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Survival outcome analysis of genetic mutation and gene pathway alteration in metastatic colorectal cancer using next generation sequencing

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노 용 호

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지도 교수 김 동 완

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부위원장 _____ (인)

위 원 _____ (인)

Abstract

Survival outcome analysis of genetic mutation and gene pathway alteration in metastatic colorectal cancer using next generation sequencing

Yongho Noh
Department of medicine
The Graduate School
Seoul National University

Background: Colorectal cancer is a highly prevalent cancer worldwide. Understanding genetic background of cancer has shown to be important for predicting treatment response and clinical prognosis. Next generation sequencing (NGS) provides easily accessible diverse genetic information to clinicians. We studied NGS result of metastatic colorectal cancer patient to identify association of survival outcomes and genetic mutations.

Methods: This is a retrospective single center study analyzing targeted panel sequencing results in metastatic colorectal cancer (mCRC). The association between genetic mutation and progression free survival (PFS) of palliative first line treatment and overall survival (OS) was assessed. Genetic alteration was classified into two key cellular signaling pathway in colorectal cancer, RAS-RAF-MAPK and PI3K-Akt-mTOR pathway, for analysis of pathway

alteration and PFS association.

Results: 171 metastatic colorectal cancer patients were enrolled in this study. The most frequent pathogenic or likely pathogenic mutations were detected in TP53 150 (87.7%), APC 128 (74.9%), KRAS 70 (40.9%), SMAD4 24 (14.0%), and FBXW7 21 (12.3%) cases of patients. The RAS-RAF-MAPK pathway was mutated in 81 (47.4%) patients and the PI3K-Akt-mTOR pathway was mutated in 14 (8.2%) patients of the study population. KRAS (adjusted HR 1.69, 95% CI; 1.10-2.60), NF1 (adjusted HR 11.56, 95% CI; 3.98-33.55) and PTEN (adjusted HR 3.72, 95% CI; 1.06-13.01) mutation was associated with poor first line chemotherapy progression free survival (PFS) outcome. SMAD4 (adjusted HR 7.74, 95% CI; 2.71-22.14) and NF1 (adjusted HR 7.53, 95% CI; 1.14-49.70) mutation revealed adverse overall survival (OS) outcome. For pathway analysis, RAS-RAF-MAPK pathway gene alteration (adjusted HR 1.92, 95% CI; 1.30-2.85) was poor prognostic factor for PFS. Patients harboring both RAS-RAF-MAPK pathway alteration and PI3K-Akt-mTOR pathway alteration showed more adverse outcome (adjusted HR 3.16, 95% CI 1.45-6.87).

Conclusion: This study shows that KRAS, NF1, and PTEN mutation is associated with poor first line chemotherapy PFS in metastatic colorectal cancer. In addition, SMAD4 and NF1 mutation was related with adverse OS outcome. RAS-RAF-MAPK pathway gene alteration was poor prognostic factor for PFS.

Keywords : metastatic colorectal cancer, next generation sequencing, genetic mutation, gene pathway alteration, prognosis

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Abbreviations

APC: Adenomatous polyposis coli
ARID1A: AT-rich interactive domain-containing protein 1A
CI: Confidence Interval
CNV: Copy number variation
CRC: Colorectal cancer
EGFR: Epidermal growth factor receptor
FBXW7: F-box/WD repeat-containing protein 7
HR: Hazard ratio
KEGG: Kyoto encyclopedia of genes and genomes
KRAS: Kirsten rat sarcoma viral oncogene homolog
MAPK: Mitogen activated protein kinase
mCRC: metastatic colorectal cancer
mTOR: Mechanistic target of rapamycin
MYC: Myelocytomatosis
NGS: Next generation sequencing
NF1: Neurofibromin 1
OS: Overall survival
PFS: Progression free survival
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA: Posphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit a
PTEN: Phosphatase and tensin homolog
RAS: Rat sarcoma
RAF: Rapidly accelerated fibrosarcoma
RTK: Receptor tyrosine kinase
SNV: Single nucleotide variant
SMAD4: Suppressor of mothers against decapentaplegic family member 4
TGF-b: Transforming growth factor b
TMB: Tumor mutation burden
TP53: Tumor protein 53
TSC: Tuberous sclerosis complex
WNT: Wingless/Integrated

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer mortality globally.[1] In Republic of Korea, CRC is third most commonly diagnosed cancer and ranks third as the leading cause of cancer related death.[2]

Despite advances in chemotherapy, there are still limitations in treating metastatic CRC. Anti-epidermal growth factor receptor (EGFR) targeted monoclonal antibodies are effective either with conventional chemotherapy combination or as monotherapy in treating metastatic CRC. However only 10% to 20% of patients showed clinical benefit from this treatment.[3] KRAS mutation was found to be prognostic and predictive for response to anti EGFR targeted antibodies. Furthermore aberrations in the RAS-RAF-MAPK signaling pathway and PIK3CA-AKT pathway, both of which are an EGFR downstream effectors, predicted adverse response to anti EGFR monoclonal antibodies.[3-5] Currently anti EGFR monoclonal antibody, such as cetuximab or panitumumab, is used in combination with cytotoxic chemotherapy for palliative first line treatment specifically for patients with RAS wild-type metastatic colorectal cancer (mCRC).[6]

The Cancer Genome atlas (TCGA) project defined genetic alteration as somatic mutations, homozygous deletions, focal amplifications, and gene expression exploring relationship with clinical importance.[7] The study identified recurrent alterations in five key cellular signaling pathways, WNT, TGF-b, RTK/RAS, PI3K,

and TP53 pathway, for CRC development. The PI3K and RAS-MAPK pathways were commonly affected. Also, co-occurrence of PI3K and RAS-MAPK pathway alterations were found in one-third of tumors. Previous genetic studies in CRC have uncovered frequently mutated genes. These gene alterations have significant implications for cellular signaling and target intracellular protein, leading to distinct biological modification. Later on other less frequent genetic mutations exhibiting similar actions for tumorigenesis were identified in CRC. [8] Notably, the BRAF gene in the RAS-MAPK pathway predominantly experiences mutation in KRAS wild-type CRC.[9] Analyzing genetic alterations as a cellular signaling pathway groups will provide further insights into the prognosis of metastatic CRC.

The purpose of this study is to enhance our comprehension of the mutation profiles in mCRC by using next generation sequencing (NGS) performed in routine clinical practice. Targeted gene panel sequencing using NGS technology allows us to detect multiple genetic alterations within a single test. NGS has recently become integrated into standard clinical practice.[10] Association between genetic alteration and palliative first line therapy progression free survival (PFS) and overall survival (OS) was investigated. Additionally, the study evaluates prognostic role of two critical pathways in the context of mCRC.

2. Method

2.1. Study design

Adult patients with pathologically confirmed metastatic CRC diagnosis were enrolled in this study. The next generation sequencing data performed from August 2018 to July 2021 was collected. Medical charts from electronic medical record system of Seoul National University Hospital (SNUH) database were reviewed for the population. The most recent update of clinical records was conducted in April 2023. Response evaluation was made in accordance to RECIST 1.1[11]

The protocol of this study was reviewed and approved by the Institutional Review Board (IRB) of SNUH (IRB number H-2108-074-1244). The study was conducted in accordance with the Declaration of Helsinki in biomedical research involving human subjects.

2.2. Targeted sequencing and interpretation

The SNUH FiRST solid cancer panel, a next-generation sequencing (NGS) based tailored target gene panel, was employed in this study. The panel is designed to detect a wide range of therapy-related somatic mutations, including single nucleotide variations (SNVs), copy number variations (CNVs), and gene fusions. The SNUH FiRST solid cancer panel version 3 comprises a total of 194 genes.

The initial sequencing dataset was generated using 50 ng of DNA extracted from cancer tissue. The libraries were prepared with Agilent SureSelect target enrichment protocol (Agilent, Santa Clara, CA, USA) for Illumina paired-end sequencing library protocol. The sequencing was carried out on the Illumina NextSeq 550Dx platform (Illumina, San Diego, CA, USA).

The sequencing dataset was analyzed using the SNUH FiRST panel analysis pipeline. In brief, paired-end alignment to the hg19 reference genome was performed using BWA-mem (v0.7.17) and GATK Best Practice. The identified mutations were then classified by tier system based on an internal mutation classification database. In general, the tiers A, B, C, and D correspond to the levels 1, 2/3A, 3B, and 4 of OncoKB, respectively. The panel also calculates Tumor Mutational Burdens (TMB) utilizing known cancer-related mutations while excluding synonymous mutations. TMB high colorectal cancer is associated with better prognosis than those with TMB low tumors.[12]

The microsatellite status of each tumor was determined by evaluating 5 microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26). We classified the microsatellite instability (MSI) status into three group. MSI high (instability of 2 or more microsatellite markers), MSI low (instability of 1 microsatellite marker), and MSS (microsatellite stable; no instability seen at any microsatellite marker).

