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Clinical Implication of *KRAS* Mutation Variants in Patients With Resected Colon Cancer

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Abstract. *Aim: This study evaluated the clinical implication of KRAS proto-oncogene, GTPase (KRAS) mutation variants in patients with resected colon cancer (CC). Patients and Methods: We retrospectively reviewed 482 patients diagnosed with CC who underwent curative surgical resection at Kyungpook National University Chilgok Hospital. The inclusion criteria were: Pathologically diagnosed with primary CC; stage I-III CC according to the 7th edition of American Joint Committee on Cancer staging system; and with available test results for KRAS mutation status. In total, 345 patients met these criteria and were included in this study. Results: Among the 345 patients, 140 (40.6%) exhibited KRAS mutations, with their incidences as follows: 90/140 (64.3%) in exon 2 codon 12, 37/140 (26.4%) in exon 2 codon 13, 1/140 (0.1%) in exon 3 codon 59, 7/140 (5.0%) in exon 3 codon 61, and 5/140 (3.6%) in exon 4 codon 146. KRAS mutation status was not a significant prognostic factor for disease-free survival or overall survival. Although there were no significant differences in survival between patients with exon 2 codon 12 and exon 2 codon 13 mutations, poorer disease-free survival (p=0.085) and overall survival (p=0.005) were seen in those with exon 3 codon 61 mutation than in others. Conclusion: KRAS mutation status was not correlated with survival, but exon 3 codon 61 mutation might be a factor for poor prognosis in patients after resection of CC.*

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Key Words: Colon cancer, *KRAS*, PNAClamp™ , prognosis.

The KRAS proto-oncogene GTPase (*KRAS*) also known as the Kirsten ras gene, is considered to participate in the progression of colorectal neoplasms from adenoma to carcinoma. Studies have demonstrated that *KRAS* is mutated in ~50% of cases of colorectal cancer (CRC) and that it is a key gene driving CRC progression (1, 2). Single nucleotide mutations in specific hotspot regions are able to change the conformation of the RAS active site, controlling various activities including angiogenesis, proliferation, and apoptosis (3).

It is well known that RAS-mutated CRC cells are resistant to monoclonal epidermal growth factor receptor antibody-based treatments (cetuximab or panitumumab) in metastatic CRC (mCRC), so their use is recommended only for patients with wild-type *KRAS* (wt*KRAS*). Moreover, some studies have suggested that mutated-*KRAS* (m*KRAS*) was associated with worse prognosis compared with wt*KRAS* mCRC in patients treated with standard first-line chemotherapies (4-6).

Recently, several studies have reported the prognostic value of *KRAS* mutation, with a focus on its variants. Specific mutation variants were associated with clinical outcomes in mCRC (7-9). However, the correlation between m*KRAS* variants and the prognosis of stage I-III CRC is still controversial. Accordingly, the present study evaluated the clinical implications of *KRAS* mutation variants in patients with resected colon cancer (CC).

Patients and Methods

Patients and treatment. This study retrospectively reviewed 482 patients who were diagnosed with CC and underwent curative surgical resection at Kyungpook National University Chilgok Hospital (KNUCH) between September 2016 and March 2019. The patients were enrolled according to the following inclusion criteria: Pathologically diagnosed with primary CC; stage I-III CC according to the seventh edition of American Joint Committee on Cancer staging system (10), and with available test results for *KRAS* mutation status. A total of 345 patients met these criteria, and thus were included in this study. Moreover, the patient records were reviewed for data on their medical history, age, sex, adjuvant chemotherapy regimen, surgical methods, and pathological results. This study was approved by the Institutional Review Board of KNUCH (KNUCH 2020-08-019), and informed consent was obtained from all study participants.

