SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 20: 655-666, 2020

Histology and its prognostic effect on *KRAS*-mutated colorectal carcinomas in Korea

HYE SEUNG LEE[1], DAE YONG HWANG[2] and HYE SEUNG HAN[3]

[1]Department of Pathology, Korea Clinical Laboratory, Seoul 05396; Departments of [2]Surgery and [3]Pathology, Konkuk University School of Medicine, Seoul 05030, Republic of Korea

Received August 29, 2019; Accepted April 15, 2020

DOI: 10.3892/ol.2020.11606

Abstract. *KRAS* mutation is frequently identified in advanced colorectal carcinoma (CRC); however, its prognostic significance and the associated histological features have remained to be clarified. In the present study, the precise histological results and prognostic value of *KRAS*-mutated CRCs were investigated in patients from South Korea. A retrospective review of the results from *KRAS* mutation testing, as well as evaluation of the histology of 310 cases of CRC at various stages, were performed. Cross-tabulation and survival analysis were performed according to the *KRAS* status. Patients with *KRAS* mutation more frequently exhibited serrated and papillary architectures (P=0.009 and P=0.014, respectively). *KRAS* mutation was an independent unfavorable prognostic factor for overall survival (OS) according to multivariate analysis (P=0.001), whereas no association was observed with disease-free survival (DFS) (P=0.611). Of note, in the subgroup of *KRAS*-mutated carcinomas, the presence of a solid component on histology was associated with less favorable OS (P=0.032). Furthermore, among the wild type cases, patients with a micropapillary component had a worse OS than those who did not (P=0.018). However, no subgroup or specific histological features were associated with DFS. In summary, *KRAS*-mutated CRCs had a moderate association with particular histological features, and according to the *KRAS* mutational status, there was a certain degree of association between histology and prognosis.

Correspondence to: Dr Hye Seung Han, Department of Pathology, Konkuk University School of Medicine, 120-1 Neungdong-ro, Gwang-Jin Gu, Seoul 05030, Republic of Korea
E-mail: aphsh@kuh.ac.kr

Abbreviations: CRC, colorectal carcinoma; KUMC, Konkuk University Medical Center; TIL, tumor-infiltrating lymphocytes; OS, overall survival; DFS, disease-free survival; HR, hazard ratio

Key words: KRAS, colorectal carcinoma, mutation, histology, prognosis

Introduction

Colorectal carcinoma (CRC) is the third most common cancer type worldwide, and its prevalence is rapidly increasing in the Republic of Korea with an annual increment of ~6.5% in males and females (1). Despite the decrease in the associated mortality rate and moderate advances in treatment, CRC remains the third highest cause of cancer-associated mortality in South Korea, with a 5-year overall survival (OS) rate of 59% (2,3).

To date, molecular genetic studies have identified several dominant somatic or germline genetic alterations associated with an underlying predisposition to CRCs. There are three common pathways responsible for sporadic CRCs: Chromosomal instability, microsatellite instability and CpG island methylator phenotype pathways (4). Most cases of CRCs result from the chromosomal instability pathway, which is characterized by the traditional adenoma-carcinoma sequence, including early loss of the adenomatous polyposis coli gene, *KRAS* point mutation, 18q loss of heterozygosity and late tumor protein (TP)53 inactivation. *KRAS* mutation has a role in the chromosomal instability pathway, mainly in the progression from early to late advanced adenoma in ~30% of adenomas and 30-50% of CRCs (5,6).

Ras family proteins are the prototypes of the small guanosine triphosphatases (GTPases) and regulate multiple intracellular processes, including growth, differentiation, immunity and survival. The *KRAS* oncogene belongs to the Ras family, which also includes *HRAS* and *NRAS*. *KRAS* encodes a 21-kDa GTPase protein called K-Ras, which is, under normal circumstances, temporarily activated as a response to certain signals, including cytokines, hormones, growth factors and external stimuli (7). However, *KRAS* mutation leads to continuous activation of the mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling pathways by the K-Ras protein, which may potentially stimulate tumorigenesis or tumor progression. *KRAS* point mutation frequently occurs at exon 2, particularly in codons 12 and 13. Clinically, *KRAS* is a well-established biomarker of resistance to anti-epidermal growth factor receptor (EGFR) antibody treatment in advanced CRCs (7). However, the prognostic significance of *KRAS* mutation remains controversial (8,9).

A number of studies have demonstrated an association between certain molecular subtypes and histology of CRCs;

for instance, a previous study revealed that the majority of sessile serrated adenomas harbor a B-Raf proto-oncogene serine/threonine kinase (*BRAF*) mutation or a CpG island hypermethylation (9). Microsatellite instability-high CRCs frequently exhibit poorly differentiated and mucinous features (10). Regarding *KRAS* mutation, an association with well-to moderately differentiated conventional adenocarcinoma histology has been suggested, but as yet there is no consensus on this (8,11). Thus, the present study investigated the prognostic value of *KRAS*-mutation in CRCs and its association with histologic features.

Materials and methods

Patient cohort. Using Systematized Nomenclature of Medicine-Clinical Terms (SNOMED-CT) (12), 310 patients who had been diagnosed with adenocarcinoma of the colon or rectum at various stages were selected, and *KRAS* mutation testing was performed between January 2011 and December 2014 at the Konkuk University Medical Center (KUMC; Seoul, Republic of Korea). Clinicopathological data including age, sex, patient history and reports of imaging, surgery and pathology, were obtained from the electronic medical records. Based on the patients' age, they were divided into 2 groups according to a study by Yang *et al* (13): Those under 60 years and those ≥60 years. Based on the tumor location, the patients were stratified into the following subgroups: 'Right-sided' (from the cecum to the transverse colon) and 'left-sided' (from the descending colon to the rectum). According to tumor size, the patients were also divided into 2 groups, <5 cm and ≥5 cm, based on previous studies (14-16).

Hematoxylin and eosin (H&E) stained slides were reviewed for 267/310 patients who underwent surgical resection for CRC. For survival analyses, 22/267 patients were excluded due to inter-hospital transfer during the follow-up period. The median follow-up period of the 245 patients was 37.4 months (range, 1.0-60.0 months).