TCGA network conducted an integrated analysis of mutations and copy number change to identify critical pathways deregulation in CRC.[7] Various mutations were grouped to better understand molecular mechanism contributing to CRC development. In this study we incorporated two modified signaling pathway which plays important role in tumor progression.[13] ERBB gene in the RTK/RAS TCGA network study group has various downstream signaling pathway including PI3K-Akt pathway. For the purposes of this study, the RTK/RAS group was further specified to the RAS-RAF-MAPK pathway which is known to control cell proliferation, differentiation, and survival.[14] PI3K-Akt pathway is also an important intracellular signal pathway responsible for a variety of cellular activities. Although PI3K is triggered either via EGFR dimerization or phosphorylated RAS, co-mutation seen in PIK3CA

in CRCs with KRAS mutations implicates that KRAS protein might not be highly efficient in activating PI3K signaling.[8] Among the numerous downstream targets of Akt, preclinical data suggest tumorigenic potential for the mTOR gene mutation.[15] As a result, we designed to include PI3K-Akt-mTOR pathway for survival analysis as well.

To define RAS-RAF-MAPK pathway and PI3K-Akt-mTOR pathway, we reviewed the Kyoto encyclopedia of genes and genomes (KEGG) pathway database (map04010, map04151)[16] in alliance with relevant previously published studies. The defined genes were matched with our target sequencing panel genes. Patients were categorized into specific pathway if any gene alterations from the matched gene criteria were found. In our study, RAS-RAF-MAPK pathway consists KRAS, NRAS, BRAF, MAP2K4, and MAP2K1. Meanwhile, PI3K-Akt-mTOR pathway includes PIK3CA, PTEN, AKT1, TSC1, TSC2, mTOR.

2.3. Statistical analysis

PFS is defined as the time interval between the first date of palliative 1st line chemotherapy and the date of documented disease progression or death from any cause. If a patient received surgical resection of localized metastasis, leaving the patient no evidence of cancer, such case was censored at the time of surgery. Patients who underwent surgery before the first date of chemotherapy were excluded in PFS analysis. Patients who did not experience disease progression or death at the time of data collection was censored at the last date known to be progression free and alive. As for the analysis of OS, the time interval is measured from the initiation of palliative 1st line chemotherapy to the date of death from any cause. Patients without documentation of death at the time of data collection was censored at the last follow up date.

Kaplan–Meier method survival curve plot with log rank test comparisons were used for PFS and OS analysis. Hazard ratios (HR) were calculated by means of Cox proportional hazards model. Clinical variables such as age (continuous), sex, tumor location (left vs right), tumor stage, lymph node stage, micro satellite status (MSI high vs MSI low and MSS), and TMB status (TMB≥10mut/kb vs <10mut/kb) were adjusted while calculating HR. Two-sided P value less than 0.05 were considered statistically significant. The statistical analysis was performed using R version 4.2.2 (http://www.r-project.org) using *survival*, *survminer* packages and oncoplot was drawn by *complexheatmap* package.

3. Results

3.1. Patient characteristics

171 metastatic colorectal cancer patients were enrolled in this study. The baseline characteristics are summarized in Table 1.

127 (74.3%) patients had left (distal) colorectal cancer defined as descending colon, sigmoid colon, and rectal cancer. 42 (24.6%) patients had right (proximal) colorectal cancer defined as cecum, ascending colon, and transverse colon. An MSI high status and TMB high (≥10mut/Mb) was found in 4.1% and 8.2% of the patients, respectively. All MSI high patients was included in TMB high cancer patients in our cohort.

Most common metastasis site was liver (119 patients, 69.6%) followed by lung (104 patients, 60.8%) and lymph node (101 patients, 59.1%). 72 patients (42.1%) had peritoneal carcinomatosis, 23 patients (13.5%) had metastatic lesion in bone and 2 patients (1.2%) in brain.

Palliative first line chemotherapy response evaluation was performed in 151 patients. 20 patients were ineligible for response evaluation because metastatic lesions were resected before initiating chemotherapy resulting in no remaining evidence of cancer. This population (20 patients) is included in overall survival analysis. 65 (43%) patients received anti EGFR antibody and chemotherapy combination for first line treatment option. 94 (62.3%) patients received FOLFOX and 51 (33.8%) patients received FOLFIRI for chemotherapy backbone. Among the 151 patients, the best responses observed were, complete response (CR) in 4 patients (2.6%), partial response (PR) in 90 patients (59.6%), stable disease (SD) in 48 patients (31.8%) and progressive disease (PD) in 9 patients (6.0%).

Table 1. Baseline characteristics

Characteristic	N=171
Age – years, median (range)	
Median	59 (23–85)
Sex – no. (%)	
Male	98 (57.3%)
Female	73 (42.7%)
Primary tumor site – no. (%)	
Left	127 (74.3%)
Right	42 (24.6%)
Rectal cancer – no. (%)	
Rectum	50 (29.2%)
Others	121 (70.8%)
Metastasis sites – no. (%)	
Liver	119 (69.6%)
Lung	104 (60.8%)
Lymph nodes	93 (54.4%)
Peritoneum	71 (41.5%)
Brain	2 (1.2%)
Bone	23 (13.5%)
MSI status – no. (%)	
MSI high	7 (4.1%)
MSI low, MSS	164 (95.9%)
TMB status – no. (%)	
TMB high (≥10mut/Mb)	14 (8.2%)
TMB low (< 10mut/Mb)	155 (90.6%)
Palliative first line chemotherapy	**N=151**
Treatment regimen – no. (%)	
Anti EGFR Ab + chemotherapy	65 (43%)
Bevacizumab + chemotherapy	45 (29.8%)

Chemotherapy alone	41 (27.2%)
Chemotherapy backbone – no. (%)	
FOLFOX	94 (62.3%)
FOLFIRI	51 (33.8%)
Others	6 (4.0%)
First line response – no. (%)	
CR	4 (2.6%)
PR	90 (59.6%)
SD	48 (31.8%)
PD	9 (6.0%)

MSI: Microsatellite instability, TMB: Tumor mutation burden, EGFR: Epidermal growth factor receptor, FOLFOX: 5-fluorouracil, leucovorin, and oxaliplatin, FOLFIRI: 5-fluorouracil, leucovorin, and irinotecan. CR: Complete response, PR: Partial response, SD: Stable disease, PD: Progressive disease

3.2. Sequencing profile

The median depth of coverage of the total samples was 614fold. A total number of 619 pathogenic or likely pathogenic single nucleotide variants (SNVs; missense, nonsense, frameshift, insertion/deletion, splicing) and 26 pathogenic or likely pathogenic copy number variations (CNVs; amplification, gain, deletion, loss) were detected in 171 samples. 67 genes were detected to harbor at least 1 SNV or CNV. The average numbers of mutations per patient was 3.77 and average number of mutated genes per patient was 3.34. When excluding MSI high and TMB high tumors, average number of mutated genes per patient was 2.79. All of the study population showed at least 1 mutation (**Figure 1**).

The most frequently mutated genes were TP53 150 (87.7%), APC 128 (74.9%), KRAS 70 (40.9%), SMAD4 24 (14.0%), FBXW7 21 (12.3%), BRAF 11 (6.4%), ARID1A 9 (5.3%), PIK3CA 9 (5.3%) and NRAS 9 (5.3%) patients. Among tumors excluding MSI high and TMB high cases, the most frequently mutated genes were TP53 141 (89.8%), APC 119 (75.8%), KRAS 64 (40.8%), SMAD4 20 (12.7%), FBXW7 16 (10.2%), BRAF 8 (5.1%), PIK3CA 7 (4.5%), MYC 7 (4.5%) and NRAS 7 (4.5%) patients. Despite a small difference in TP53 and APC mutation rates, our data showed TP53 mutation as the most frequent single nucleotide variant. **Figure 2** depicts a Venn diagram of commonly mutated genes. Notably, the mutation of KRAS gene and BRAF gene was mutually exclusive in our cohort (**Figure 2D**).

Patients were classified into the pathway alteration if they had any gene mutation in predefined RAS-RAF-MAPK or PI3K-Akt-mTOR pathway gene list. The RAS-RAF-MAPK pathway was mutated in 93 (54.4%) patients, and the PI3K-Akt-mTOR pathway was mutated in 17 (9.9%) patients in the study population (**Figure 2E**).

Out of the sequencing specimens, 133 (77.8%) were obtained at the time of metastasis diagnosis, while 42 (24.6%) specimen were obtained from sites other than colon or rectum.

