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항암 화학 요법으로 치료한 대장암에서 예외적으로 좋은 반응을 보인 환자들의 유전체 특성

The genomic characteristics of exceptional responders in palliative chemotherapy-treated colorectal cancer

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의 과 학 과

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The genomic characteristics of exceptional responders in palliative chemotherapy-treated colorectal cancer

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이 논문을 이학석사 학위 논문으로 제출함

2022 년 2 월

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Abstract

Among patients with metastatic colorectal cancer (mCRC), the occasional presence of exceptional responders (ERs) who achieved deep and/or sustained response to chemotherapy has been reported. This study aimed to identify the genomic characteristics of ERs by targeted sequencing.

Between January 2016 and December 2018, mCRC patients who were treated with palliative chemotherapy and had targeted sequencing results from tumor tissues were included in this study. Patients who received curative resection and/or metastasectomy were excluded. ERs were defined as those having complete response as the best overall response or sustained partial response for ≥28 months to the palliative first- or second-line chemotherapy.

Among the 340 patients included, 15 (4.4%) were classified as ERs. Clinical characteristics did not differ between groups. No patient in the ER group had BRAF V600 mutation. The proportion of patients with DNA damage response and repair gene

mutations and the tumor mutational burden were not different between groups. We identified 19 gene and 1 pathway mutations that were more frequent in the ERs, but they were not associated with favorable survival outcomes individually and did not provide plausible mechanisms for the exceptional responses observed.

Targeted sequencing data collected from mCRC patients treated with palliative chemotherapy suggested that ERs had a higher proportion of BRAF wild-type MSI-H patients. However, tumor genomics alone is insufficient to predict treatment response. Multi-factorial analysis might be needed to identify factors beyond tumor genomics.

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Introduction

Colorectal Cancer (CRC)

Colorectal cancer (CRC) is cancer that occurs in the colon or rectum, and signs and symptoms may include bloody stools, changes in bowel movements, weight loss, and fatigue ("Colon Cancer Treatment (PDQ®)–Patient Version - National Cancer Institute," 2021).

CRC is one of the most common cancers worldwide, female breast cancer is the most often diagnosed cancer in both sexes (11.7 % of total cases), followed by lung (11.4 %) and colorectal (10.0 %) cancers. Lung cancer is the most common cancer killer (18.0 % of all cancer deaths), followed by colorectal cancer (9.4 %) (Figure 1) (Sung et al., 2021). As such, CRC occurs frequently in the upper ranks, and the mortality rate is also high.

CRC was one of the first tumor forms to be recognized as a genetic illness, with the progression to carcinoma and invasion owing to the accumulation of genetic

abnormalities (Vogelstein et al., 1988). More recently, large-scale sequencing projects like The Cancer Genome Atlas (TCGA) have identified genomic events that distinguish ultra-mutated, microsatellite instability-high (MSI-H)/hypermutated, and microsatellite stable (MSS) CRCs (Cancer Genome Atlas, 2012; Donehower et al., 2013; Giannakis et al., 2016).

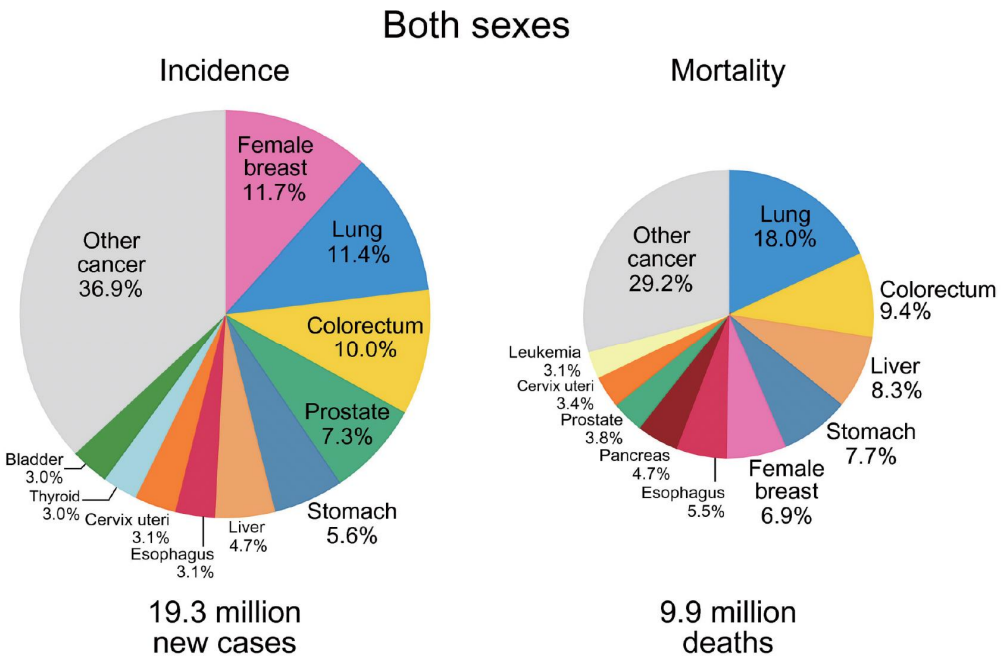

Figure 1. Distribution of Cases and Deaths in both sexes

Cases and Deaths for the Top 10 Most Common Cancers in both Sexes in 2020

(Adapted from Global Cancer Statistics 2020) (Sung et al., 2021)

Palliative chemotherapy for metastatic colorectal cancer

Fluoropyrimidine + oxaliplatin or irinotecan two-drug regimen (FOLFOX, XELOX, FOLFIRI) is the standard for cytotoxic chemotherapy, and there is no difference in overall effect according to the exposure sequence of oxaliplatin and irinotecan. FOLFOX and XELOX are equivalent, but in the case of XELIRI, there are reports that the efficacy is lower than that of FOLFIRI and the side effects may be high, so caution is required. The use of fluoropyrimidine alone (FL or capecitabine) from the beginning can be used when the patient's general condition is poor, but in other cases, it is not recommended.