KRAS mutation analysis. All DNA extraction and pyrosequencing were performed according to methods routinely used at KUMC (17). In all cases, tumor-rich areas detected on microscopic examination were marked on the formalin-fixed, paraffin-embedded tissue slides by a pathologist (HSL). After removing the cover glass, tumor cells were scraped using a 26-gauge needle and 50-100 μl of DNA extraction buffer solution [including 50 mM Tris buffer, pH 8.3; 1 mM EDTA, pH 8.0; 5% Tween-20 (Sigma-Aldrich; Merck KGaA, Germany), 200 μg/ml proteinase K; and 10% resin] was added to the scraped cells. After incubation for at least 1 h at 56˚C, each tube was heated for 20 min at 100˚C, followed by centrifugation at 4˚C for 10 min at 13,000 x g to pellet the debris. The recovered supernatant was used for PCR. The amount of genomic DNA was spectrophotometrically determined using a Qubit assay kit 3.0 (Thermo Fisher Scientific, Inc.).

PCR primer sequences used for the amplification of *KRAS* gene were as follows: Codons 12 and 13, 5'-CTGGTGGAGTAT TTGATAGTGTA-3' (forward) and 5'-biotin-TGGTCCTGC ACCAGTAATAT-3' (reverse); and codon 61, 5'-biotin-TCC AGACTGTGTTTCTCCCTTC-3' (forward) and 5'-TACTGG TCCCTCATTGCACTGT-3' (reverse). A total of 10-20 ng/μl of DNA were added to each 50 μl of PCR solution mixture [0.2 mmol each of deoxynucleoside triphosphate, 1.5 mmol/l $MgCl_2$, 1X PCR buffer, 1.5 U of Immolase™ DNA polymerase (Bioline) and 20 pmol of each primer]. PCR was performed with an initial denaturation for 5 min at 95˚C followed by 40 cycles of 30 sec at 95˚C, annealing of the primers for codons 12, 13 and 61 for 30 sec at 55˚C, primer extension for 30 sec at 72˚C, and a final incubation for 10 min at 72˚C. Electrophoresis of the PCR products was performed in an agarose gel to confirm amplification. Immobilization of biotinylated PCR products onto streptavidin-coated beads (GE Healthcare) using the solution from the PSQ™ 96 sample Preparation kit (Qiagen) was performed, according to the manufacturer's instructions. Following 20-fold dilution of 3 μl beads in binding buffer (37 μl) and distilled water (20 μl) with 10 μl biotinylated PCR products, incubation for 10 min at room temperature was performed. The beads were then transferred to a filter probe and the liquid was removed by vacuum filtration. The DNAs were separated in PyroMark denaturation solution (Qiagen GmbH) for 2 min at room temperature. The templates were washed in PyroMark wash buffer (Qiagen GmbH), transferred to a PSQ 96 single nucleotide polymorphism (SNP) plate and then annealed with sequencing primers 5'-ATAAACTTGTGG TAGTTGG-3' (codons 12 and 13) and 5'-CCTCATTGCACT GTAC-3' (codon 61), in PyroMark annealing buffer (Qiagen GmbH) at room temperature. Finally, pyrosequencing was performed using the PyroMark Q96 ID system (Qiagen), with PyroMark Gold Q96 Reagents (Qiagen GmbH), according to the manufacturer's instructions.

Histological evaluation. One pathologist (HSL) reviewed all H&E and immunohistochemistry slides according to the 2010 World Health Organization (WHO) classification and American Joint Committee on Cancer (AJCC) staging manual, 8th edition TNM staging (18,19). Histological features were comprehensively evaluated for tumor border, differentiation, degree of patterns (cribriform, serrated, mucinous, signet ring cell, solid, papillary and micropapillary), degree of inflammatory reactions associated with CRCs [Crohn's-like lymphoid reaction, neutrophilic infiltration and tumor-infiltrating lymphocytes (TILs)], dirty necrosis, tumor budding counts, lymphatic invasion, vascular invasion and perineural invasion.

Specifically, the microscopic tumor border was divided into two groups according to the fraction of the infiltrative border area: <50 and ≥50%. Tumor differentiation was graded into three groups according to the 2010 WHO classification: Well, moderately and poorly differentiated. Each histological feature was divided into two or three categories, depending on the degree of each pattern: Cribriform (absent, mild or moderate), serrated (absent, mild or moderate), signet ring cell (absent or present), solid (absent or present), papillary (absent or present) and micropapillary (absent or present).

Mucinous components were divided into two groups by the quality of mucin (low or high grade) (20). The Q score for mucinous components was calculated by multiplying the quality of mucin by the proportion of the area. Crohn's-like lymphoid reaction was defined as the degree of peritumoral lymphoid aggregate ≥1 mm (absent to mild or moderate) (21). Intraluminal necrotic debris (dirty necrosis) was graded into five degrees: Absent, low, moderate, high or confluent.

Neutrophil infiltration was graded into four degrees: Absent, small numbers and scattered in the stroma, focal abscesses within tumor glands or numerous abscesses disrupting tumor glands (22). The density of TILs at the hot spot area of the tumor border was evaluated at a magnification of x20 and subdivided into two groups: <50 and ≥50% (23). The tumor budding count was determined, and cases were subdivided into low, intermediate and high groups, according to the recommendations of the International Tumor Budding Consensus Conference in 2016 (24). The presence of lymphatic, vascular and perineural invasion was also re-evaluated. For *TP53*, expression in at least 50% of the tumor nuclei was regarded as positive, according to previous studies (25,26).