Determination of KRAS mutation status. Representative formalin-fixed, paraffin-embedded tissue blocks were used for DNA extraction. Assays for detecting the *KRAS* mutant variants were performed using the PNAClamp™ KRAS Mutation Detection kits (Panagene Inc., Daejeon, Korea), following the manufacturer's instructions. The assay that was used was the peptide nucleic acid (PNA)-based polymerase chain reaction (PCR) clamping that selectively amplifies only the mutated target DNA sequence. PNA is a synthetic DNA analog wherein the phosphodiester backbone is replaced with a peptide-like repeat formed by (2-aminoethyl)-glycine unit. The PNA probe was complementary to the wt*KRAS* allele and suppressed amplification of wt*KRAS*, resulting in the preferential amplification of mutant sequences. PCR efficiency was determined by measuring the threshold cycle values for control and mutation assays obtained from SYBR® Green amplification plots. Between September 2016 and February 2018, the PNAClamp™ KRAS Mutation Detection Kit Ver. 2 was used, which can detect 14 *KRAS* mutation variants in *KRAS* codons 12, 13, and 61. From March 2018, the PNAClamp™ KRAS Mutation Detection Kit Ver. 4 was also used; it can detect 40 *KRAS* mutation variants in *KRAS* codons 12, 13, 59, 61, 117, and 146 (Table I).

Statistical analysis. Categorical variables are summarized as counts with proportions, whereas continuous variables are presented as their median and range. The categorical variables were evaluated using chi-square and Fisher's exact tests, as appropriate. Disease-free survival (DFS) was calculated from the time of surgery to the time of tumor recurrence or death from any cause. Overall survival (OS) was measured from the date of surgery to death from any cause. The data were censored if patients were free of recurrence or alive at the last follow-up. The Kaplan–Meier method was used to estimate DFS and OS. The survival curves were compared using a log-rank test according to the *KRAS* mutation status or *KRAS* mutation subtype. Statistical significance was set at $p<0.05$. The statistical analyses were performed using SPSS for Windows (version 21.0; IBM, Armonk, NY, USA).

Table I. *Mutations detected by PNAClamp™ KRAS Mutation Detection Kit Ver. 2 and Ver. 4*.

Exon	Codon	Mutations	
		Ver. 2	Ver. 4
2	12	p.G12S (c.34 G>A)	p.G12S (c.34 G>A)
		p.G12R (c.34 G>C)	p.G12R (c.34 G>C)
		p.G12C (c.34 G>T)	p.G12C (c.34 G>T)
		p.G12D (c.35 G>A)	p.G12D (c.35 G>A)
		p.G12A (c.35 G>C)	p.G12A (c.35 G>C)
		p.G12V (c.35 G>T)	p.G12V (c.35 G>T)
	13	p.G33D (c.38 G>A)	p.G13S (c.37 G>A)
			p.G13R (c.37 G>C)
			p.G13C (c.37 G>T)
			p.G13D (c.38 G>A)
			p.G13A (c.38 G>C)
			p.G13V (c.38 G>T)
3	59	--	p.A59S (c.175 G>T)
			p.A59T (c.175 G>A)
			p.A59E (c.176 C>A)
			p.A59G (c.176 C>G)
			p.A59del (c.176_178delCAG)
	60	--	p.G60D (c.179 G>A)
			p.G60A (c.179 G>C)
			p.G60V (c.179 G>T)
			p.G60G (c.180 T>A)
			p.G60G (c.180 T>G)
	61	p.Q61E (c.181 C>G)	p.Q61E (c.181 C>G)
		p.Q61K (c.181 C>A)	p.Q61K (c.181 C>A)
		p.Q61L (c.182 A>T)	p.Q61L (c.182 A>T)
		p.Q61R (c.182 A>G)	p.Q61R (c.182 A>G)
		p.Q61P (c.182 A>C)	p.Q61P (c.182 A>C)
		p.Q61H (c.183 A>T)	p.Q61H (c.183 A>T)
		p.Q61H (c.183 A>C)	p.Q61H (c.183 A>C)
4	117	--	p.K117E (c.349 A>G)
			p.K117R (c.350 A>G)
			p.K117N (c.351 A>G)
			p.K117N (c.351 A>T)
	146	--	p.A146T (c.436 G>C)
			p.A146T (c.436 G>A)
			p.A146G (c.43T C>G)
			p.A146V (c.437 C>T)
			p.A146A (c.438 A>G)
			p.A146A (c.438 A>C)
			p.A146A (c.438 A>T)

Results

Patients and tumor characteristics. The patients and their tumor characteristics are summarized in Table II. Their median age was 67 (range: 25-86) years at the time of surgery, and 153 (44.3%) patients were male. Primary tumors were located at the right-sided colon in 150 (43.5%) patients and at the left-sided colon in 195 (56.5%) patients. According to the test results for mismatch repair status, 35 (10.1%) patients exhibited high microsatellite instability. In this study, 71 (20.6%), 127 (36.8%), and 147 (42.6%) patients had stage I, II, and III disease, respectively. Adjuvant therapy was administered to 190 (55.1%) patients, among whom, 75 (39.5%) received capecitabine alone or 5-fluorouracil/leucovorin, and 115 (60.5%) received folinic acid/fluorouracil/oxaliplatin or capecitabine/oxaliplatin.