In the case of bevacizumab, it is recommended to be used in combination with cytotoxic chemotherapy as the 1st or 2nd line therapy. However, multi-faceted consideration is required in terms of cost-effectiveness, and there is no clear evidence for the 3rd line or higher. Cetuximab can be used at any time, but it is allowed to use only when there is no *KRAS* mutation after checking whether there is a *KRAS* mutation (Hong & Kim, 2009).

Risk factors

Being overweight or obese, drinking excessive alcohol, having a family history of CRC, having inflammatory bowel disease, smoking cigarettes, and eating processed and red meat all appear to increase the chance of developing CRC, according to published data. (Johnson et al., 2013).

Moreover, Lee and colleagues studied the risk factors affecting survival in CRC patients in Taiwan. As a result, the five-year survival rate was 68.7%, with a mean survival duration of 71.27 months after cancer diagnosis. Perineural nerve invasion, distant metastasis, age, pathological differentiation grade, obstruction, and regional lymph node metastasis have all been demonstrated to be independent predictors of CRC patient survival and prognosis. Among them, Perineural nerve invasion was found to be a significant influence in CRC patients' survival. As a consequence, earlier identification of CRC may enhance survival. (Lee et al., 2018)

Biomarker and therapeutic target

The pathogenesis of CRC has been linked to several different pathways. Pathways implicated in aberrant DNA methylation, gene expression control via microRNA (miRNA), and the CIN pathway are among them. Through the participation of CRC risk factors, changes in these pathways may emerge. Mutations that cause CRC may be identified in the genes that code for the proteins implicated in these processes. The formation of abnormal crypt foci (ACF) is regarded to be the first stage in colorectal carcinogenesis. The WNT pathway is activated during ACF, which is caused by inactivating mutations in the adenomatous polyposis coli (*APC*) gene. Inactivating mutations in the adenomatous polyposis coli (*APC*) gene cause the WNT pathway to be activated during ACF. The development to adenoma and carcinoma is usually mediated by activation of *KRAS* mutations and loss of *TP53* expression. Mutations in *PIK3CA* and the deletion of 18q cause adenoma to develop (P. J. Kim, Plescia, Clevers, Fearon, & Altieri, 2003).

According to oncokb, there are genes in CRC that the FDA has recognized as specific changes to predict response to level 1, FDA-approved drugs. The genes and drugs include *BRAF* V600E and Encorafenib plus Cetuximab, *KRAS* wildtype and either Cetuximab as monotherapy or in combination with Cetuximab and chemotherapy, other drugs are either Panitumumab as monotherapy or in combination with Panitumumab and chemotherapy, *NRAS* wildtype and either Panitumumab as monotherapy or Panitumumab and chemotherapy combination.

NTRK1, NTRK2, and *NTRK3* fusions include Entrectinib or Larotrectinib, MSI-H and either Pembrolizumab as monotherapy or in combination with Nivolumab and Ipilimumab, tumor mutation burden-high (TMB-H) and Pembrolizumab(Chakravarty et al., 2017). Moreover, patients with deficient DNA mismatch repair (dMMR) in CRC, ipilimumab, nivolumab, and pembrolizumab have shown effectiveness (Kusnoor et al., 2016).

Furthermore, colorectal cancer treatment targets HER2 activating mutations. In 7% of colorectal cancer patients, the Cancer Genome Atlas project discovered HER2 somatic

mutations and gene amplification. Oncogenic transformation of colon epithelial cells is caused by HER2 mutations found in CRC patients. In CRC cells, HER2 mutations also cause resistance to EGFR monoclonal antibodies. Furthermore, HER2 mutations are very vulnerable to the irreversible HER2/EGFR tyrosine kinase inhibitors neratinib and afatinib. (Kavuri et al., 2015)

Next generation sequencing

The procedure of finding the exact order of nucleotides present in a DNA or RNA molecule is known as nucleic acid sequencing. As the ability to sequence has become accessible to research and clinical labs all over the world, the usage of nucleic acid sequencing has expanded tremendously in the last decade. The Human Genome Project, a $3 billion, 13-year project finished in 2003, was the first major step into DNA sequencing. First-generation sequencing, also known as Sanger sequencing, was used to complete the Human Genome Project. Sanger sequencing (the chain-termination method), which was introduced in 1975, was regarded the gold standard for nucleic acid sequencing (Sanger, Nicklen, & Coulson, 1977).

The desire for cheaper and quicker sequencing methods has risen dramatically since the first human genome sequence was completed. As a result of this need, second-generation sequencing methods, also known as next-generation sequencing, have been developed (NGS). Massively parallel sequencing is performed on NGS platforms, in which millions of fragments of DNA from a single sample are sequenced simultaneously.

High-throughput sequencing allows a complete genome to be sequenced in less than a day by massively parallel sequencing technology. Several NGS technologies that allow low-cost, high-throughput sequencing have been developed over the past. The Ion Torrent Personal Genome Machine (PGM), as well as the Illumina MiSeq and other NGS platforms, have made sequencing more accessible, resulting in a dramatic increase in nucleic acid sequencing research and clinical diagnoses (Figure 2) (Grada & Weinbrecht, 2013).