Statistical analysis. Statistical analysis was performed using SPSS version 19 (IBM, Corp.). Comparison of clinicopathological factors between patients with *KRAS* mutation and wild type *KRAS* was performed using Pearson's χ^2 test (n=310). Histological findings between patients with *KRAS* mutation and wild type *KRAS* were also compared using Pearson's χ^2 test (n=267). In survival analyses (n=245), the OS rate was calculated as the rate of survivors from the onset of typical CRC symptoms, including bowel habit changes, bloody stool, continuously localized abdominal pain or anemia until the date of the last clinical follow-up. The DFS rate was calculated based on the date of symptom onset until the date of the detection of local recurrence or metastasis by imaging observation. The Cox proportional hazards model was used for univariate and multivariate survival analyses to estimate the hazard ratios (HRs) for patients. The results are presented as estimated HRs with 95% confidence intervals (CIs) and Wald test P-values. The parameters used in the multivariate analysis of 245 patients are as follows: *KRAS* mutation, age, sex, grade of regression after neoadjuvant therapy, tumor location, tumor size, T stage, N stage, M stage, total TNM stage, *TP53* expression, tumor border and differentiation, and comprehensive histological features including lymphatic, vascular, and perineural invasions. The parameters used in the separate multivariate analysis in each '*KRAS*-mutated' (n=91) and 'wild type' (n=154) subgroup are as follows: Age, sex, tumor location, tumor size, M stage, tumor border, tumor differentiation, and comprehensive histological features except lymphovascular and perineural invasions. The proportional hazards assumption was assessed using graphical methods and tests based on Schoenfeld residuals. The Kaplan-Meier method and the log-rank test was used to estimate the OS curves. A two-tailed P-value was used for all analyses, with P<0.05 considered to indicate statistical significance.

Results

Patient characteristics and KRAS mutation subtypes. The clinicopathological characteristics stratified by *KRAS* mutation status are presented in Table I. Of all patients, 195 (62.9%) had wild type *KRAS* and 115 (37.1%) had mutations of *KRAS*. There were 176 males and 134 females, with a median age of 62 years (age range, 27-88 years). The samples were predominantly acquired from the primary tumor sites (n=298, 96.1%). A total of 28 patients (9.0%) received neoadjuvant therapy and exhibited minimal to near complete regression. A total of 34 patients (11.0%) received adjuvant anti-EGFR therapy (cetuximab) in combination with irinotecan (n=14, 41.2%), folinic acid + fluorouracil + irinotecan (FOLFIRI; n=19, 55.9%) or folinic acid + fluorouracil + oxaliplatin (FOLFOX; n=1, 2.9%). The mean tumor size was 4.83 cm (range, 1-13 cm) and the number of samples with ≥5 cm was 140 (45.3%). The initial distribution of TNM stages in the cohort was as follows: 0 (1.6%), I (9.4%), II (14.2%), III (28.1%) and IV (46.8%). There were no significant differences in age, sex, acquisition site, grade of regression after neoadjuvant therapy, tumor size, T stage, N stage, M stage, and total TNM stage between the groups with different *KRAS* mutation status. Generally, the tumors were in the left colon in both *KRAS*-mutated and wild type groups. However, *KRAS*-mutated CRCs were more likely to be in the right colon than wild type (P=0.014). In addition, absence of *TP53* expression was apparent in only wild type CRCs (P=0.038).

The distribution of *KRAS* mutational changes is presented in Table II. Most of the mutations occurred in exon 2 (114/115, 99.1%), and among these, mutations in codon 12 were more common compared with those in codon 13 (76.3% vs. 23.7%, respectively). The three most common amino acid changes were G12D (n=44, 38.3%), G12V (n=26, 22.6%) and G13D (n=24, 20.9%).

Association of KRAS mutation with survival. The results of univariate and multivariate survival analyses in 245 patients are presented in Table SI (OS) and Table SII (DFS), respectively. The factors for the multivariate analysis included *KRAS* mutation, age, sex, tumor location, tumor size, TNM stage and comprehensive histological features. Overall, *KRAS* mutation was an unfavorable prognostic factor in terms of OS, according to the univariate (P=0.023; HR, 1.593; 95% CI, 1.065-2.382) and multivariate analyses (P=0.001; HR, 2.49; 95% CI, 1.427-4.343) (Table SI). The cumulative OS rate of patients with *KRAS* mutation using log-rank test was lower compared with that of patients with wild type *KRAS* (P=0.021, 40.9 vs. 53.8%) (Fig. 1A). However, the *KRAS* mutation status was not associated with DFS in univariate (P=0.611; HR, 1.097; 95% CI, 0.769-1.564) and multivariate (P=0.365; HR, 1.221; 95% CI, 0.793-1.878) analyses (Table SII). Of the common mutation types, patients with G12D and G13D mutations had significantly lower OS rates compared with those with wild type *KRAS* (P=0.003; Fig. 1B). However, there was no significant difference between the OS curves for G12V and wild type *KRAS* (P=0.999; Fig. 1C).

Histological features according to KRAS mutation status. The cross-tabulation analysis results of the comprehensive histological examination are summarized in Table III. For accurate morphological analysis, only surgically resected specimens (n=267) were included. In general, similar histological findings were revealed in patients with mutations and those with wild type *KRAS*; however, samples from those with mutations exhibited more prominent serrated (Fig. 2A) and/or papillary (Fig. 2B) patterns (P=0.009 and P=0.014, respectively).

Influence of clinicopathological features on OS according to KRAS mutation status. Further univariate and multivariate survival analyses for OS were performed on the '*KRAS*-mutated' and 'wild type' CRC subgroups.

Table I. Clinicopathological features of 310 patients with primary colorectal cancer stratified by KRAS mutation.