Table II. *Patient characteristics*.

Characteristic	Total N=345	Mutated KRAS	Exon 2 codon 12	Exon 2 codon 13	Exon 3 codon 59	Exon 3 codon 61	Exon 4 codon 146
		N=140/345 (40.6%)	N=90/345 (26.1%)	N=37/345 (10.7%)	N=1/105 (1.0%)	N=7/345 (2.0%)	N=5/105 (4.8%)
Age, years							
Median (range)	67 (25-86)	66 (25-86)	65.5 (25-86)	67 (50-83)	41	74 (43-77)	60 (51-72)
Gender, n (%)							
Male	153 (44.3)	72 (51.4)	45 (50.0)	22 (59.5)	0 (0.0)	4 (57.1)	1 (20.0)
Female	192 (55.7)	68 (48.6)	45 (50.0)	15 (40.5)	1 (100.0)	3 (42.9)	4 (80.0)
		p=0.192					
Primary tumor location, n (%)							
Right	150 (43.5)	70 (50.0)	43 (47.8)	20 (54.1)	0 (0.0)	3 (42.9)	4 (80.0)
Left	195 (56.5)	70 (50.0)	47 (52.2)	17 (45.9)	1 (100.0)	4 (57.1)	1 (20.0)
		***p*=0.043**					
MMR status, n (%)							
MSS or MSI-low	310 (89.9)	129 (92.1)	84 (93.3)	36 (97.3)	0 (0.0)	6 (85.7)	3 (60.0)
MSI-high	35 (10.1)	11 (7.9)	6 (6.7)	1 (2.7)	1 (100.0)	1 (14.3)	2 (40.0)
		p=0.245					
Stage, n (%)							
I	71 (20.6)	34 (24.3)	20 (22.2)	12 (32.4)	0 (0.0)	1 (14.3)	1 (20.0)
II	127 (36.8)	40 (28.6)	26 (28.9)	10 (27.0)	0 (0.0)	2 (28.6)	2 (40.0)
III	147 (42.6)	66 (47.1)	44 (48.9)	15 (40.5)	1 (100.0)	4 (57.1)	2 (40.0)
		p=0.029					
Adjuvant chemotherapy, n (%)							
Yes	190 (55.1)	77 (55.0)	51 (56.7)	16 (43.2)	1 (100.0)	5 (71.4)	4 (80.0)
No	155 (44.9)	63 (45.0)	39 (43.3)	21 (56.8)	0 (0.0)	2 (28.6)	1 (20.0)
		p=0.982					
Oxaliplatin-containing, n (%)							
Yes	115 (60.5)	49 (63.6)	32 (62.7)	12 (75.0)	1 (100.0)	1 (20.0)	3 (75.0)
No	75 (39.5)	28 (36.4)	19 (37.3)	4 (25.0)	0 (0.0)	4 (80.0)	1 (25.0)
		p=0.469					
Relapse, n (%)							
Yes	37 (10.7)	15 (10.7)	10 (11.1)	3 (8.1)	0 (0.0)	2 (28.6)	0 (0.0)
No	308 (89.3)	125 (89.3)	80 (88.9)	34 (91.9)	1 (100.0)	5 (71.4)	5 (100.0)
Death, n (%)							
Yes	19 (5.5)	8 (5.7)	3 (3.3)	3 (8.1)	0 (0.0)	2 (28.6)	0 (0.0)
No	326 (94.5)	132 (94.3)	87 (96.7)	34 (91.9)	1 (100.0)	5 (71.4)	5 (100.0)

MMR: Mismatch repair; MSI: microsatellite instability; MSS: microsatellite stable. No cases were found with exon 4 codon 117 mutation. *p*-Values in bold indicate statistical significance.