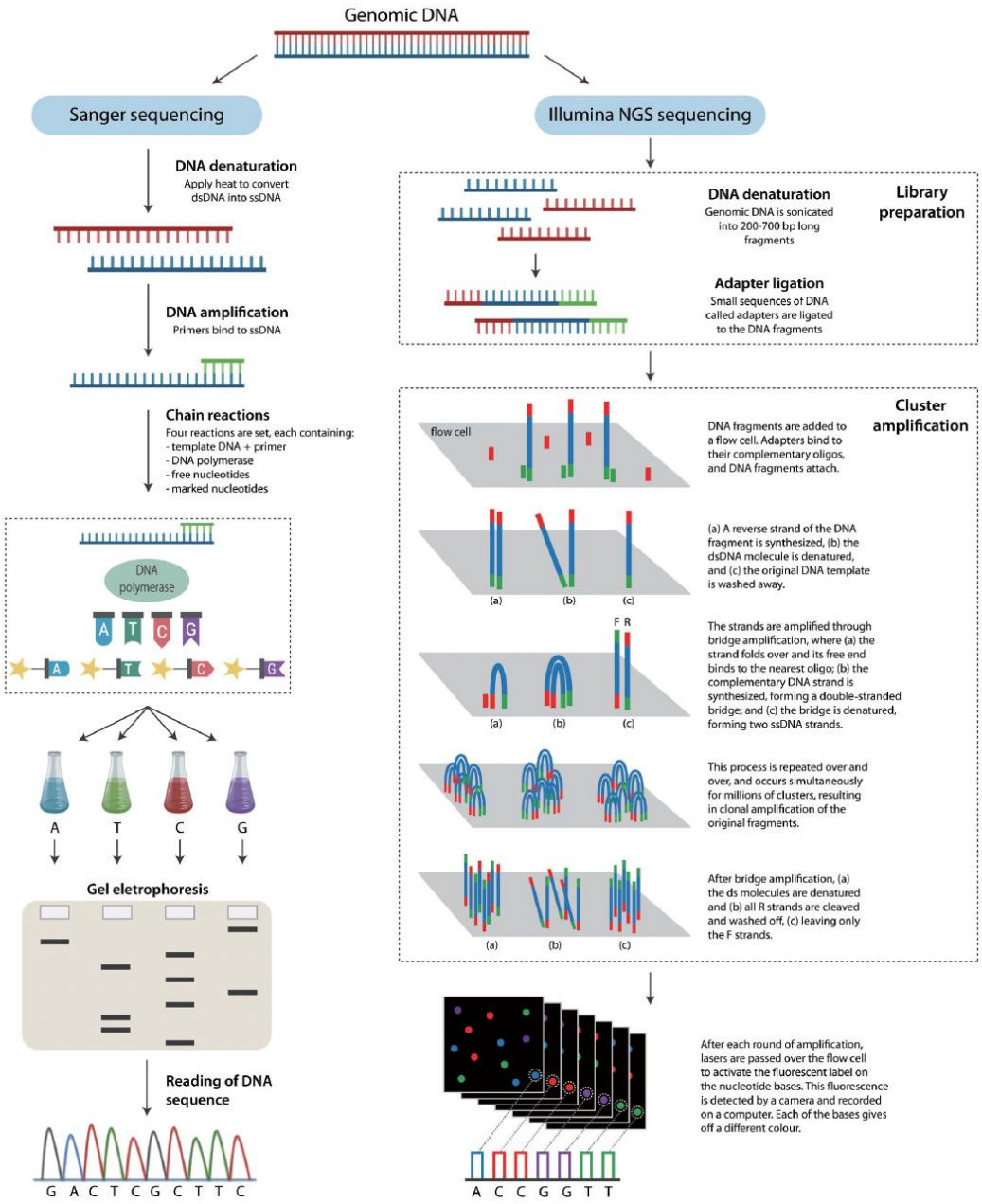

Figure 2. Comparison of Sanger sequencing (left) and Illumina next-generation sequencing (right).

A flow cell is a glass slide with lanes, each lane being a channel coated with two types of oligos, which are complementary to the adapters ligated in the DNA fragments. dsDNA,

double-stranded DNA; F, forward DNA strand; R, reverse DNA strand; ssDNA, single-stranded DNA.

(Adapted from 2020 Systematic Entomology) (Young & Gillung, 2020)

Previous studies with next generation sequencing

Table 1 summarizes the NGS-based investigations of CRC. The mutational spectrum and novel targets of genomic changes in CRC were discovered in these investigations, which have biological and therapeutic implications.

The Cancer Genome Atlas (TCGA) project initially published the largest NGS-based analysis of the CRC genome (Cancer Genome Atlas, 2012). Similar to previous studies, the majority of the highly recurring mutations were discovered in known cancer-related genes such as *APC* (81%), *TP53* (60%), *KRAS* (43%), *PIK3CA* (18%), *FBXW7* (11%), *SMAD4* (10%), and *NRAS* (9%). After manual evaluation of sequencing data for 30 known MSI loci, they found frequent coding MSI on *ACVR2A* (63%), *TGFBR2* (51%), *MSH3* (40%), and *MSH6* (40%) (Figure 3).