Characteristic	Total (n=310)	*KRAS* mutation status		P-value
		Wild type (n=195, 62.9%)	Mutated (n=115, 37.1%)	
Sex				0.587
Female	134 (43.2)	82 (42.1)	52 (45.2)	
Male	176 (56.8)	113 (57.9)	63 (54.8)	
Age (years)				0.281
<60	136 (43.9)	81 (41.5)	55 (47.8)	
≥60	174 (56.1)	114 (58.5)	60 (52.2)	
Acquisition site				0.738
Primary	298 (96.1)	188 (96.4)	110 (95.7)	
Metastatic	12 (3.9)	7 (3.6)	5 (4.3)	
Grade of regression after neoadjuvant therapy				0.778
Not received	282 (91.0)	178 (91.3)	104 (90.4)	
Minimal	16 (5.2)	11 (5.6)	5 (4.3)	
Moderate	10 (3.2)	5 (2.6)	5 (4.3)	
Near complete	2 (0.6)	1 (0.5)	1 (0.9)	
Anti-EGFR therapy				<0.001
Not received	276 (89.0)	161 (82.6)	115 (100.0)	
Cetuximab + Irinotecan	14 (4.5)	14 (7.2)	0 (0.0)	
Cetuximab + FOLFIRI	19 (6.1)	19 (9.7)	0 (0.0)	
Cetuximab + FOLFOX	1 (0.3)	1 (0.5)	0 (0.0)	
Tumor location				0.014
Right-sided	78 (25.2)	40 (20.5)	38 (33.0)	
Left-sided	232 (74.8)	155 (79.5)	77 (67.0)	
Tumor size (cm)				0.463
<5	169 (54.7)	103 (53.1)	66 (57.4)	
≥5	140 (45.3)	91 (46.9)	49 (42.6)	
T stage				0.713
Tis	5 (1.6)	3 (1.5)	2 (1.7)	
T1	20 (6.5)	13 (6.7)	7 (6.1)	
T2	48 (15.5)	32 (16.4)	16 (13.9)	
T3	177 (57.1)	114 (58.5)	63 (54.8)	
T4	60 (19.4)	33 (16.9)	27 (23.5)	
N stage				0.511
N0	111 (35.8)	71 (36.4)	40 (34.8)	
N1	96 (31.0)	56 (28.7)	40 (34.8)	
N2	103 (33.2)	68 (34.9)	35 (30.4)	
M stage				0.645
M0	167 (53.9)	107 (54.9)	60 (52.2)	
M1	143 (46.1)	88 (45.1)	55 (47.8)	
TNM stage				0.692
0	5 (1.6)	3 (1.5)	2 (1.7)	
I	29 (9.4)	18 (9.2)	11 (9.6)	
II	44 (14.2)	25 (12.8)	19 (16.5)	
III	87 (28.1)	60 (30.8)	27 (23.5)	
IV	145 (46.8)	89 (45.6)	56 (48.7)	
TP53 expression				0.038
Negative (<50%)	221 (78.6)	140 (79.5)	81 (77.1)	
Positive (≥50%)	8 (2.8)	8 (4.5)	0 (0.0)	
Not determined	52 (18.5)	28 (15.9)	24 (22.9)	

Values are expressed as n (%). EGFR, epidermal growth factor receptor; FOLFIRI, folinic acid + fluorouracil + irinotecan; FOLFOX, folinic acid + fluorouracil + oxaliplatin; TP53, tumor protein 53; T, tumor; N, nodes; M, metastasis.

Table II. Distribution of *KRAS* mutation variants in colorectal carcinoma (n=115).

A, Exon 2 (n=114)		
Codon/position/ base change	Amino acid change	n (%)
Codon 12 (n=87)		
Position 34 (n=12)		
G>T	G12C	8 (7.0)
G>A	G12S	3 (2.6)
G>C	G12R	1 (0.9)
Position 35 (n=75)		
G>A	G12D	44 (38.3)
G>T	G12V	26 (22.6)
G>C	G12A	5 (4.3)
Codon 13 (n=27)		
Position 37 (n=3)		
G>T	G13C	2 (1.7)
G>C	G13R	1 (0.9)
Position 38 (n=24)		
G>A	G13D	24 (20.9)

B, Exon 3 (n=1)		
Codon/position/ base change	Amino acid change	n (%)
Codon 61		
Position 182		
A>G	Q61R	1 (0.9)

On the basis of the univariate analysis of '*KRAS*-mutated' subgroup (Table SIII), tumor size ≥5 cm (P=0.030), high initial TNM stage (P<0.001), presence of signet ring cells (P=0.021), absent to mild Crohn's-like lymphoid reaction (P=0.020), absence of dirty necrosis (P=0.021) and paucity of neutrophilic infiltration (P=0.002) were associated with lower OS rate. However, in multivariate analysis (Table IV), those who were aged ≥60 years (P=0.023; HR, 3.058; 95% CI, 1.169-7.995), male (P=0.034; HR, 2.747; 95% CI, 1.079-6.995), M1 stage at diagnosis (P=0.029; HR, 5.608; 95% CI, 1.197-26.279) and particularly the presence of solid component (representative histology image, see Fig. 2C) were associated with a lower OS rate (P=0.032; HR, 4.040; 95% CI, 1.127-14.488).

By contrast, in the 'wild type' subgroup, right-sided tumor location (P=0.021), high initial TNM stage (P<0.001), infiltrative tumor border ≥50% (P=0.046), poorly-differentiated tumor (P=0.006), moderate cribriform pattern (P=0.006), presence of signet ring cells (P=0.002), absence of dirty necrosis (P=0.002), absence of neutrophilic infiltration (P=0.041) and high tumor budding grade (P=0.005) were the independent prognostic factors for worse OS rate in univariate analysis (Table SIII). However, in the multivariate analysis (Table IV), initial M1 stage (P<0.001) and the presence of a micropapillary component (representative histology image, see Fig. 2D) were associated with a lower OS rate (P=0.018; HR, 2.908; 95% CI, 1.205-7.017).

Influence of clinicopathological features on DFS according to KRAS mutation status. Further univariate and multivariate survival analyses for DFS were performed on the '*KRAS*-mutated' and 'wild type' CRC subgroups.

On the basis of the univariate analysis in the '*KRAS*-mutated' subgroup (Table SIV), high initial TNM stage (P<0.001), mild or absence of Crohn's-like lymphoid reaction (P=0.033) and paucity of neutrophilic infiltration (P=0.004) were associated with lower DFS rate. However, only initial M1 stage (P<0.001) exhibited statistical significance following multivariate analysis (Table SV).

