kawakami T, et al. Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors. *Cancer Res* 2003; 63: 8132-7.
4. Zlobec I, Bihl MP, Schwarb H, Terracciano L, Lugli A. Clinicopathological and protein characterization of BRAF- and K-RAS-mutated colorectal cancer and implications for prognosis. *Int J Cancer* 2010; 127: 367-80.
5. Bogaert J, Prenen H. Molecular genetics of colorectal cancer. *Ann Gastroenterol* 2014; 27: 9-14.
6. Mao C, Liao RY, Chen Q. BRAF mutation predicts resistance to anti-EGFR monoclonal antibodies in wild-type KRAS metastatic colorectal cancer. *J Cancer Res Clin Oncol* 2010; 136: 1293-4.
7. Yuan ZX, Wang XY, Qin QY, Chen DF, Zhong QH, Wang L, Wang JP. The prognostic role of BRAF mutation in metastatic colorectal cancer receiving anti-EGFR monoclonal antibodies: a meta-analysis. *PLoS One* 2013; 8: e65995.
8. Cui D, Cao D, Yang Y, Qiu M, Huang Y, Yi C. Effect of BRAF V600E mutation on tumor response of anti-EGFR monoclonal antibodies for first-line metastatic colorectal cancer treatment: a meta-analysis of randomized studies. *Mol Biol Rep* 2014; 41: 1291-8.
9. Herr R, Köhler M, Andrlová H, Weinberg F, Möller Y, Halbach S, Lutz L, Mastroianni J, Klose M, Bittermann N, et al. B-Raf inhibitors induce epithelial differentiation in BRAF-mutant colorectal cancer cells. *Cancer Res* 2015; 75: 216-29.
10. Yaeger R, Saltz LB. RAF plus EGFR inhibition for BRAF-mutant metastatic colorectal cancer-response. *Clin Cancer Res* 2015; 21: 2188.
11. Thiel A, Ristimäki A. Toward a molecular classification of colorectal cancer: the role of BRAF. *Front Oncol* 2013; 3: 281.
12. French AJ, Sargent DJ, Burgart LJ, Foster NR, Kabat BF, Goldberg R, Shepherd L, Windschitl HE, Thibodeau SN. Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. *Clin*

Cancer Res 2008; 14: 3408-15.
13. Mouradov D, Domingo E, Gibbs P, Jorissen RN, Li S, Soo PY, Lipton L, Desai J, Danielsen HE, Oukrif D, et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. *Am J Gastroenterol* 2013; 108: 1785-93.
14. Kim JH, Bae JM, Oh HJ, Lee HS, Kang GH. Pathologic factors associated with prognosis after adjuvant chemotherapy in stage II/III microsatellite-unstable colorectal cancers. *J Pathol Transl Med* 2015; 49: 118-28.
15. Ueno H, Mochizuki H, Hashiguchi Y, Shimazaki H, Aida S, Hase K, Matsukuma S, Kanai T, Kurihara H, Ozawa K, et al. Risk factors for an adverse outcome in early invasive colorectal carcinoma. *Gastroenterology* 2004; 127: 385-94.
16. Klintrup K, Mäkinen JM, Kauppila S, Väre PO, Melkko J, Tuominen H, Tuppurainen K, Mäkelä J, Karttunen TJ, Mäkinen MJ. Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 2005; 41: 2645-54.
17. Kaserer K, Schmaus J, Bethge U, Migschitz B, Fasching S, Walch A, Herbst F, Teleky B, Wrba F. Staining patterns of p53 immunohistochemistry and their biological significance in colorectal cancer. *J Pathol* 2000; 190: 450-6.
18. Chung HW, Lee SY, Han HS, Park HS, Yang JH, Lee HH, So Y. Gastric cancers with microsatellite instability exhibit high fluorodeoxyglucose uptake on positron emission tomography. *Gastric Cancer* 2013; 16: 185-92.
19. Kim SK, Kim DL, Han HS, Kim WS, Kim SJ, Moon WJ, Oh SY, Hwang TS. Pyrosequencing analysis for detection of a BRAFV600E mutation in an FNAB specimen of thyroid nodules. *Diagn Mol Pathol* 2008; 17: 118-25.
20. Kim JH, Kang GH. Molecular and prognostic heterogeneity of microsatellite-unstable colorectal cancer. *World J Gastroenterol* 2014; 20: 4230-43.
21. Kang GH. Four molecular subtypes of colorectal cancer and their precursor lesions. *Arch Pathol Lab Med* 2011; 135: 698-703.
22. Chen D, Huang JF, Liu K, Zhang LQ, Yang Z, Chuai ZR, Wang YX, Shi DC, Huang Q, Fu WL. BRAFV600E mutation and its association with clinicopathological features of colorectal cancer: a systematic review and meta-analysis. *PLoS One* 2014; 9: e90607.
23. Sinicrope FA, Rego RL, Foster N, Sargent DJ, Windschitl HE, Burgart LJ, Witzig TE, Thibodeau SN. Microsatellite instability accounts for tumor site-related differences in clinicopathologic variables and prognosis in human colon cancers. *Am J Gastroenterol* 2006; 101: 2818-25.
24. Lavotshkin S, Jalas JR, Torisu-Itakura H, Ozao-Choy J, Lee JH, Sim MS, Stojadinovic A, Wainberg Z, Bifulco CB, Fox BA, et al. Immunoprofiling for prognostic assessment of colon cancer: a novel complement to ultrastaging. *J Gastrointest Surg* 2015; 19: 999-1006.
25. Gatalica Z, Torlakovic E. Pathology of the hereditary colorectal carcinoma. *Fam Cancer* 2008; 7: 15-26.
26. Jenkins MA, Hayashi S, O'Shea AM, Burgart LJ, Smyrk TC, Shimizu D, Waring PM, Ruszkiewicz AR, Pollett AF, Redston M, et al. Pathology features in Bethesda guidelines predict colorectal cancer microsatellite instability: a population-based study. *Gastroenterology* 2007; 133: 48-56.
27. Yoon HH, Orrock JM, Foster NR, Sargent DJ, Smyrk TC, Sinicrope FA. Prognostic impact of FoxP3+ regulatory T cells in relation to CD8+ T lymphocyte density in human colon carcinomas. *PLoS One* 2012; 7: e42274.

 https://doi.org/10.3346/jkms.2017.32.1.38