KRAS mutation status and KRAS mutation subtypes. According to the test results for *KRAS* mutation status, 140 (40.6%) patients exhibited m*KRAS* (Table II). *KRAS* mutation had a higher incidence among those with right-sided CC and with stage I CC. Exon 2 codon 12 mutation was the most frequent mutation, whereas exon 4 codon 117 mutation was not detected in our series. The incidences of *KRAS* mutations were as follows: 90/140 (64.3%) in exon 2 codon 12, 37/140 (26.4%) in exon 2 codon 13, 1/140 (0.1%) in exon 3 codon 59, 7/140 (5.0%) in exon 3 codon 61, and 5/140 (3.6%) in exon 4 codon 146.

Survival outcomes. With a median follow-up duration of 37.3 (range=0.6-55.2) months, the estimated 3-year DFS and OS rates were 88.1% and 93.8%, respectively. During the analyses, 37 (10.7%) patients experienced disease relapse, and 19 (5.5%) patients died. Among the patients with m*KRAS*, 15 (10.7%) experienced relapse and 8 (5.7%) died. *KRAS* mutation status was not a significant prognostic factor for DFS (3-year DFS: wt*KRAS* 87.2% *vs*. m*KRAS* 89.3%, *p*=0.676) nor OS (3-year OS: wt*KRAS* 93.8% *vs*. m*KRAS* 93.7%, *p*=0.828) (Figure 1). There was also no significant survival difference between patients with exon

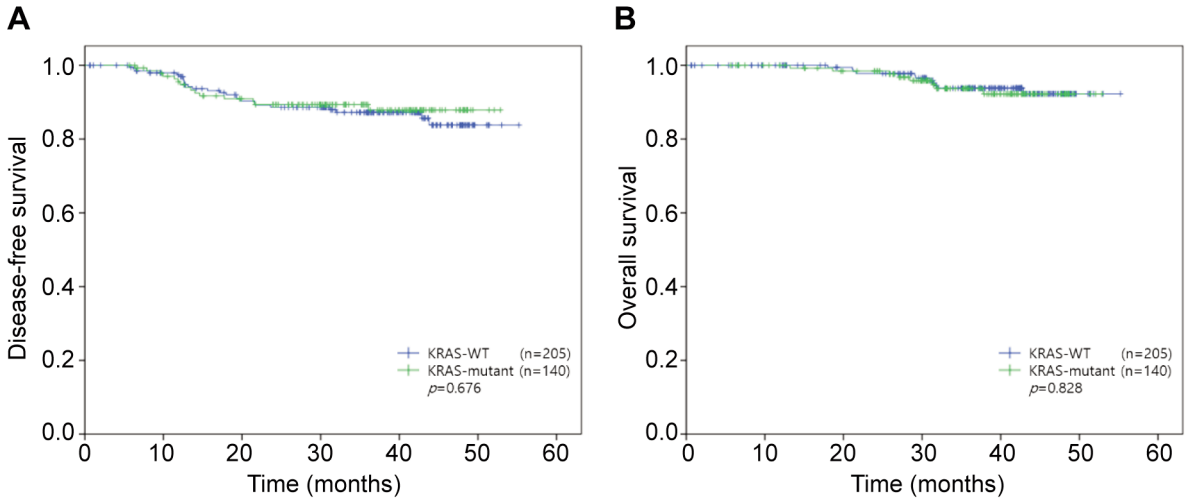

Figure 1. *Kaplan–Meier survival curves for disease-free (A) and overall (B) survival of patients with colon cancer according to KRAS mutation status. WT: Wild-type.*