On the basis of univariate analysis in the 'wild type' subgroup (Table SIV), high initial TNM stage (P<0.001), presence of signet ring cells (P=0.020), paucity of neutrophilic infiltration (P=0.027) and TIL<50% (P=0.018) were independent prognostic factors for DFS. However, only initial M1 stage (P<0.001) was associated with lower DFS in multivariate analysis (Table SV).

Influence of clinicopathological factors on OS. The results of univariate and multivariate analyses of OS are presented in Table SI. Right-sided tumor location (P=0.016), tumor size ≥5 cm (P=0.025), high initial TNM stage (P<0.001), *TP53* positivity (P=0.005), high-grade tumor differentiation (P=0.006), a moderate cribriform pattern (P=0.047), a signet ring cell pattern (P<0.001), absence of Crohn's-like lymphoid reaction (P=0.009), absence of dirty necrosis (P<0.001), paucity of neutrophilic infiltration (P=0.003), a high tumor budding grade (P=0.050) and presence of lymphatic or vascular or perineural invasion (P=0.001, P<0.001, and P<0.001, respectively) were unfavorable prognostic factors regarding OS, according to univariate analysis. However, according to the multivariate analysis, age ≥60 (P=0.001), right-sided tumor location (P=0.036), tumor size ≥5 cm, a high initial TNM stage (P=0.001), a high tumor budding grade (P=0.021) and vascular invasion (P=0.049) were associated with poor OS.

Influence of clinicopathological factors on DFS. Local recurrence or distant metastasis occurred in 128 (47.8%) patients during the follow-up period, but there was no statistical significance (30.692 vs. 35.013 months, respectively). The cumulative DFS was 44.9% for the *KRAS*-mutated group and 49.1% for the wild type group (P=0.518). The results of univariate and multivariate analyses between clinicopathological factors and DFS are summarized in Table SII. Tumor size ≥5 cm (P=0.025) and high initial TNM stage (P<0.001), a signet ring cell pattern (P=0.006), absence of Crohn's-like lymphoid reaction (P=0.038), less neutrophilic infiltration (P=0.001), TIL <50% (P=0.013) and presence of lymphatic or vascular or perineural invasions (P<0.001, P=0.001 and P=0.009, respectively) were associated with a lower DFS rate based on univariate analysis. However, only initial M1 stage (P<0.001) reached significance in the multivariate analysis.

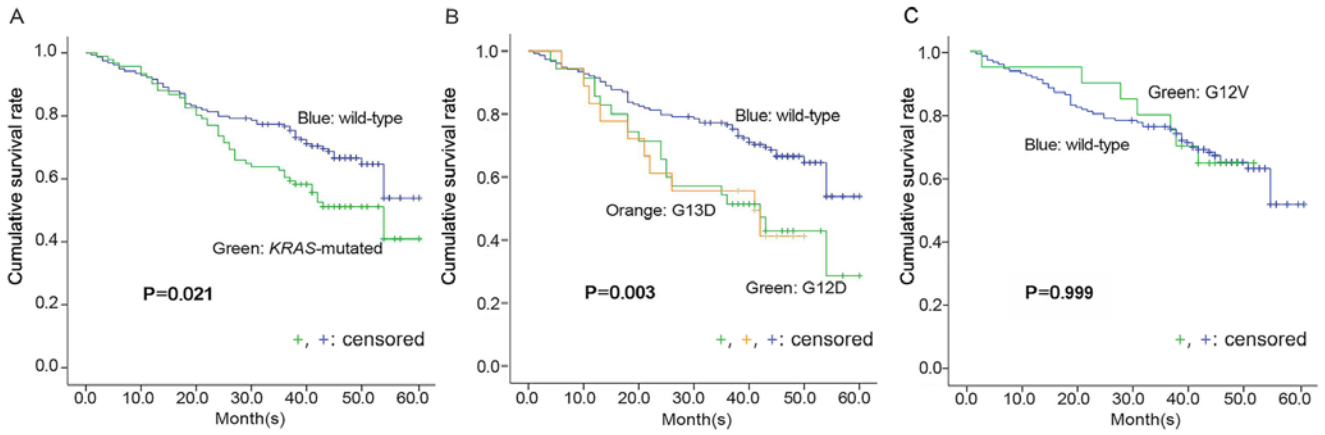

Figure 1. Kaplan-Meier plots for overall survival rates in patients with colorectal carcinoma stratified by *KRAS* mutation status. (A) Those with *KRAS* mutations had a worse overall survival than those with wild type *KRAS* (P=0.021). (B) Patients with G12D and G13D had poorer overall survival than patients with wild type *KRAS* (P=0.003). (C) The overall survival curves of patients with G12V were not significantly different from those of patients with wild type *KRAS* (P=0.999).