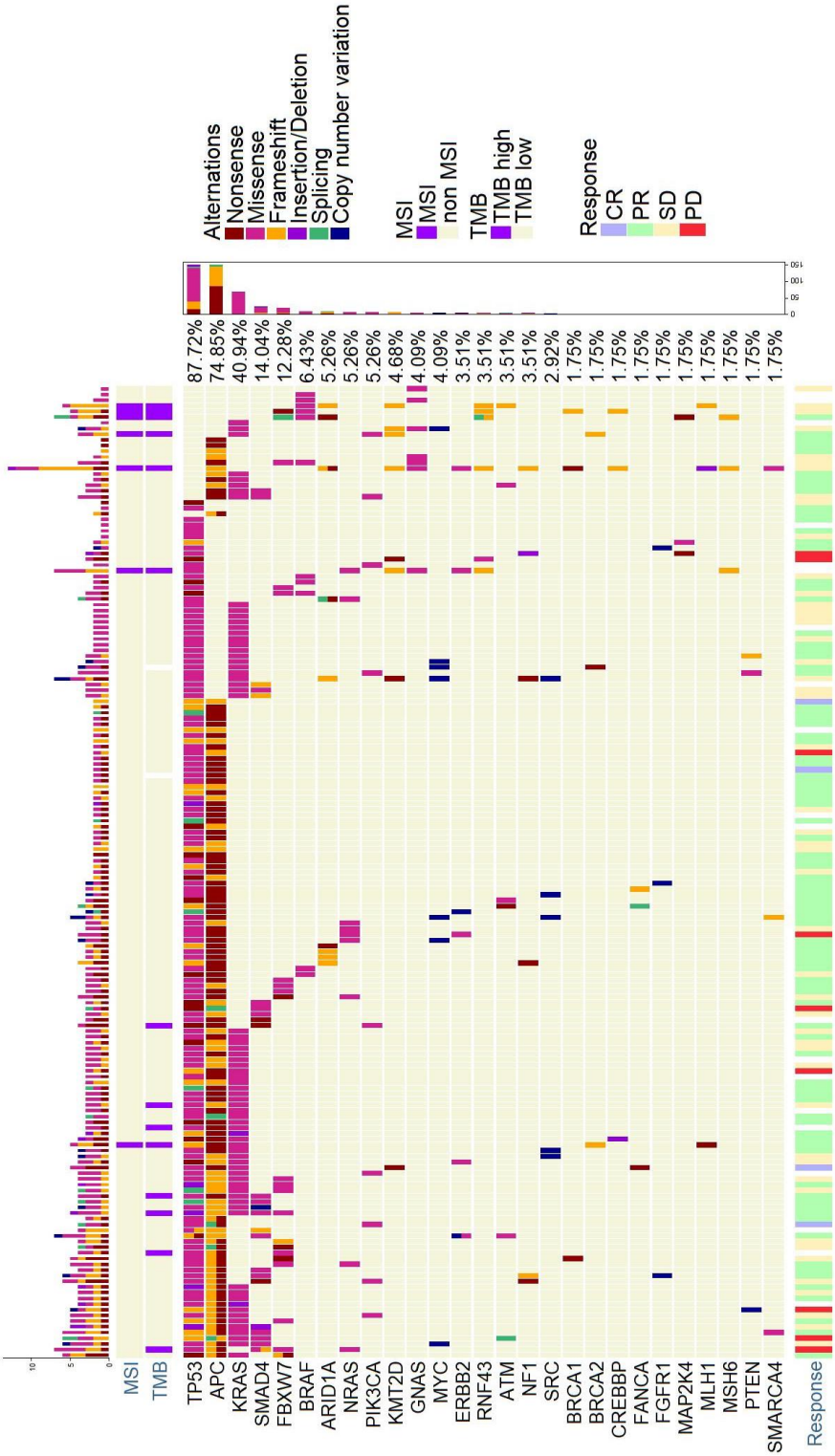


Figure 1. Genetic mutation data of 171 patients. 27 genes with at least 3 recurrences are shown in the figure.

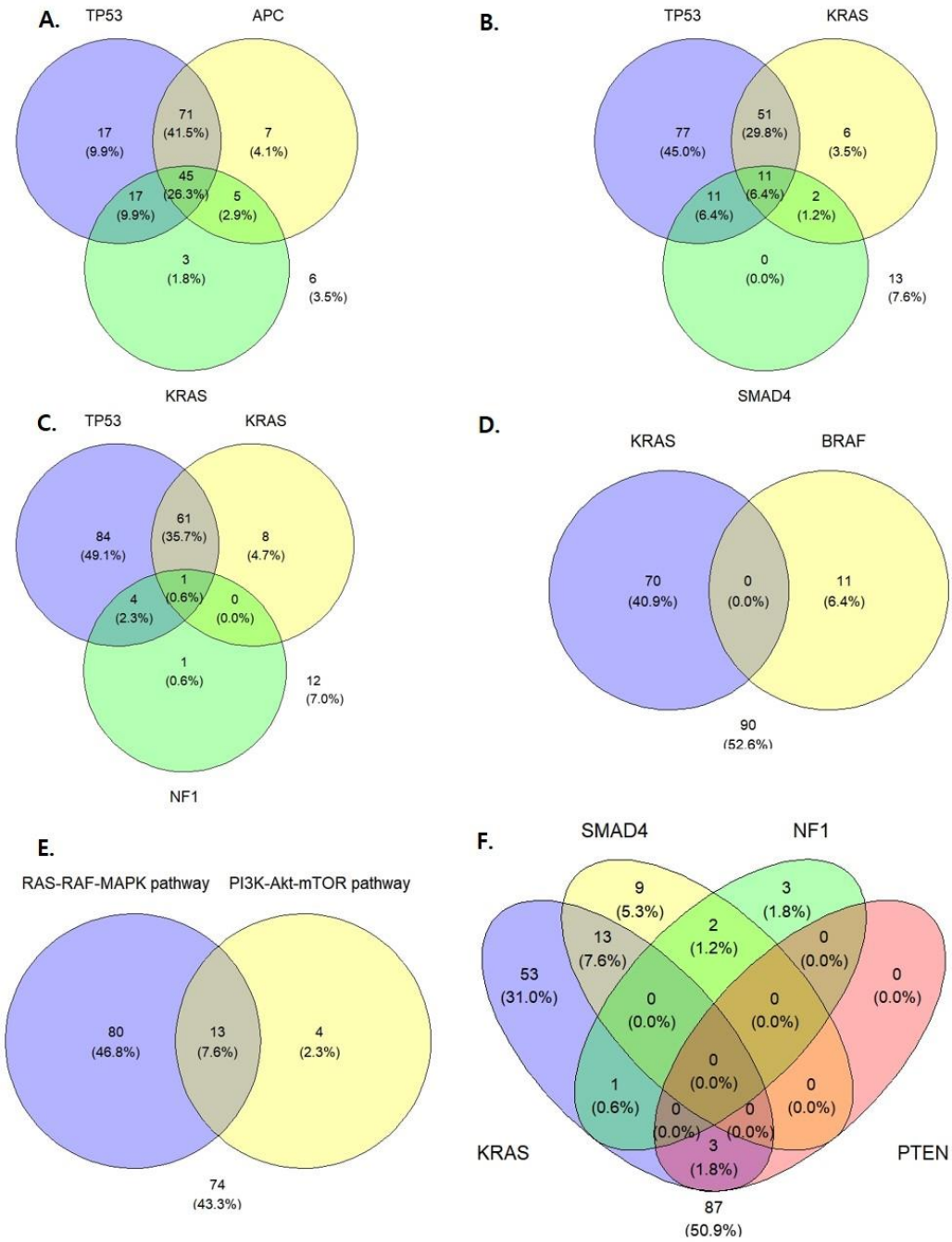


Figure 2. Venn diagram of commonly mutated genes with number of samples and percentage inserted. (A) TP53, APC, and KRAS gene, (B) TP53, KRAS, and SMAD4 gene, (C) TP53, KRAS, and NF1 gene, (D) KRAS and BRAF gene, (E) KRAS, SMAD4, NF1, and PTEN gene

3.3. Prognostic role of genetic mutation.

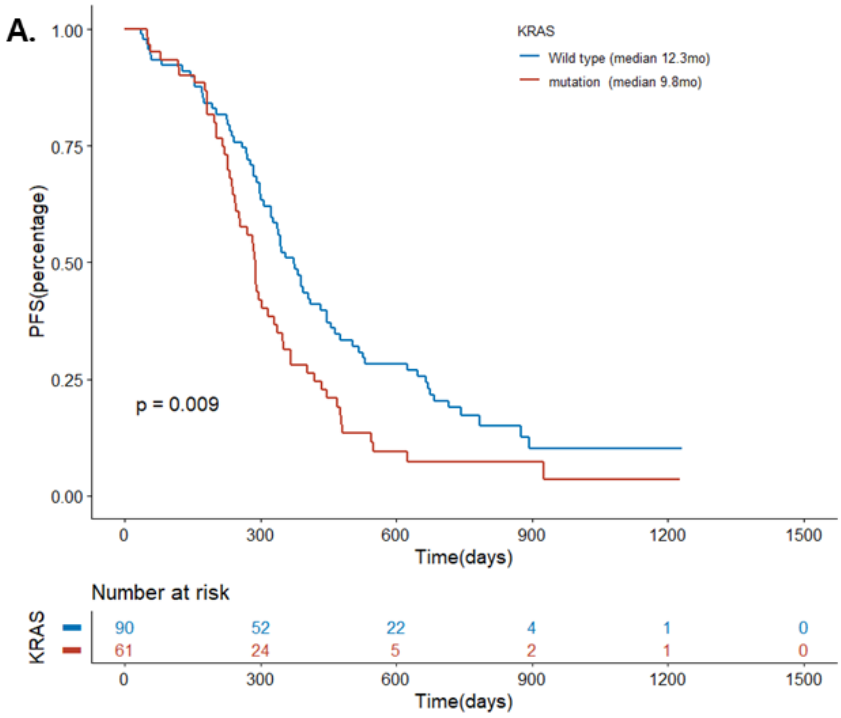
After a median follow up duration of 29.5 months, median progression free survival (PFS) of palliative first line chemotherapy was 10.8 months. The 1year PFS rate of first line palliative chemotherapy was 42.9% (95% Confidence Interval (CI), 35.4%–51.9%).