Table 1. Summary of previous studies with NGS in colorectal cancer

Group	Application	Samples	Analysis
Cancer Genome Atlas Network Nature 2012	Exome sequencing, RNA-seq, methylation, miRNA-seq	224 pairs	SNVs, CNVs, SVs, expression, methylation, miRNA
Seshagiri et al. Nature 2013	Whole genome sequencing, RNA-seq	74, 68 pairs	SNVs, CNVs, expression, fusions
Brannon et al. Genome Biology 2014	Targeted sequencing, Whole genome sequencing	69, 4 pairs	SNVs, INDELs
Guda et al. PNAS 2015	Exome sequencing, Targeted sequencing	31 pairs, 29 pairs	SNVs

Table 1. Summary of previous studies with NGS in colorectal cancer (Continue)

Group	Application	Samples	Analysis
Giannakis et al. Cell reports 2016	Exome sequencing	619 pairs	SNVs, INDELs, neoantigen load, survival
Yaeger et al. Cancer Cell 2018	Targeted sequencing	1134 pairs	SNVs, INDELs, CNVs, survival
Vasaikar et al. Cell 2019	Exome sequencing, RNA-seq, miRNA-seq, label-free shotgun proteomic analyses	110 pairs	SNVs, INDELs, CNVs, PTMs, expression
Mondaca et al. Gastroenterology 2020	Targeted sequencing	471 pairs	SNVs, survival

Summary of previous studies with NGS in colorectal cancer by year.

Abbreviations: RNA-seq, RNA sequencing; miRNA-seq, microRNA sequencing; SNVs, Single nucleotide variants; INDELs, Insertion–deletion mutations; CNVs, Copy number variants; SV, Structural variations; PTM, post-translational modification.

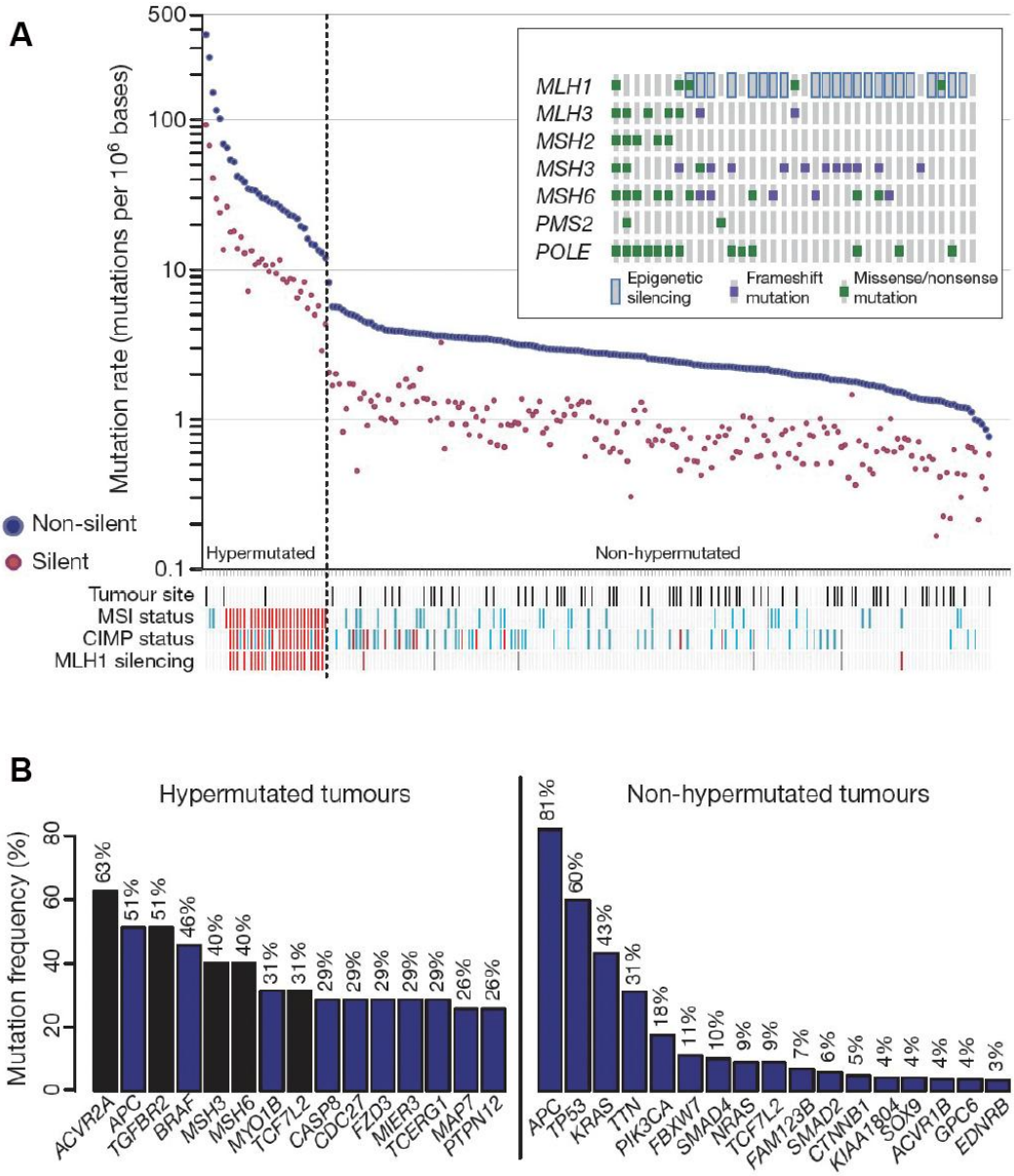

Figure 3. Mutation frequencies in human CRC

A, Mutation frequencies in each of the tumor samples from 224 patients. Note a clear separation of hypermutated and non-hypermutated samples. Red, MSI high, CIMP high or MLH1 silenced; light blue, MSI low, or CIMP low; black, rectum; white, colon; grey,

no data. Inset, mutations in mismatch-repair genes and POLE among the hypermutated samples. The order of the samples is the same as in the main graph. B, Significantly mutated genes in hypermutated and non-hypermutated tumors. Blue bars represent genes identified by the MutSig algorithm and black bars represent genes identified by manual examination of sequence data.