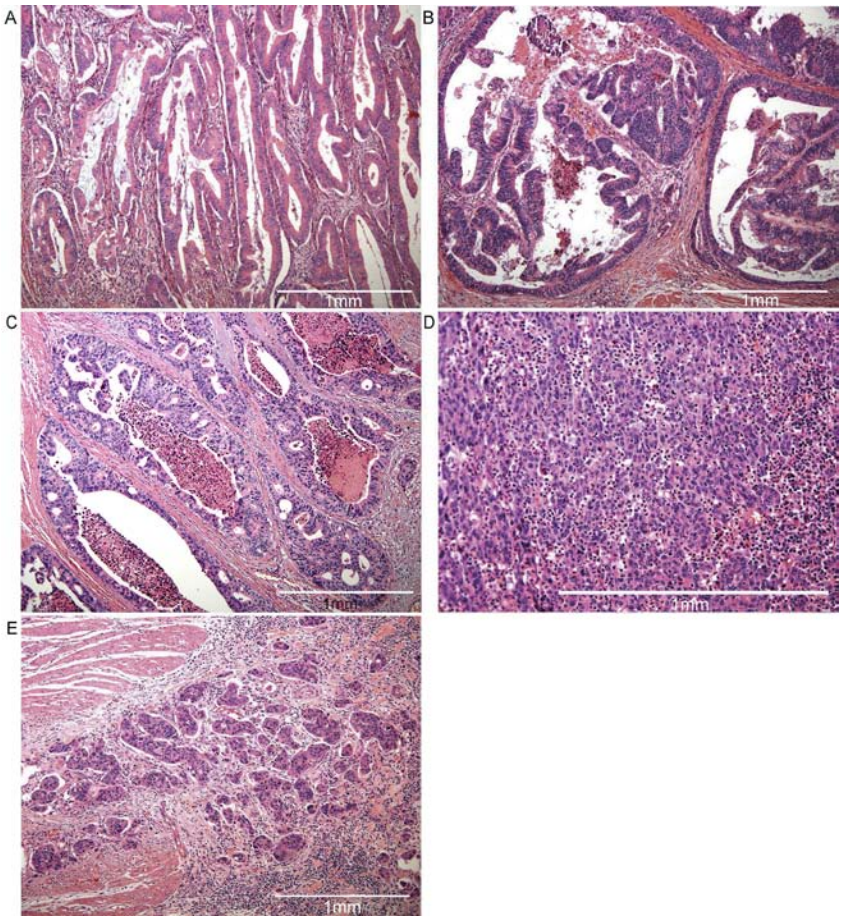

Figure 2. Representative histologic features and independent prognostic factors according to *KRAS* mutation status (H&E stain; scale bar, 1 mm). The *KRAS*-mutated subgroup exhibited more degree of (A) serrated (original magnification, x100) and (B) papillary architectures (magnification, x100) than the histology of wild types. (C) In contrast, the wild type subgroup exhibited less degree of serrated architecture and/or absence of papillary component compared with the *KRAS*-mutated subgroup (magnification, x100). (D) *KRAS*-mutated colorectal carcinoma with a prominent solid component were associated with poor overall survival (magnification, x200). (E) Survival analysis of the 'wild type' subgroup (n=154) demonstrated that patients with a micropapillary pattern (n=26, 16.9%) had a worse overall survival rate than those without a micropapillary pattern (n=128, 83.1%) (magnification, x100).

Discussion

In the present study, the prevalence of *KRAS* mutation and common amino acid changes determined were consistent with the results of previous studies (27,28). The results of the present study support those of previous studies, with a more frequent right-sided tumor location in patients with mutations and rare simultaneous *TP53* and *KRAS* mutation (29,30). Of note, *KRAS* mutation was a significant prognostic marker of OS with a 2.5-fold increased HR. There has been controversy

Table III. Comparison of histologic findings according to *KRAS* mutation status.

Morphologic characteristic	Total (n=267)	*KRAS* mutation status		P-value
		Wild type	Mutated	
Infiltrative tumor border (%)				0.932
<50	48 (18.0)	31 (18.1)	17 (17.7)	
≥50	219 (82.0)	140 (81.9)	79 (82.3)	
Degree of differentiation				0.208
Well	35 (13.1)	20 (11.7)	15 (15.6)	
Moderate	208 (77.9)	132 (77.2)	76 (79.2)	
Poor	24 (9.0)	19 (11.1)	5 (5.2)	
Cribriform pattern				0.734
Absent	35 (13.1)	22 (12.9)	13 (13.5)	
Mild	172 (64.4)	108 (63.2)	64 (66.7)	
Moderate	60 (22.5)	41 (24.0)	19 (19.8)	
Serrated pattern				0.009
Absent	104 (39.0)	75 (43.9)	29 (30.2)	
Mild	142 (53.2)	88 (51.5)	54 (56.3)	
Moderate	21 (7.9)	8 (4.7)	13 (13.5)	
Quality of mucin (n=66)				0.190
Low	50 (75.8)	25 (69.4)	25 (83.3)	
High	16 (24.2)	11 (30.6)	5 (16.7)	
Q score of mucin (n=66)				0.327
<60	42 (63.6)	21 (58.3)	21 (70.0)	
≥60	24 (36.4)	15 (41.7)	9 (30.0)	
Signet ring cells				0.245
Absent	253 (94.8)	160 (93.6)	93 (96.9)	
Present	14 (5.2)	11 (6.4)	3 (3.1)	
Solid component (%)				0.195
<50	208 (77.9)	129 (75.4)	79 (82.3)	
≥50	59 (22.1)	42 (24.6)	17 (17.7)	
Papillary component				0.014
Absent	149 (55.8)	105 (61.4)	44 (45.8)	
Present	118 (44.2)	66 (38.6)	52 (54.2)	
Micropapillary component				0.763
Absent	220 (82.4)	140 (81.9)	80 (83.3)	
Present	47 (17.6)	31 (18.1)	16 (16.7)	
Crohn's-like lymphoid reaction				0.923
Absent to mild	177 (66.3)	113 (66.1)	64 (66.7)	
Moderate	90 (33.7)	58 (33.9)	32 (33.3)	
Dirty necrosis				0.345
Absent	17 (6.4)	11 (6.4)	6 (6.3)	
Low	51 (19.1)	32 (18.7)	19 (19.8)	
Moderate	81 (30.3)	51 (29.8)	30 (31.3)	
High	48 (18.0)	26 (15.2)	22 (22.9)	
Confluent	70 (26.2)	51 (29.8)	19 (19.8)	
Neutrophilic infiltration				0.055
Absent	58 (21.7)	45 (26.3)	13 (13.5)	
Low and scattered	88 (33.0)	56 (32.7)	32 (33.3)	
Focal abscesses	65 (24.3)	35 (20.5)	30 (31.3)	
Numerous abscesses	56 (21.0)	35 (20.5)	21 (21.9)	

Table III. Continued.

Morphologic characteristic	Total (n=267)	*KRAS* mutation status		P-value
		Wild type	Mutated	
Tumor-infiltrating lymphocytes (%)				0.133
<50	194 (72.7)	119 (69.6)	75 (78.1)	
≥50	73 (27.3)	52 (30.4)	21 (21.9)	
Tumor budding grade				0.872
Low	150 (56.2)	97 (56.7)	53 (55.2)	
Intermediate	73 (27.3)	45 (26.3)	28 (29.2)	
High	44 (16.5)	29 (17.0)	15 (15.6)	

over the prognostic value of *KRAS* mutation, but a recent review strengthens the evidence of a negative clinical effect of the mutation in metastatic CRC (P<0.001; HR, 1.674; 95% CI, 1.341-2.089) (8,31). In addition, the cumulative OS rate of patients with *KRAS* mutations was lower compared with that reported previously and this difference may be due to low patient numbers or shorter observational periods compared with those in the present study (32-34).