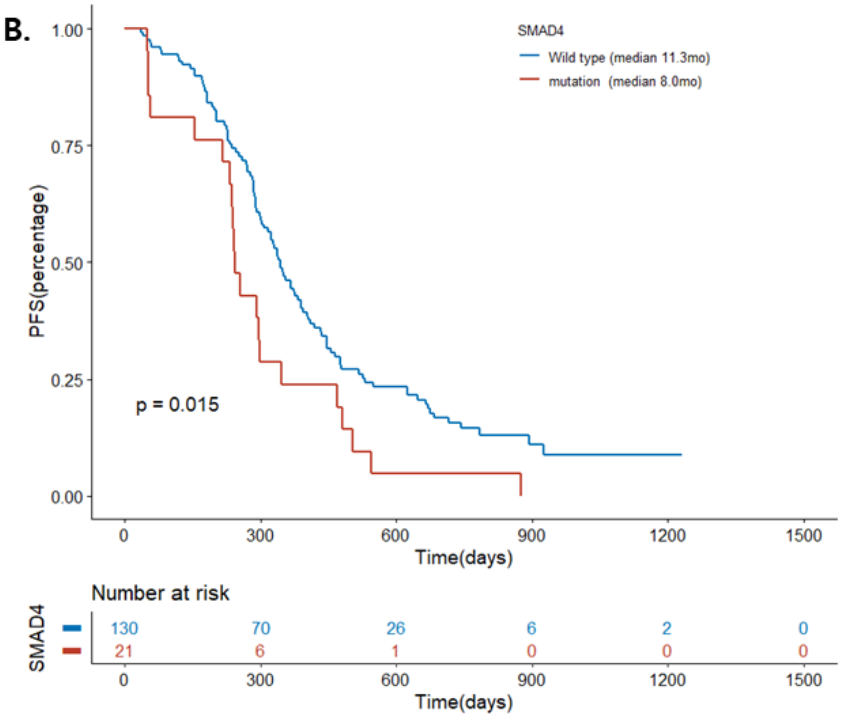
We analyzed 27 frequently mutated genes shown in **Figure 1** to determine whether individual genetic mutations were associated with palliative first line chemotherapy PFS. Patients with KRAS (HR 1.6, 95% CI; 1.1–2.3), SMAD4 (HR 1.8, 95% CI; 1.1–2.9), NF1 (HR 8.2, 95% CI; 3.4–20), and PTEN (HR 3.6, 95% CI; 1.1–12) mutations were associated with poor prognosis compared to patients without these mutations (**Figure 3**). We than constructed multivariate cox proportional hazards model to find adjusted HRs for each gene. Clinical variables such as age (continuous), sex, tumor location (left vs right), tumor stage, lymph node stage, microsatellite status (MSI high vs MSI low and MSS), and TMB status (TMB≥10mut/kb vs <10mut/kb) were adjusted as predesigned. After the adjustment, KRAS (adjusted HR 1.69, 95% CI; 1.10–2.60), NF1 (adjusted HR 11.56, 95% CI; 3.98–33.55) and PTEN (adjusted HR 3.72, 95% CI; 1.06–13.01) mutations were found to be prognostic mutations for poor first line chemotherapy PFS outcome (**Table 2**). SMAD4 mutation showed a tendency towards an adverse PFS outcome but was not statistically significant in the multivariate adjustment model. Primary tumor location on the left side was associated with favorable outcome, whereas MSI high and TMB high status did not show a significant association with the PFS outcome.

Mutation profiles of KRAS, SMAD4, NF1, and PTEN gene are described in **Table 3**. Concurrently mutated genes in each of the four genes are also shown. **Figure 2F** depicts a Venn diagram for

the four genes. Among the KRAS mutations, the most frequent mutation site was KRAS G12D (32.4%) followed by KRAS G13D (22.5%). The five most commonly identified KRAS mutation sites were further analyzed with worse PFS associated with KRAS G12V (univariate HR 2.3, 95% CI; 1.1−4.7) point mutation (**Table 4**).

We next analyzed association of these genetic mutation with overall survival (OS) outcome. Patients who were excluded during the PFS analysis due to surgical resection of metastasis site before initial chemotherapy were included. Patients with SMAD4 (adjusted HR 7.74, 95% CI; 2.71−22.14) and NF1 (adjusted HR 7.53, 95% CI; 1.14−49.70) mutation revealed adverse survival outcome (**Figure 4**). KRAS and PTEN mutations which were related with poor PFS outcome showed a tendency towards adverse survival outcome, but the increase in HR was not statistically significant. NF1 mutation is a strong prognostic mutation for both PFS and OS adverse outcome. Primary tumor location, MSI/TMB status were not significantly related to overall survival.


A.
KRAS
Wild type (median 12.3mo)
mutation (median 9.8mo)
PFS(percentage)
1.00
0.75
0.50
0.25
0.00
p = 0.009
0
300
600
900
1200
1500
Time(days)
Number at risk
KRAS
90 52 22 4 1 0
61 24 5 2 1 0
Time(days)


B.
SMAD4
Wild type (median 11.3mo)
mutation (median 8.0mo)
PFS(percentage)
1.00
0.75
0.50
0.25
0.00
p = 0.015
0
300
600
900
1200
1500
Time(days)
Number at risk
SMAD4
130 70 26 6 2 0
21 6 1 0 0 0
Time(days)

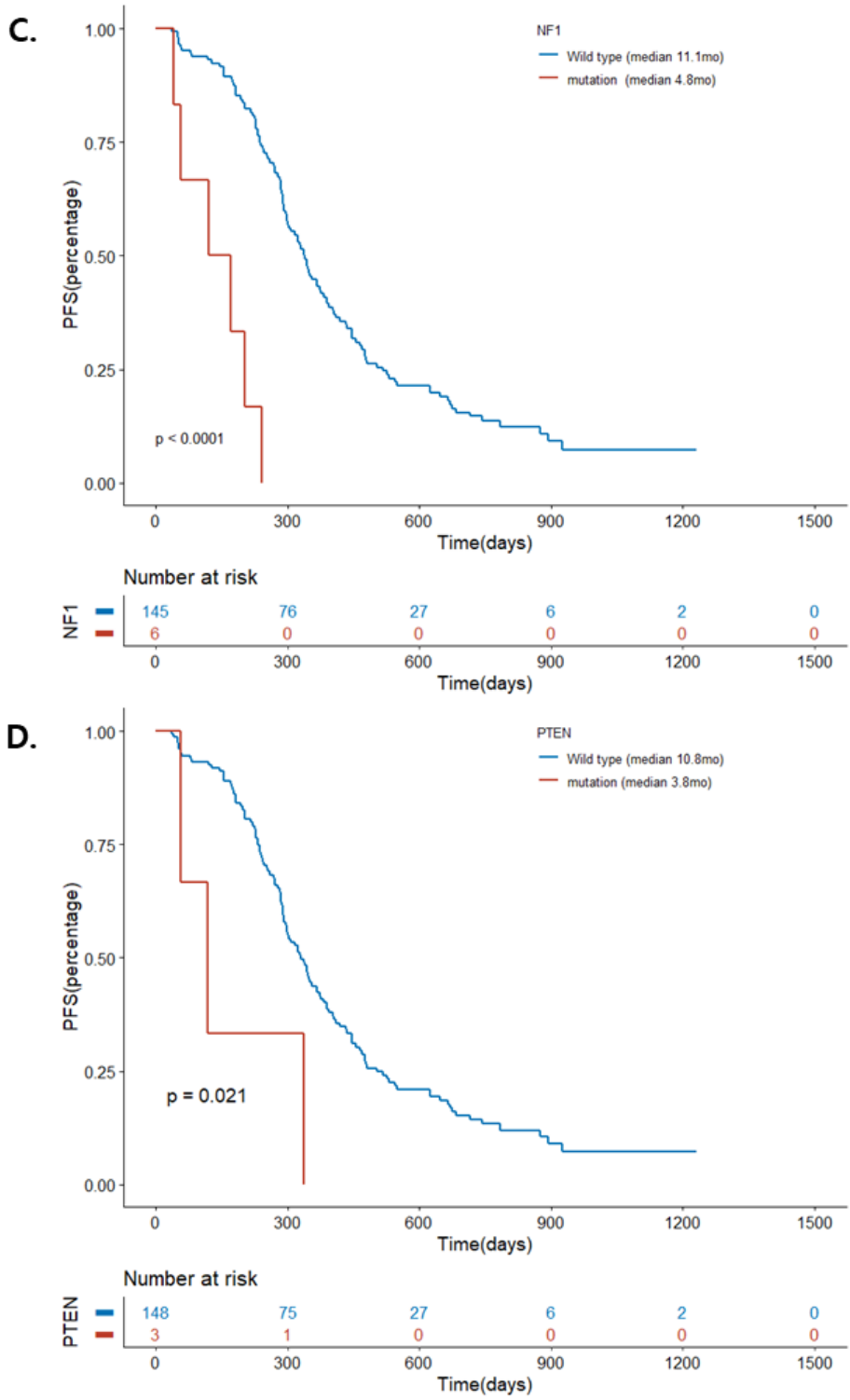


Figure 3. Association of progression free survival and genetic mutation. (A) KRAS (N=61), (B) SMAD4 (N=21), (C) NF1 (N=6), (D) PTEN (N=3)