(Adapted from The Cancer Genome Atlas Network, 2012) (Cancer Genome Atlas, 2012)

Seshagiri and colleagues published CRC paper related NGS (Seshagiri et al., 2012). They identified frequently mutated genes including *KRAS, APC, SMAD4, FBXW7* and *EP400*, as well as genes involved in chromatin remodeling such as *SIN3A, SMARCA5, NCOR1* and histone modifying enzyme JARID2. Also, they discovered known amplifications linked like *KRAS* (13 %, 10 of 74) and *MYC* (23 %, 17 of 74) and Deletion involving a tumor-suppressor gene *FHIT* was observed in 30% (22 out of 74) of the samples. They identified 36 gene fusion rearrangements using RNA-seq data, including two recurrent ones which is the R-spondin family members RSPO2 (3 %, 2 of 68) and RSPO3 (8 % , 5 out of 68).

Brannon et al performed targeted sequencing on 69 normal and tumor pairs of CRC (Brannon et al., 2014). The mutation profile was in line with the expected mutation frequency of non-hypermutated samples as reported by The Cancer Genome Atlas (TCGA), with very good agreement (93%, 229/247) between primary tumor and consistent metastases among mutations that had been reported as significantly mutated (247/434, 57%).

Guda and colleagues conducted whole exome and targeted sequencing to analyze somatic mutations in 103 African-American CRCs, discovering 20 novel genes that were significantly altered in CRCs (Guda et al., 2015). Among all ethnic groups in the United States, African Americans have the highest incidence and fatality rates for colon cancer. Nevertheless, due to the lack of knowledge about the genetic mechanisms underlying the development of colon cancer in African Americans, they set out to characterize the mutational landscape of African American colon cancer. These findings imply that different ethnic groups have diverse colon carcinogenesis pathways, which could explain why race-related tumor incidence and prognosis differ.

Giannakis and colleagues used whole-exome sequencing of 613 CRCs (Giannakis et al., 2016). They discovered additional CRC driver genes and correlated high neoantigen load with increased lymphocytic infiltration and improved survival. Also, they found positive selection for *HLA* mutations in immune-cell infiltrated tumors. These results may inform immunotherapeutic approaches in CRCs.

Yaeger et al. performed prospective targeted sequencing of 1134 CRCs (Yaeger et al., 2018). Splice alterations in *APC's* intron region and in-frame deletions in *CTNNB1* were found to increase carcinogenic WNT pathway modifications to 96 percent of CRC cases. When compared to the left primary site, the right primary site of microsatellite stable mCRC was linked to shorter survival, aging at diagnosis, higher mutations, and enrichment of oncogenic alterations in *KRAS, BRAF, PIK3CA, AKT1, RNF43*, and *SMAD4*. Their findings point to alternative carcinogenesis routes in right and left microsatellite stable CRC, which could explain clinical variations.

Vasaikar et al. conducted the first prospectively collected colon cancer cohort proteogenomic analysis (Vasaikar et al., 2019). A list of colon cancer-associated proteins and phosphosites was discovered using comparative proteome and phosphoproteomic examination, including known and putative novel biomarkers, therapeutic targets, and cancer antigens. Phosphoproteomics findings in colon cancer linked Rb phosphorylation to enhanced proliferation and decreased apoptosis, explaining why this traditional tumor suppressor is amplified in colon cancers and suggesting a justification for targeting Rb

phosphorylation in colon cancer. In MSI-H cancers, proteomics found a link between decreased CD8 T cell infiltration and enhanced glycolysis, suggesting glycolysis as a possible target to overcome MSI-H tumor resistance to immune checkpoint blockade. Proteogenomics opens new possibilities for biological research and drug development.

Mondaca and colleagues published paper related to WNT and DDR pathways in CRC (Mondaca et al., 2020). In mCRC, changes in the Wnt and DNA damage response (DDR) pathways have been suggested as outcome modifiers and therapeutic targets. The DDR pathway is crucial for maintaining genomic stability. Platinum compounds and poly (ADP) polymerase inhibitors have been linked to changes in this pathway. Still, the functional significance of DDR changes in mCRC is unknown.

NGS-driven genomic studies are already revealing novel characteristics of cancer genomes that go beyond known mutational categories (T.-M. Kim, Lee, & Chung, 2013). Overall, NGS technology will improve our understanding of CRC genomes, resulting in better diagnosis and personalized CRC treatments.

Exceptional responders in mCRC

Despite the limited survival of patients with metastatic solid cancers, the presence of "exceptional responders (ERs)" showing a deep and/or durable response to systemic antitumor therapy has been reported. These ERs have helped in providing a better understanding of the molecular mechanisms underlying tumor response and resistance to anticancer agents (Chau & Lorch, 2015; Iyer et al., 2012). Recently, further attempts to explain these exceptional responses by multiplatform genomic tools in a large nationwide cohort were made, which impressively identified plausible molecular backgrounds for the exceptional response in 23% of the study population (Wheeler et al., 2021). However, in most instances of clinical practice, such rare exceptional responses remain unexplained.

For patients with metastatic colorectal cancer (mCRC), despite recent advances in systemic treatments, survival outcomes have remained poor with a 5-year survival rate of <15% except in those who were eligible for complete tumor resection (Siegel et al., 2020). However, the rare presence of patients who achieved complete response with

palliative chemotherapy was reported in approximately 1-3% of mCRC cases (Zhang, Zhou, & Lin, 2015), and far less frequently, durable complete responses were also reported (Ferrarotto et al., 2011). Due to their scarcity, the genomic features of these exceptional responders among mCRC patients have been rarely discussed. In addition, data on the role of targeted exome sequencing for the prediction of tumor responses to palliative chemotherapy are limited despite its widespread use in daily practice.

In this study, we aimed to identify the genomic characteristics of ERs among mCRC patients who were treated with palliative chemotherapy only and discuss the prognostic implications of these genomic features identified by targeted exome sequencing with the data acquired from routine clinical practice.