Furthermore, G12D and G13D mutations resulted in a worse OS rate compared with wild type *KRAS* (P=0.035). While mutations of codons 12 or 13 have been widely studied in CRCs, their impact on clinical action has been debated (35). Thus far, the prevailing view is that codon 12 mutation results in poor clinical outcome, contrary to the case with codon 13 (36,37). There is more evidence of decreased sensitivity or reduced survival in patients with G12D and G13D mutations (38,39). Different results were reported for G12V, which has been indicated to result in lower DFS and OS rates (32,37). However, further verification is required, since, unlike the present study, most studies involved only metastatic CRCs.

The prognostic role of *KRAS* mutation in DFS has been debated. In particular, most studies, including those on non-metastatic CRCs, concluded that *KRAS* mutations are associated with poor DFS (40-43). However, studies including metastatic CRCs indicated no association between *KRAS* mutations and DFS (44-46). Inoue *et al* (47) also revealed that the *KRAS* genotype had no effect on DFS of patients at stage IV, but G13D was a poor prognostic factor for DFS of patients at stage I-III. Similarly, the present study revealed no significant prognostic value of *KRAS* mutation for DFS, including metastatic CRCs (stage 0-IV). However, stage 0-III CRCs exhibited a trend of association between the KRAS-mutated genotype and lower DFS (P=0.059), in accordance with the results of previous studies (40-43).

The present study was performed on a specific ethnic group as the cohort. Most of the studies from South Korea have indicated that *KRAS* is not associated with either OS or DFS (48,49) except for that by Lee *et al* (42), according to which DFS was shorter in stage II and III patients treated with FOLFOX. However, the present results are in accordance with those obtained by certain other studies in East Asian countries, which indicated an association between *KRAS* mutation and a lower OS rate (32,47). By contrast, *KRAS* was not associated with prognosis in most studies from in Southwest Asia and South America (34,50-52). However, in Caucasians, the results were controversial. Studies performed in Italy and Spain suggested shorter OS and DFS of patients with *KRAS* mutation (53,54). However, studies performed in Sweden and Australia indicated no association between *KRAS* mutation and OS (8,55). Furthermore, a study from Austria reported that *KRAS*-mutated patients had better OS, except for those with G12V mutation (56).

Of note, patients with *KRAS* mutation presented with more serrated and/or papillary features compared with those with wild type *KRAS*. A previous study also demonstrated that *KRAS* is involved in the traditional serrated pathway, in addition to the conventional pathway of the pathogenesis of CRCs (57). However, in the mutation group, the serrated feature itself was not associated with prognosis (P=0.242) and this result was similar to that in the whole population (P=0.329). The biological behavior of CRCs with marked serrated features has been debated; however, their prognostic impact appears to be defined by molecular traits rather than by morphology (58,59). Taken together, *KRAS* mutation may be involved in the morphogenesis of serration, but the prognostic value of the serrated pattern in patients with mutations remains uncertain.

Similarly, certain studies reported on CRCs with papillary or villous features and designated them as papillary, adenoma-like or villous adenocarcinoma (60-62). These tumors were less likely to be associated with lymph node metastasis, absence of aberrant p53 expression and frequent *KRAS* mutation (62,63). However, the papillary architecture was not associated with survival in patients with mutations and the whole population (P=0.852 and P=0.135, respectively). Overall, *KRAS* mutation has a moderate association with papillary architecture but the prognostic effect of the predominant papillary architecture in CRC requires further verification.

The present study also demonstrated the association of solid architecture with a lower OS rate in the group with *KRAS* mutation, whereas micropapillary features were associated with a lower OS rate in the wild type group. A possible explanation is that the solid pattern, by definition, includes poorly differentiated tumors, resulting in a more accurate estimate of high-grade differentiated tumors than the original diagnoses, which are directed toward moderately differentiated tumors.

Table IV. Results of the multivariate analysis of the influence of various factors on overall survival according to *KRAS* mutation status.