Table 2. Multivariate Analysis of Progression Free Survival

	Number of patients (n)	HR (95% CI)	P value	Median PFS (month)
Gene				
KRAS	61	1.69 [1.10−2.60]	0.016	9.8 vs. 12.3
NF1	6	11.56 [3.98−33.55]	<0.001	4.8 vs. 11.1
PTEN	3	3.72 [1.06−13.01]	0.040	3.8 vs. 10.8
Adjusted factor				
Primary tumor site (Left) vs. reference (Right)	113	0.50 [0.31−0.82]	0.006	11.3 vs. 8.09

Table 3. Gene mutation profile and frequently co-mutated genes

Mutated gene	Amino acid change	Coding sequence	Frequency (%)*	Comutated gene	Count (%)+
KRAS	p.Gly12Asp	c.35G>A	23 (32.4)	TP53	62 (88.6)
	p.Gly13Asp	c.38G>A	16 (22.5)	APC	50 (71.4)
	p.Gly12Val	c.35G>T	11 (15.5)	SMAD4	13 (18.6)
	p.Gly12Cys	c.34G>T	5 (7.0)	FBXW7	6 (8.6)
	p.Ala146Thr	c.436G>A	5 (7.0)	PIK3CA	5 (7.1)
	p.Gly12Ser	c.34G>A	3 (4.2)	MYC	5 (7.1)
SMAD4	p.Arg361His	c.1082G>A	2 (8.0)	TP53	22 (91.7)
	p.Arg496His	c.1487G>A	2 (8.0)	APC	21 (87.5)
				KRAS	13 (54.2)
				PIK3CA	3 (12.5)
NF1	p.Arg192*	c.574C>T	1 (14.3)	TP53	5 (83.3)
	p.Ser1567*	c.4700C>G	1 (14.3)	APC	4 (66.7)
	p.Arg461*	c.1381C>T	1 (14.3)	ARID1A	3 (50.0)
	p.Ile679fs	c.2033dupC	1 (14.3)		
	p.Phe945fs	c.2835delT	1 (14.3)		
	p.Ser1868fs	c.5594_5600dupCTGACCC	1 (14.3)		
	p.Arg1066_Asp1067delinsSer	c.3198-3_3200delCAGAGA	1 (14.3)		
PTEN	CNV (Deletion)		1 (33.3)	KRAS	3 (100)
	p.Gly20Arg	c.58G>A	1 (33.3)	TP53	3 (100)
	p.Tyr188fs	c.561_574delTTATAGACCAGTGG	1 (33.3)		

* Amino acid change frequency percentage is calculated as proportion of specific site amino acid change count to total amino acid change count in the mutated gene. (Total amino acid change: KRAS 71, SMAD4 25, NF1 7, PTEN 3). Amino acid change with more than 2 occurrence per site is shown except for NF1 and PTEN gene which have only 1 amino acid change per site.

+ Frequently co-mutated gene count percentage is calculated as proportion of sample count of co-mutated gene to total sample count of the mutated gene. (Total sample count: KRAS 70, SMAD4 24, NF1 7, PTEN 3). Co-mutated gene with More than 3 alterations are shown.

Table 4. Univariate analysis of Progression Free Survival according to KRAS mutation site

KRAS mutation	Number of patients (n)	HR (95% CI)	P value
WT	90	1 (Reference)	
p.Gly12Asp	21	1.70 [1.01−2.87]	0.048
p.Gly13Asp	16	1.15 [0.65−2.04]	0.634
p.Gly12Val	10	2.28 [1.12−4.66]	0.023
p.Gly12Cys	5	1.94 [0.47−8.05]	0.360
p.Ala146Thr	2	1.99 [0.80−4.97]	0.140

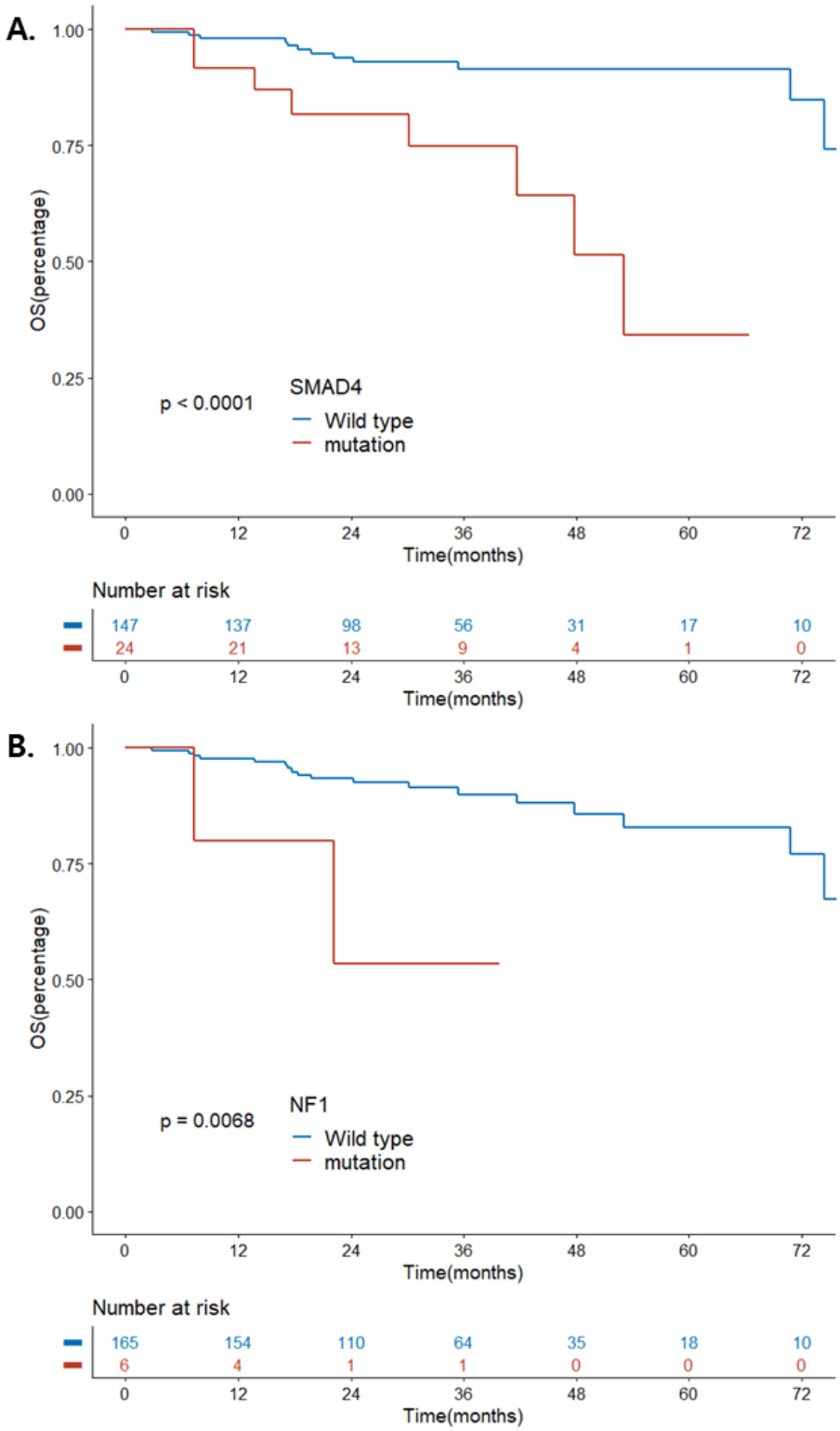


Figure 4. Association of overall survival and genetic mutation. (A) SMAD4 (N=24), (B) NF1 (N=6)

3.4. Prognostic role of gene pathway alteration.

Two key cellular signaling pathways, RAS−RAF−MAPK pathway and PI3K−Akt−mTOR pathway, were assessed to identify prognostic role in first line palliative chemotherapy PFS. A multivariate analysis using the Cox proportional hazards model showed RAS−RAF−MAPK pathway gene alteration (adjusted HR for PFS 1.92, 95% CI; 1.30−2.85, P 0.0011) as an independent prognostic factor. On the other hand, PI3K−Akt−mTOR pathway (adjusted HR for PFS 1.77, 95% CI; 0.89−3.50, P 0.1039) showed tendency towards worse outcome, but did not reach statistical significance (**Figure 5A/B**).