Materials and methods

Study population

Between January 2016 and December 2018, mCRC patients who were treated with palliative chemotherapy and had targeted sequencing results from tumor tissues were identified from the mCRC dataset of the medical oncology department. Among those, patients who received curative resection after starting palliative chemotherapy were excluded. This study was approved by the Institutional Review Board (IRB) of the Asan Medical Center (AMC) and was conducted as per the Declaration of Helsinki. The IRB granted a waiver of informed consent for this retrospective study. For correlative survival analysis, an independent public cohort of mCRC patients treated in Memorial Sloan Kettering Cancer Center (MSKCC) was used in addition to the original study population, excluding those who received metastasectomy (Yaeger et al., 2018).

Assessments

Patients were classified into the ER or non-ER groups according to their best overall response to the first- or second-line palliative chemotherapy, which was assessed according to the Response Evaluation Criteria in Solid Tumors version 1.1. ERs were defined as 1) patients who achieved complete response, or 2) patients with sustained partial response for ≥28 months, which was more than three times longer than the median time-to-progression, to the palliative first-line chemotherapy of the study population (9.4months) (Conley et al., 2021). Patients who did not meet any of the abovementioned criteria were considered non-ERs.

Targeted exome sequencing

Genomic DNA was extracted from archival formalin-fixed, paraffin-embedded tis-sue specimens. Sequencing panels used in this study comprised in-house panels developed at the AMC (OncoPanel AMC, versions 3 and 4). The OncoPanel AMC version 3 and 4 were run using the MiSeq and NextSeq platforms (Illumina; San Diego,

CA, USA) and captured 383 (199 genes for entire exons, eight genes for partial introns, and 184 genes for hotspots) and 322 (225 genes for entire exons, six genes for partial introns, and 99 for hotspots) cancer-related genes, respectively.

Bioinformatics analysis

The sequence mapping methods were described elsewhere (Hwang et al., 2018; Jeong E. Kim et al., 2019; Jeong Eun Kim et al., 2018). Sequenced reads were aligned to human reference genome (GRCh37) using BWA (v0.5.9) with additional options (-q 5 -l 32 -k 2 -o 2) (Li & Durbin, 2009). MarkDuplicates of Picard package (v2.5.0) was used to remove PCR duplicates from the aligned reads. Deduplicated reads were realigned at known indel positions with the GATK IndelRealigner (1.6.5). Then base quality was recalibrated using GATK Table Recalibration. Somatic single nucleotide variants and indels were detected using Vardict (1.4.10) with qual 20, total depth 30, variant allele depth 3, variant allele frequency 0.03 options (Lai et al., 2016). These variants were filtered out with common dbSNP (v137) (Sherry et al., 2001), genomAD, Korean

Reference Genome da-tabase, and a ~600 in-house panel of normal, and were then annotated using vcf2maf. All candidates of somatic mutation were manually reviewed using Integrative Geno-mics Viewer (IGV). Somatic mutations and clinical features were plotted using R package maftools (Mayakonda, Lin, Assenov, Plass, & Koeffler, 2018).

DNA damage response and repair (DDR) gene mutations included mutations in *ATM, ATR, BAP1, BARD1, BRCA1, BRCA2, BRIP1, CHEK2, FAM175A, GEN1, MLH1, MSH2, MSH6, MRE11A, NBN, PALB2, PMS2, RAD50, RAD51, RAD51C, RAD51D*, and *XRCC2* in this study. MSI status and tumor mutational burden (TMB) were assessed and calculated using targeted sequencing results by previously described methods (Jeong E. Kim et al., 2019). Specifically, MSI status in colorectal cancer was determined by tumor mutation burden (TMB) and indel index (I index). TMB was divided by tumor panel size of 0.64Mb (For example, if the total mutation counts were 10, then dividing 0.64 means that 15.6 is the resulting TMB). The Indel index was calculated by dividing the indel count by the total number of mutations. MSI-H was defined as a TMB cut-off of ≥40, and

an I index of ≥9%. Also, ACVR2A frame-shift deletion (K437Rfs*5) was used biomarker of MSI.

Statistical Methods

Overall survival (OS) was defined as the time from the date of starting palliative first-line chemotherapy to the date of death from any cause. Baseline characteristics of the patients were compared using a descriptive method. Survival outcomes were estimated by the Kaplan–Meier method and compared by a log-rank test between the groups. A Cox proportional hazards model was used to estimate the effect of genomic features on the survival outcomes. All tests were two-sided, and a p value less than 0.05 was considered statistically significant. Statistical analyses were performed using R version 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Patients

A total of 340 patients were included in the study, excluding eight patients who received curative resection after starting palliative intent chemotherapy (Figure 4). Among the 340 patients, 15 (4.4%) were classified as ERs, of whom seven had complete responses and eight had sustained partial responses to the first- (n=14) or second-line (n=1) palliative chemotherapy. The baseline demographics including age at diagnosis, sex, or tumor characteristics including primary tumor location or tumor grade did not differ between the groups (Table 2). The patients in the ER group had a tendency of more microsatellite instability-high (MSI-H) tumors (13.3% [n=2] vs. 2.5% [n=8]).

With the median follow-up duration of 29.9 months (95% confidence interval [CI], 26.9–31.1), the median OS from the start of palliative first-line chemotherapy was 57.3 months (95% CI; 33.1–not estimated [NE]) in the ER group and 21.5 months (95% CI; 19.9–24.6) ($p<0.001$) in the non-ER group (Figure 5).