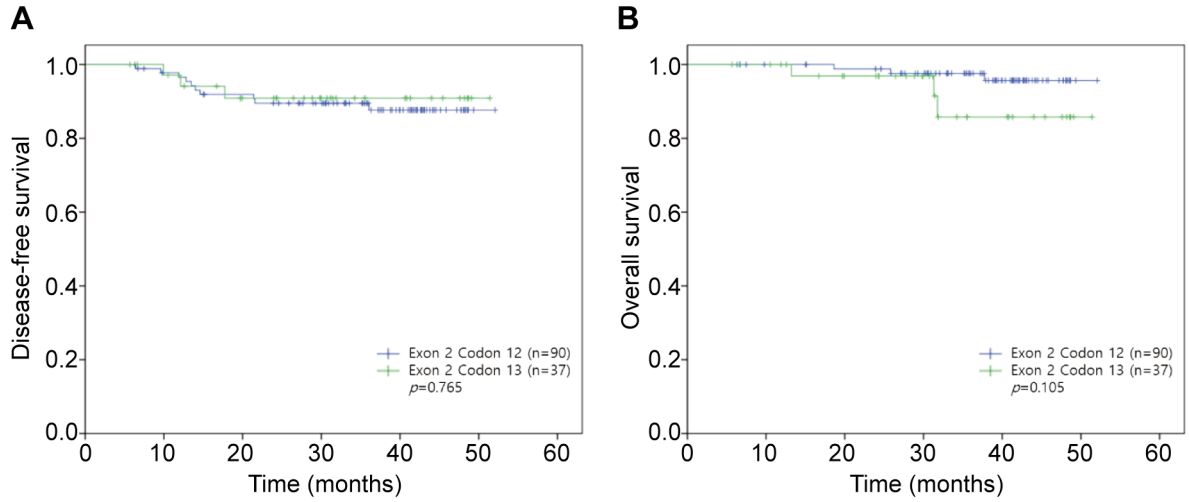

Figure 2. *Kaplan–Meier survival curves for disease-free (A) and overall (B) survival of patients with colon cancer according to KRAS exon 2 codon 12 and codon 13 mutations.*

2 codon 12 and exon 2 codon 13 mutations (3-year DFS: exon 2 codon 12 mutation 89.5% *vs*. exon 2 codon 13 mutation 90.9%, p=0.765; 3-year OS: exon 2 codon 12 mutation 97.6% *vs*. exon 2 codon 13 mutation 85.8%, p=0.105) (Figure 2). Despite the small number of patients, patients with exon 3 codon 61 mutation had poorer DFS (3-year: exon 3 codon 61 mutation 71.4% *vs*. non-exon 3 codon 61 mutation 88.8%, p=0.085) and OS (3-year: exon 3 codon 61 mutation 62.5% *vs*. non-exon 3 codon 61 mutation 95.2%, p=0.005) than those with other subtypes (Figure 3).

Discussion

The present analysis investigated *KRAS* mutational status and the prognostic impact of each mutation variant in Korean patients with resected CC. Similarly to previous studies, *KRAS* mutations occurred in 40.6% of patients, with the majority being observed in exon 2 codon 12 (1). Although there were no significant differences in survival between patients with wt*KRAS* and those with m*KRAS*, it is worth noting that exon 3 codon 61 mutation correlated with poorer DFS and OS compared with other mutations in our study patients.