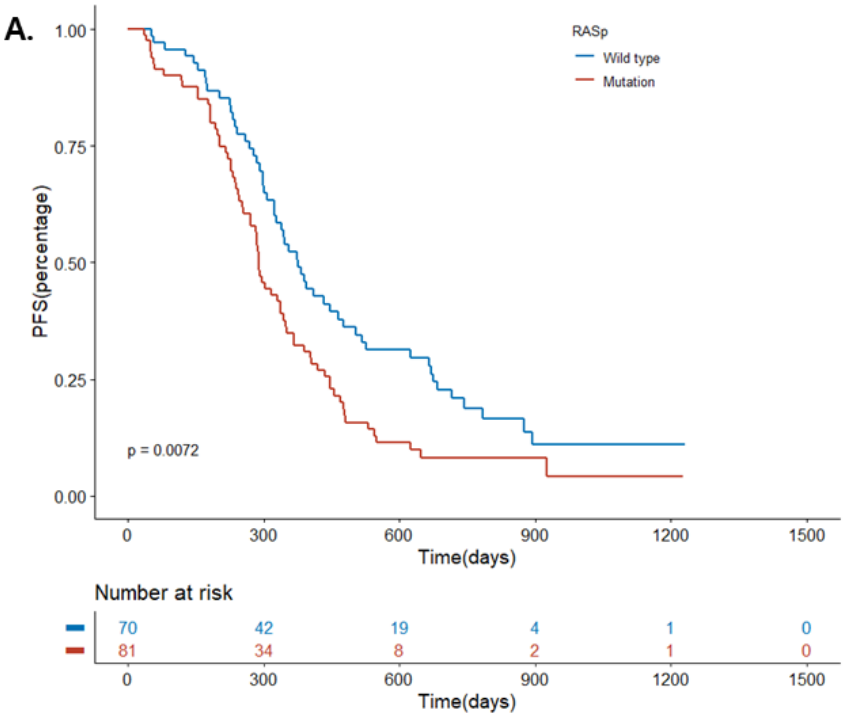
RAS−RAF−MAPK and PI3K−Akt−mTOR cellular signaling pathways are known to influence each other during downstream activation.[13] To study the combination effect of the two pathways, patients were divided into three groups based on their pathway alterations. RAS−RAF−MAPK wild type and PI3K−Akt−mTOR wild type group (RASp(−)PI3Kp(−)), alteration only in RAS−RAF−MAPK pathway but not in the PI3K−Akt−mTOR pathway group(RASp(+)PI3Kp(−)), and alteration in both RAS−RAF−MAPK and PI3K−Akt−mTOR (RASp(+)PI3Kp(+)) pathway group. Adjusted HR for PFS in both the RAS and PI3K pathway alteration group to the RAS and PI3K wild type group was 3.16 (95% CI 1.45−6.87, P 0.0038) which was higher than adjusted HR for PFS of alteration only in the RAS pathway group to the RAS and PI3K wild type group (adjusted HR 1.83 95% CI 1.22−2.76, P 0.0037). Kaplan–Meier method survival curve plot of the three groups show this trend (**Figure 5C**). Median PFS for the RAS−RAF−MAPK pathway alteration was 9.5 months (vs. 12.3 months in wild type), PI3K−Akt−mTOR pathway alteration was 8.5 months (vs. 11.3 months in wild type), whereas those who had alteration in the both pathways was 8.0 months (**Table 5**).

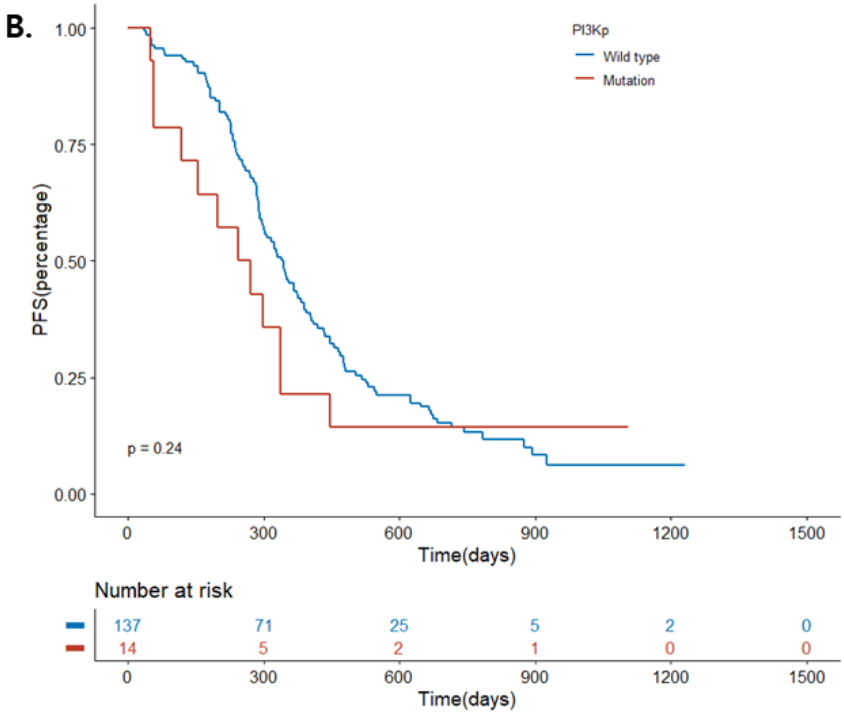
Both the RAS−RAF−MAPK and PI3K−Akt−mTOR pathways are

triggered by upstream RTK (Receptor Tyrosine Kinase) activation. To analyze the gene pathway as a whole, we defined a new RTK-RAS-PI3K pathway. The patient was considered to have an RTK-RAS-PI3K pathway alteration if any of the genes from the RAS-RAF-MAPK pathway, PI3K-Akt-mTOR pathway, or EGFR, ERBB2, ERBB3, NF1 gene were altered. RTK-RAS-PI3K pathway gene alteration was an independent prognostic factor to first line palliative chemotherapy PFS (adjusted HR for PFS 2.08, 95% CI; 1.39-3.11, P 0.0003) (**Figure 5D**). Median PFS for RAS-RAF-MAPK pathway alteration was 9.5 months (**Table 5**).

Multivariate analysis in both the RAS-RAF-MAPK pathway and the PI3K-Akt-mTOR pathway revealed primary tumor location as statistically significant adjustment factor. Regardless of the pathway, tumors originating from distal side had lower hazard ratios. We addressed the prognostic role of each pathway separately according to the tumor location.

The RAS-RAF-MAPK pathway alteration in distal tumor showed poor prognosis in both univariate and multivariate adjusted HR (Univariate HR 1.71, 95% CI; 1.13-2.59, P 0.0109, adjusted HR 1.94, 95% CI; 1.25-3.03, P 0.0034). As for the PI3K-Akt-mTOR pathway, univariate HR in distal tumor location was not significant (Univariate HR 1.68, 95% CI; 0.81-3.50, P 0.163) but exhibited an adverse trend in Kaplan Meier curve. After adjusting with clinical variables, distal tumor with PI3K-Akt-mTOR pathway alteration demonstrated a significantly poor prognosis (adjusted HR 2.49, 95% CI; 1.03-6.06, P 0.0439) (**Figure 6**). There was minimal to no association between gene pathway alteration and right sided tumor. Neither pathway alteration univariate analysis nor multivariate analysis showed a considerable association in proximal tumor.