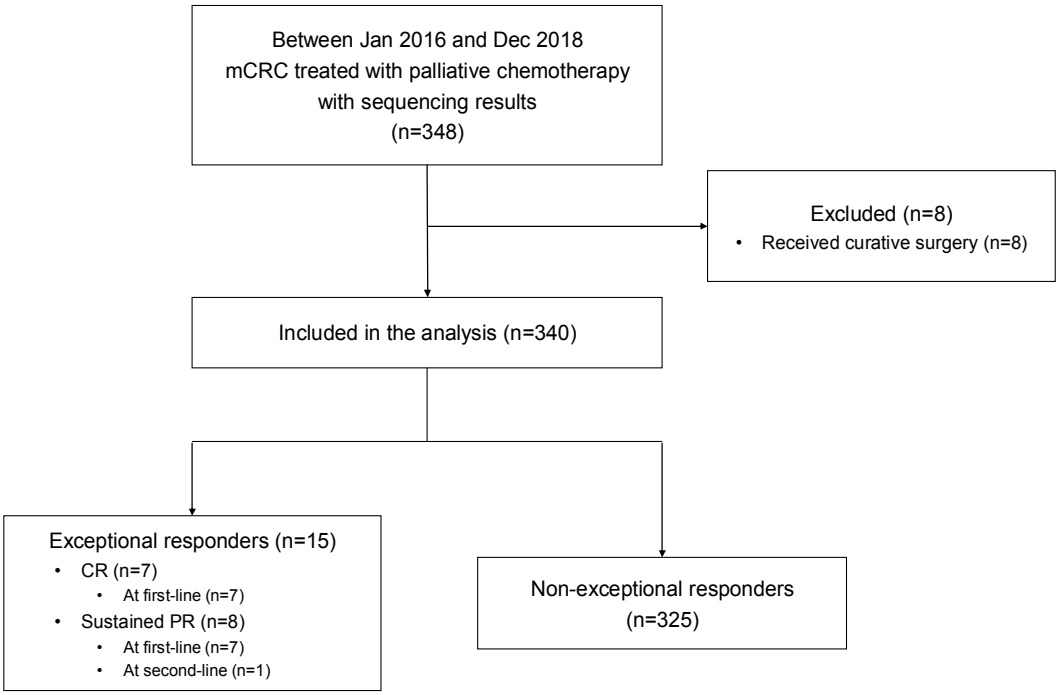

Figure 4. Flow diagram

Abbreviations: CR, complete response; mCRC, metastatic colorectal cancer; PR, partial response.

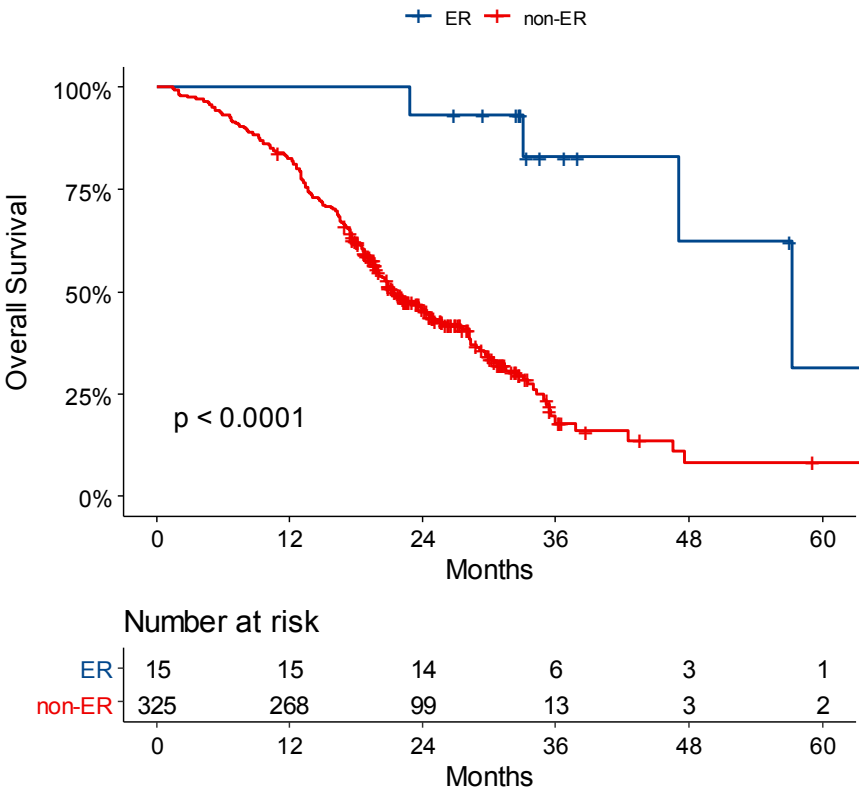

Figure 5. Kaplan-Meier survival plot for patients included in this study, according to the responder group

Table 2. Baseline characteristics

	Exceptional responders (n=15)	Regular responders (n=325)	*p*-value
Age			0.531
Median (range)	59.0 (39-68)	58.0 (25-83)	
Sex			1.000
Male	9 (60.0%)	200 (61.5%)	
Female	6 (40.0%)	125 (38.5%)	
ECOG PS at palliative 1st line chemotherapy			0.423
0-1	14 (93.3%)	314 (96.6%)	
≥2	1 (6.7%)	11 (3.4%)	
Primary tumor location			1.000
Right	3 (20.0%)	77 (23.7%)	
Left/Rectal	12 (80.0%)	248 (76.3%)	
Tumor grade			0.780
W/D	2 (13.3%)	27 (8.3%)	
M/D	11 (73.3%)	247 (76.0%)	
P/D	1 (6.7%)	33 (10.2%)	
Grade unidentified	1 (6.7%)	18 (5.5%)	
Disease status			0.185
Recurrent after curative surgery	6 (40.0%)	71 (21.8%)	
Initially metastatic	9 (60.0%)	254 (78.2%)	