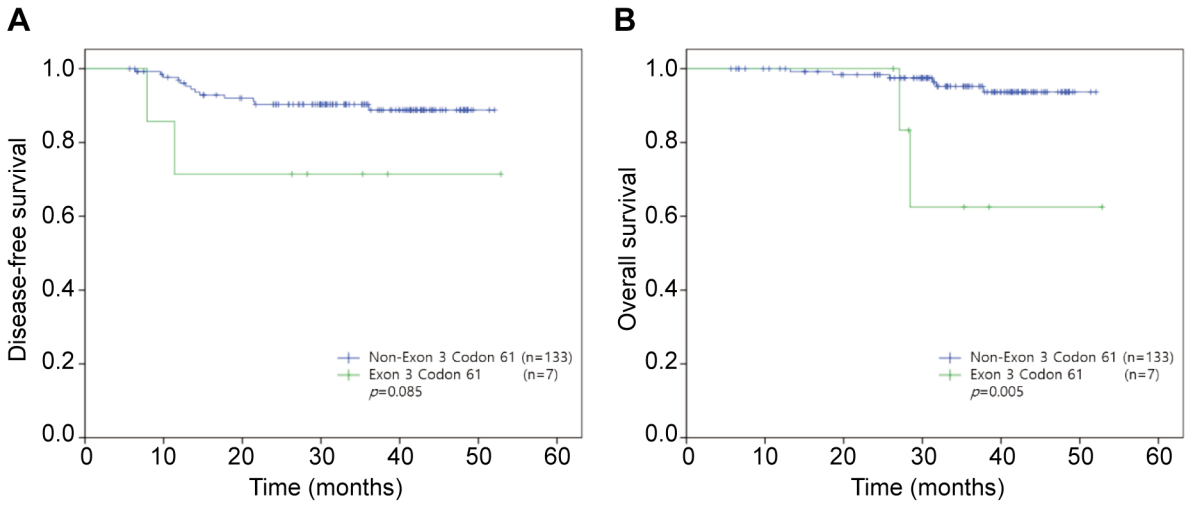

Figure 3. *Kaplan–Meier survival curves for disease-free (A) and overall (B) survival of patients with colon cancer according to KRAS exon 3 codon 61 and non-exon 3 codon 61 mutations.*

It is well known that activating mutations in the *KRAS* gene are observed in 30-50% of CRC cases (1). Single base substitutions in codons 12 and 13 are very common mutations that affect glycine residues in the GTP-binding pocket, leading to changes in the normal regulation of GTPase (11). In particular, exon 2 codon 12 mutations are the most dominant mutations in CRC (12), as confirmed by the results of the current study. In a study of Korean patients, 36.2% had m*KRAS*, with 26% having a mutation in codon 12 (13). Another study conducted in Japan reported *KRAS* mutations in exons 2 and 3 for 34.1% and 3.8% of cases, respectively (14). We found that *KRAS* mutations in exons 3 and 4 were less frequent, similarly to the findings of previous studies (15).

Growing evidence suggests that *KRAS* mutation might be a negative prognostic factor for CRC, being associated with advanced disease, liver metastasis, poor tumor differentiation, and right-sided colon tumors (16). However, the impact of *KRAS* mutation in patients with resected CRC has been controversial across various studies. The current study was consistent with two large randomized studies which demonstrated that *KRAS* mutation had no significant effect on survival and relapse in patients with stage II/III CC or CRC treated with adjuvant chemotherapy (17, 18). Conversely, Hutchins *et al*. reported that *KRAS* mutations had an adverse effect on recurrence and OS in patients with stage II CRC (19). These conflicting results might be due to differences in stage, location of primary tumors, inclusion of the rectum, use of adjuvant treatment, subsequent treatment after recurrence, and the retrospective nature of the studies. Thus, further large-scale studies and more comprehensive analyses using prospective, balanced, and homogeneous data are required to validate our results.

In our series, exon 3 codon 61 mutation was associated with poorer DFS even in a small number of patients. Several studies have already found that some specific codon mutations were associated with survival in CRC. For example, Li *et al*. reported that codon 12 mutations were related to poorer progression-free survival and OS, especially for G12D and G12V mutations (20). Codon 13 mutations demonstrated no prognostic significance. Other studies suggested that G12C was correlated with inferior survival compared with other mutants or wild type (7, 8). Interestingly, a recent study on 138 patients with metastatic CRC who had mutations in *KRAS* codons 61 (n=7) and 146 (n=1) showed that these mutations were associated with lower response rate and worse progression-free survival (21). These findings point to the possibility of exon 3 codon 61 as a biomarker in CRC treatment, suggesting that different specific mutations in *KRAS* can induce alterations in the multiple signal pathways regulated by *KRAS* downstream. Unfortunately, as this study used PNAClamp™ technology, we were unable to detect the specific site of variants among *KRAS* mutations, and identification of specific mutations was not available for our analysis.

In summary, *KRAS* mutation status was not correlated with survival in Korean patients with resected CC. Exon 3 codon 61 mutation might be a poor prognostic factor in these patients.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

Study conception and design: Jin Ho Baek, Byung Woog Kang, and Jong Gwang Kim. Acquisition of data: Juhyung Kim, Dong Won

Baek, Eunhye Chang, Hye Jin Kim, Su Yeon Park, Jun Seok Park, and Gyu Seog Choi. Analysis and interpretation of data: Jin Ho Baek, Byung Woog Kang, and Jong Gwang Kim. Drafting of article: Jin Ho Baek, Byung Woog Kang, and Jong Gwang Kim.

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