A.
RASp
Wild type
Mutation
PFS(percentage)
1.00
0.75
0.50
0.25
0.00
p = 0.0072
0
300
600
900
1200
1500
Time(days)
Number at risk
70 42 19 4 1 0
81 34 8 2 1 0
0 300 600 900 1200 1500
Time(days)


B.
PI3Kp
Wild type
Mutation
PFS(percentage)
1.00
0.75
0.50
0.25
0.00
p = 0.24
0
300
600
900
1200
1500
Time(days)
Number at risk
137 71 25 5 2 0
14 5 2 1 0 0
0 300 600 900 1200 1500
Time(days)

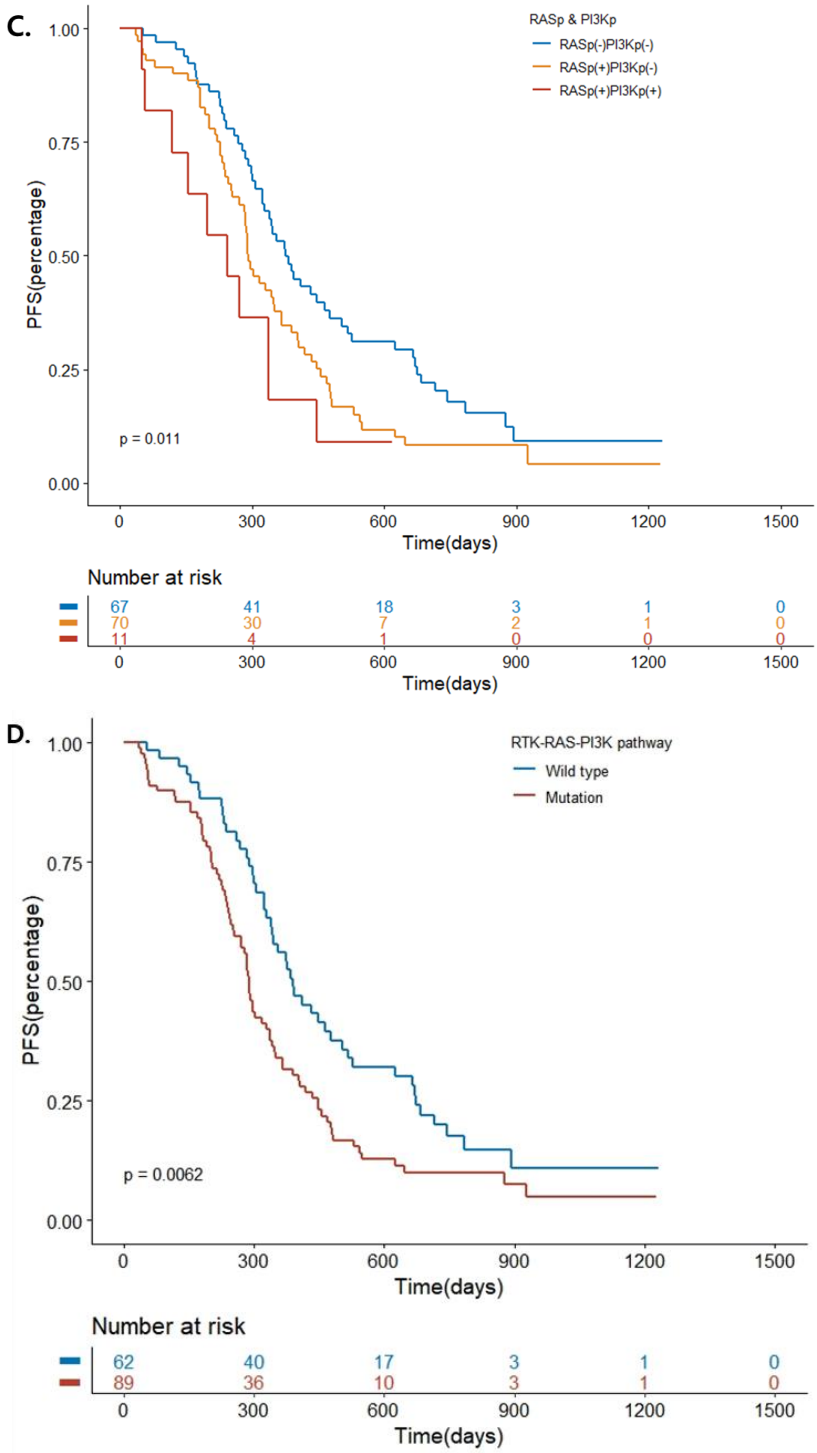


Figure 5. Association of gene pathway alteration with progression free survival. (A) RAS-RAF-MAPK pathway, (B) PI3K-Akt-mTOR pathway, (C) RAS-RAF-MAPK and PI3K-Akt-mTOR pathway, (D) RTK-RAS-PI3K pathway

Table 5. Multivariate Analysis of Progression Free Survival by Gene Pathway Alteration

Gene pathway alteration	Number of patients (n =)	HR (95% CI)	P value	Median PFS (month)
RAS-RAF-MAPK (RASp)	81	1.92 [1.30-2.85]	0.0011	9.5 vs. 12.3
PI3K-Akt-mTOR (PI3Kp)	14	1.77 [0.89-3.50]	0.1039	8.5 vs. 11.3
RAS-RAF-MAPK (RASp) & PI3K-Akt-mTOR (PI3Kp)	11	3.16 [1.45-6.87]	0.0038	8.0 vs. 12.3
RTK-RAS-PI3K	89	2.08 [1.39-3.11]	0.0003	9.5 vs. 12.8

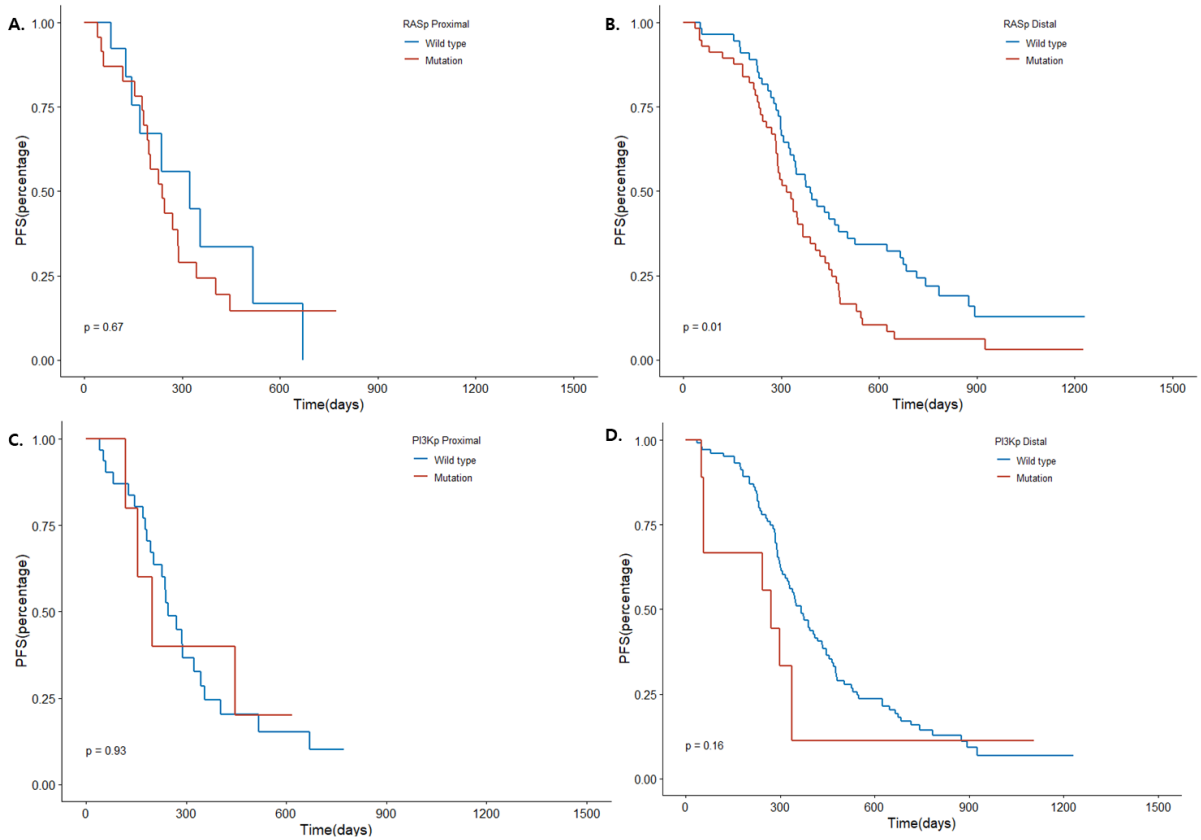


Figure 6. Correlation between gene pathway alteration and progression free survival according to the primary tumor site. RAS-RAF-MAPK pathway (A) proximal, (B) distal, PI3K-Akt-mTOR pathway (C) proximal, and (D) distal

4. Discussion

This study analyzed 171 mCRC patients targeted gene panel sequencing data to identify prognostic role of genetic mutation and gene pathway alteration. Of the participants, 127 (74.3%) patients exhibited distal CRC and 42 (24.6%) patients had proximal CRC. The analysis focused exclusively on pathogenic or likely pathogenic mutations classified by our institutional database which is in align with OncoKB data. 619 SNVs and 26 CNVs were detected and the most frequently mutated genes were found in TP53 150 (87.7%), APC 128 (74.9%), KRAS 70 (40.9%), SMAD4 24 (14.0%), FBXW7 21 (12.3%) patients.

Generally APC gene is known to be most frequently mutated gene in mCRC followed by TP53 gene.[17] On the contrary, our study showed TP53 mutation as the most prevalent single nucleotide variant. This difference could be attributed to disparities in tumor location. Majority of our cohort had left (distal) side CRC (74.3% compared to 58.3% in TCGA data[7]) which is known to harbor more TP53 mutations than right side (proximal) CRC. Mei et al. also reported higher TP53 mutation frequency (75.8%) in 33 Chinese mCRC patients.[18] These findings may refer to the different CRC mutation profile between ethnic groups.