	KRAS-mutated		Wild type	
Characteristic	P-value	HR (95% CI)	P-value	HR (95% CI)
Age, years (≥60 vs. <60)	0.023	3.058 (1.169-7.995)	0.111	1.914 (0.862-4.250)
Sex (Male vs. Female)	0.034	2.747 (1.079-6.995)	0.123	0.544 (0.251-1.180)
Tumor location (Left vs. Right)	0.326	0.589 (0.205-1.694)	0.106	0.450 (0.171-1.185)
Tumor size, cm (≥5 vs. <5)	0.800	1.118 (0.471-2.655)	0.695	0.855 (0.391-1.869)
M stage (M1 vs. M0)	0.029	5.608 (1.197-26.279)	<0.001	10.176 (3.488-29.686)
Infiltrative tumor border, % (≥50 vs. <50)	0.658	0.729 (0.180-2.956)	0.575	1.880 (0.207-17.088)
Tumor differentiation (Poor vs. Moderate/Well)	0.644	0.660 (0.113-3.838)	0.061	3.874 (1.106-8.574)
Cribriform pattern	0.606		0.059	
Mild vs. Absent	0.564	0.677 (0.180-2.549)	0.906	0.945 (0.372-2.405)
Moderate vs. Absent	0.812	1.240 (0.211-7.298)	0.040	0.186 (0.037-0.926)
Serrated pattern	0.242		0.402	
Mild vs. Absent	0.093	0.442 (0.171-1.145)	0.392	0.675 (0.275-1.660)
Moderate vs. Absent	0.790	0.829 (0.208-3.297)	0.201	0.358 (0.074-1.727)
Signet ring cells (Present vs. Absent)	0.284	4.886 (0.268-89.090)	0.224	2.478 (0.574-10.708)
Solid component, % (≥50 vs. <50)	0.032	4.040 (1.127-14.488)	0.086	0.424 (0.159-1.129)
Papillary component (Present vs. Absent)	0.852	1.096 (0.416-2.891)	0.536	0.741 (0.286-1.918)
Micropapillary component (Present vs. Absent)	0.562	1.502 (0.379-5.956)	0.018	2.908 (1.205-7.017)
Quality of mucin	0.795		0.251	
Low-grade vs. Absent	0.501	0.659 (0.195-2.222)	0.563	1.422 (0.431-4.693)
High-grade vs. Absent	0.947	0.001 (0.000-0.000)	0.252	0.352 (0.059-2.097)
Q score of mucin (≥60 vs. <60)	0.953	0.001 (0.000-0.000)	0.357	2.237 (0.403-12.416)
Crohn's-like lymphoid reaction (Moderate vs. Absent-Mild)	0.107	0.358 (0.102-1.251)	0.987	1.008 (0.395-2.574)
Dirty necrosis	0.205		0.314	
Low vs. Absent	0.307	2.161 (0.493-9.470)	0.151	0.468 (0.166-1.318)
Moderate vs. Absent	0.115	4.583 (0.689-30.476)	0.151	0.374 (0.097-1.434)
High vs. Absent	0.061	5.055 (0.927-27.560)	0.574	0.569 (0.080-4.063)
Confluent vs. Absent	0.656	1.684 (0.170-16.668)	0.964	0.965 (0.209-4.451)
Neutrophilic infiltration	0.136		0.411	
Low vs. Absent	0.220	0.394 (0.089-1.744)	0.680	1.306 (0.368-4.626)
Focal vs. Absent	0.753	0.797 (0.195-3.266)	0.160	2.325 (0.717-7.540)
Numerous vs. Absent	0.048	0.153 (0.024-0.980)	0.208	2.141 (0.655-7.003)
Tumor-infiltrating lymphocytes, % (≥50 vs. <50)	0.759	1.185 (0.402-3.492)	0.942	1.036 (0.404-2.656)
Tumor budding grade	0.060		0.222	
Intermediate vs. Low	0.018	3.519 (1.243-9.964)	0.108	2.219 (0.839-5.865)
High vs. Low	0.530	1.948 (0.243-15.583)	0.194	2.347 (0.649-8.491)

HR, hazard ratio; CI, confidence interval; M, metastasis.

For the micropapillary feature, this has been associated with frequent lymph node metastasis in numerous solid organ tumors, including CRCs (64,65). However, the results of the present study support the fact that *KRAS* mutation has a more significant impact on prognosis compared with micropapillary histology.

Most of the results of the survival analysis for clinicopathological factors of the present study are in accordance with those of previous studies (19,66,67). In addition, in previous studies, a better OS has been indicated in stage IV/*KRAS* wild type and anti-EGFR-treated subgroups (68,69). A higher 3-year OS rate suggests that anti-EGFR treatment may have a positive effect on short-term survival.

However, there are certain differences between the present results and those of previous studies. First, an association of dirty necrosis or tumor necrosis with better OS is not reported in the literature. While there have only been a few studies that have directly investigated the role of dirty

necrosis on survival in CRC (70,71), a previous study by Pollheimer *et al* (70) reasoned that tumor necrosis reflects a hypoxic environment due to rapid proliferation of the tumor and is therefore associated with a poor prognosis. Väyrynen *et al* (71) also reported that tumor necrosis was associated with high T stage, vascular invasion and short DFS time in CRC, but the degree of necrosis was not proportional to the Ki-67 proliferation index. Overall, dirty necrosis is not merely an indicator of the tumor growth rate but may also reflect intraluminal growth rather than invasiveness. The second point is regarding neutrophilic infiltration. In the present study, the paucity of neutrophilic infiltration was associated with poor OS. Certain studies have reported neutrophil infiltration as a favorable prognostic factor, whilst other studies have proposed that the induction of immune escape by intratumoral neutrophils results in stimulation of tumor growth (72,73). The results of the present study support an activated anti-tumor immune response induced by intratumoral neutrophils rather than immune evasion.

There are certain limitations to this type of retrospective study using previously processed material for diagnostic purposes. First, only a small number of patients (2.6%, 8/310) were able to undergo *BRAF* testing, as the procedure was not covered by their health insurance. *BRAF*, like *KRAS*, is also known to cause constitutive activation of the MAPK pathway and *BRAF* mutations have been associated with adverse clinical outcomes in advanced CRC (74-76). Won *et al* (44) even concluded that *BRAF* mutations, rather than *KRAS* mutations, were significant prognostic factors in Korean patients with CRC. Accordingly, simultaneous testing for *KRAS* and BRAF is required in future studies in CRC. In addition, the detection kit used in the present study only included common codon changes. Therefore, further studies on the histological results and their prognostic value of rare *KRAS* codon variants are necessary.

In summary, the present study demonstrated a moderate association between *KRAS*-mutated CRCs and specific histology, and, to a certain degree, an association between histology and prognosis, according to *KRAS* mutation status. Due to the different prognostic value of *KRAS* mutations in patients with different ethnicities, the present study holds scientific value as the patient cohort consists of a specific ethnic group. Given that the results of the present study vary from previous findings performed in South Korea, based on the prognostic value of *KRAS* mutation, which have indicated that *KRAS* is not associated with either OS or DFS (48,49), this should be further clarified by meta-analysis.

Acknowledgements

The authors greatly appreciate the valuable work carried out by Ms Seo Young Oh at the Department of Pathology, KUMC (Seoul, Korea) for performing the *KRAS* mutation test, and her interpretation of the *KRAS* test results.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HSH designed and supervised the study. DYH contributed to data collection and data analysis. HSL contributed to data collection, histological and statistical analyses, constructed the figures and tables and drafted the initial manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This retrospective study was reviewed and a waiver of informed consent was provided by the Institutional Review Board of KUMC (Seoul, Korea; ethics approval no. KUH1210056).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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