Our study examined the association between palliative first line treatment PFS and genetic mutation. KRAS (adjusted HR 1.69), NF1 (adjusted HR 11.56) and PTEN (adjusted HR 3.72) mutation were associated with unfavorable PFS outcome. We also identified that SMAD4 (adjusted HR 7.74) and NF1 (adjusted HR 7.53) mutation were related to adverse OS outcome.

SMAD4 is a tumor suppressor gene and a pathway member of TGF-β signaling pathway, located on chromosome 18q21. Loss of function of this gene has been related with distant metastasis and

adverse outcome.[19] Alazzouzi et al. found out that relative expression level of SMAD4 by immunohistochemistry is associated with survival outcome in colorectal cancer patient who underwent surgery.[20] Oyanagi et al. utilized NGS data of SMAD4 mutation identifying relationship with adverse OS outcome in stage I–III colorectal cancer, but not in stage IV patients.[21] In our cohort, which encompasses only metastatic colorectal cancer, SMAD4 gene mutation presented adverse overall survival outcome.

NF1, located on chromosome 17q11, is infrequently mutated gene in CRC (3.51% sample alteration in our cohort) and its protein is a negative regulator of RAS signaling pathway. Previous studies show that NF1 inactivation promotes anti EGFR treatment resistance.[18,22] When NF1 overexpression is induced in cell lines with low NF1 expression anti–EGFR resistance, anti EGFR treatment sensitivity was recovery. Additionally, following anti EGFR therapy, NF1 RNA expression tended to be slightly decreased, implying acquired resistance mechanism to the treatment.[23] The current study found NF1 mutation as an adverse prognostic factor in both PFS and OS analyses. Also, RTK–RAS–PI3K pathway exhibited an unfavorable PFS outcome (adjusted HR for PFS 2.08). Together with our findings alongside preclinical data, NF1 mutation may serve as a potential biomarker for anti EGFR therapy in mCRC. Further study is needed to validate the hypothesis.

Multiple mutations across various genes are required for the formation of a mCRC.[24] During tumorigenesis, mutation of a different gene from same gene pathway family may show similar outcome. Integrated interpretation of gene pathway alteration can provide more vivid aspects of cancer tumorigenesis, and perspectives to therapeutic strategies. [12,25,26] We defined the RAS–RAF–MAPK pathway alteration as one or more mutations in KRAS, NRAS, BRAF, MAP2K4, and MAP2K1 genes. PI3K–Akt–mTOR pathway alteration was defined as one or more mutation in PIK3CA, PTEN, AKT1, TSC1, TSC2, and mTOR genes. Our multivariate

analysis revealed an association of the RAS-RAF-MAPK pathway gene alteration with adverse PFS (adjusted HR PFS 1.92) outcomes. Additionally, a tendency towards unfavorable PFS was observed in the PI3K-Akt-mTOR pathway alteration.

Tumors originating from distal side exhibited favorable hazard ratios for PFS. However, among the distal tumors, the RAS-RAF-MAPK pathway alteration was linked to a poor prognosis (adjusted HR 1.94). Lee et al. demonstrated prognostic outcomes for pathway gene mutations based on the location of surgically treated stage III or high-risk stage II CRC patients.[25] In that study, RTK-RAS pathway alteration was also identified as a negative prognostic factor in distal tumors. In contrast to our study which showed no prognostic role of the PI3K-Akt-mTOR pathway in proximal CRC, PI3K pathway mutation was a positive prognostic factor in proximal tumors in Lee et al.'s research.

PI3K can be activated through direct activation from RAS or indirectly by activated growth factor receptors SRC homology 2 (SH2) domain phosphotyrosine residues.[27] RAS-RAF pathway is known to crosstalk with PI3K-Akt-mTOR pathway.[13,27] In our study, double alteration involving both the RAS-RAF-MAPK pathway and the PI3K-Akt-mTOR pathway presented higher HR (adjusted HR 3.16) compared to the RAS-RAF-MAPK pathway alteration alone. Consequently, simultaneous inhibition of both the RAS and PI3K pathways, rather than single blockade, could potentially yield improved therapeutic efficacy. Research for combination treatment targeting PI3K-Akt-mTOR pathway is ongoing.[27,28]

This study analyzed clinical targeted panel sequencing. Omitted genetic information that cannot be captured by panel sequencing puts limitation to our study. Additionally, some important genes for analyzing signaling pathway in colorectal cancer were not included in the study. However, our primary objective was to interpret real

world data that is readily accessible in the clinical field. Recent advance in equipment for cancer genetic evaluation provided flood of information to frontline clinicians. NGS allows rapid targeted gene panel sequencing in a cost efficient manner. [29] We hope to offer insights into the prognosis of mCRC utilizing targeted gene panel sequencing by NGS method.

This is a retrospective study. Survival status was often difficult to obtain resulting in substantial amount of censored data. Additionally. throughout the treatment duration, diverse approaches including surgical options were taken to make optimal decisions for individual patients. This diversity in treatment strategies rendered heterogeneity in the treatment process. Nevertheless, despite these complexities, the study exhibited adequate statistical power to discriminate survival prognosis in some critical genes.

In conclusion, this single center retrospective study analyzed targeted gene panel sequencing of 171 mCRC patient and identified KRAS, NF1, PTEN mutation as a poor prognostic indicator for palliative first line treatment PFS. Also, SMAD4 and NF1 mutation was identified as adverse prognostic marker for overall survival. We demonstrated relationship of the RAS-RAF-MAPK pathway alteration with unfavorable outcome. Double alteration of the RAS-RAF-MAPK pathway and the PI3K-Akt-mTOR pathway result in more deleterious prognosis.

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국 문 초 록

차세대 염기서열 분석법을 활용한 전이성 직결장암의 유전 변이 및 유전자 경로 변이와 생존 성적 분석

배경: 직결장암은 전세계적으로 발생률이 높은 암이다. 치료 반응과 예후를 예측하기 위해 암 유전자 정보를 이해하는 것이 중요하다. 차세대 염기서열 분석법(Next generation sequencing, NGS)은 임상의사들이 다양한 유전 정보를 쉽게 획득할 수 있게 해준다. 본 연구는 전이성 직결장암에서 유전 변이와 생존 성적 사이의 관계를 확인하기 위해서 NGS 결과를 분석하였다.

방법: 본 연구는 전이성 직결장암의 표적 패널 시퀀싱 (target panel sequencing) 결과를 분석한 단일기관 후향적 연구이다. 유전 변이 정보와 고식적 일차 치료의 PFS (Progression Free Survival) 그리고 OS (Overall Survival) 사이의 관계를 평가하였다. 추가적으로 유전자 경로의 변이와 PFS 사이의 관계를 평가하기 위해서 유전 변이 결과를 직결장암에서 중요한 RAS-RAF-MAPK와 PI3K-Akt-mTOR 두 가지 경로로 분류하였다.

결과: 171명의 전이성 직결장암 환자가 분석되었다. 가장 흔히 발견된 병리적 또는 병리적 가능성이 있는 유전자 변이가 있는 환자는 TP53 변이에서 150명 (87.7%), APC 변이 128명 (74.9%), KRAS 변이 70명 (40.9%), SMAD4 변이 24 명 (14.0%), FBXW7 변이 21 명 (12.3%) 이였다. RAS-RAF-MAPK 경로 변이는 81 명 (47.4%), PI3K-Akt-mTOR 경로 변이는 14 명 (8.2%)에서 확인되었다. KRAS (adjusted HR 1.69, 95% CI; 1.10-2.60), NF1 (adjusted HR, 11.56 95% CI; 3.98-33.55) 그리고 PTEN (adjusted HR 3.72, 95% CI; 1.06-13.01) 변이가 나쁜 고식적 일차 치료의 PFS와 관련 있었다.

SMAD4 (adjusted HR 7.74, 95% CI; 2.71-22.14)와 NF1 (adjusted HR 7.53, 95% CI; 1.14-49.70) 변이는 나쁜 OS와 연관 있는 것으로 확인되었다. 유전 경로 변이 분석에서는 RAS-RAF-MAPK (adjusted HR for PFS 1.92, 95% CI 1.30-2.85) 경로가 불량한 PFS의 예후 인자였다. 환자가 RAS-RAF-MAPK 와 PI3K-Akt-mTOR 경로 모두에서 변이를 가지고 있는 경우에 더 좋지 못한 예후 결과를 보였다 (adjusted HR for PFS 3.16, 95% CI 1.45-6.87).

결론: 본 연구는 KRAS, NF1, 그리고 PTEN 변이가 전이성 직결장암에서 불량한 고식적 일차 치료 PFS와 관련 있음을 확인하였다. 또 SMAD4 와 NF1 변이는 나쁜 OS와 연관되어 있었다. 유전 경로 변화 분석에서는 RAS-RAF-MAPK 경로가 나쁜 PFS의 예후 인자였다.

주요어 : 전이성 직결장암, 차세대 염기서열 분석법, 유전 변이, 유전 경로 변이, 임상 예후
학 번 : 2021-26572