Table 2. Baseline characteristics (Continue)

	Exceptional responders (n=15)	Regular responders (n=325)	*p*-value
Metastatic sites at diagnosis			
Liver	7 (46.7%)	195 (60.0%)	0.448
Lung	2 (13.3 %)	117 (36.0%)	0.096
Peritoneum	3 (20.0%)	80 (24.6%)	1.000
MSI status by targeted sequencing			0.067
MSS	13 (86.7%)	317 (97.5%)	
MSI-H	2 (13.3%)	8 (2.5%)	
Palliative 1st line, chemotherapy			0.117
Oxaliplatin-based doublet	4 (26.7%)	172 (52.9%)	
Irinotecan-based doublet	11 (73.3%)	148 (45.5%)	
Others*	0 (0.0%)	5 (1.5%)	
Palliative 1st line, targeted agents			0.416
Bevacizumab	11 (73.3%)	241 (74.2%)	
Cetuximab	4 (26.7%)	58 (17.8%)	
None	0 (0.0%)	26 (8.0%)	
Immune-checkpoint inhibitors			
1st line†	0 (0.0%)	2 (0.6%)	1.000
2nd line‡	0 (0.0%)	7 (2.2%)	1.000
Best overall response to palliative 1st line regimen			<0.001
CR	7 (46.7%)	0 (0.0%)	
PR	7 (46.7%)	192 (59.1%)	
SD	0 (0.0%)	90 (27.7%)	
PD	1 (6.7%)	39 (12.0%)	
nonCR/nonPD	0 (0.0%)	4 (1.2%)	

Table 2. Baseline characteristics (Continue)

	Exceptional responders (n=15)	Regular responders (n=325)	*p*-value
Origin of tissue used for targeted sequencing			0.228
Colorectum	12 (80.0%)	290 (89.2%)	
Metastases	3 (20.0%)	35 (10.8%)	

* Others include 5-fluorouracil/capecitabine monotherapy in 3 patients, and immune-checkpoint inhibitor in 2 patients. † Includes durvalumab and pembrolizumab in 1 patient each. ‡ Includes avelumab in 3 patients, pembrolizumab in 2 patients, atezolizumab and durvalumab in 1 patient each. Abbreviations: CR, complete response; ECOG PS, Eastern Cooperative Oncology Group performance status; ER, exceptional responder; FOLFIRI, 5-fluorouracil plus irinotecan; FOLFOX, 5-fluorouracil plus oxaliplatin; M/D, moderately differentiated; MSI-H, microsatellite instability-high; MSS, microsatellite stable; P/D, poorly differentiated; PD, progressive disease; PR, partial response; SD, stable disease; W/D, well differentiated.

Genomic characteristics

Genetic mutations and copy number alterations were observed in 98.2% (n=334) and 62.9% (n=214) of the entire study population, respectively (Figure 6). The most recurrently mutated genes in the overall study population were *TP53* (81.5%), *APC* (75.9%), and *KRAS* (49.4%), without significant differences in occurrences between the groups. Nineteen genes (*PIK3CB, TOP2A, NTRK1, ZNRF3, ARID1A, ERBB3, NOTCH3, MSH6, PDGFRB, EPHB4, ASH1L, EZH2, MTOR, AR, ETV6, SEC63, CEBPZ, PTCH2,* and *BRCA2*) were significantly mutated in the ER group (Figure 7). No gene mutations were more frequent in the non-ER group. Of note, no patient vs. 18 patients (5.5%) in the ER and non-ER groups had *BRAF* V600 mutations, respectively (p=1.000).


113
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Group
Sex
Location
Grade
1L regimen
Survival
MSI status
ER (%)
Non-ER (%)
TP53 87 81
APC 73 76
KRAS 47 50
PIK3CA 20 20
SMAD4 20 18
FBXW7 27 14
BRCA2 * 33 12
LRP1B 27 12
BRAF 13 9
ATM 7 8
ARID1A * 33 7
NF1 13 7
NOTCH4 13 6
KMT2A 13 6
GNAS 7 6
BRCA1 13 6
RNF43 20 6
NOTCH1 13 6
POLE 20 6
ASXL1 20 5
MSH6 * 20 2
APC del
SMAD4 del
ERBB2 amp
MYC amp
Mutation Status
Missense Mutation
Nonsense Mutation
Frame Shift Insertion
Frame Shift Deletion
Splice Site
In Frame Insertion
In Frame Deletion
Multi Hit
Responder group
Exceptional responders
Non-exceptional responders
Sex
Male
Female
Primary tumor location
Lt sided
Multiple
Rectal
Rt sided
Tumor grade
Well Differentiated
Moderately Differentiated
Poorly Differentiated
Unknown
Palliative first-line chemotherapy regimen
FOLFOX+Bevacizumab
FOLFOX+Cetuximab
FOLFOX
FOLFIRI+Bevacizumab
FOLFIRI+Cetuximab
FOLFIRI
Others
Survival status
Alive
Deceased
MSI status
MSI-H
MSS
CNV
Deletion
Amplification

Figure 6. Genomic landscape

Note: Aligned by responder status, sex, tumor grade, and primary location. Genetic mutations and copy number alterations were observed in 334 (98.2%) and 214 (62.9%) patients, respectively. The asterisk (*) indicates *p* value <0.05.

Abbreviations: 1L, first-line; amp, amplification; CNV, copy number alteration; del, deletion; ER, exceptional responders; FOLFIRI, 5-fluorouracil and irinotecan; FOLFOX, 5-fluorouracil and oxaliplatin.

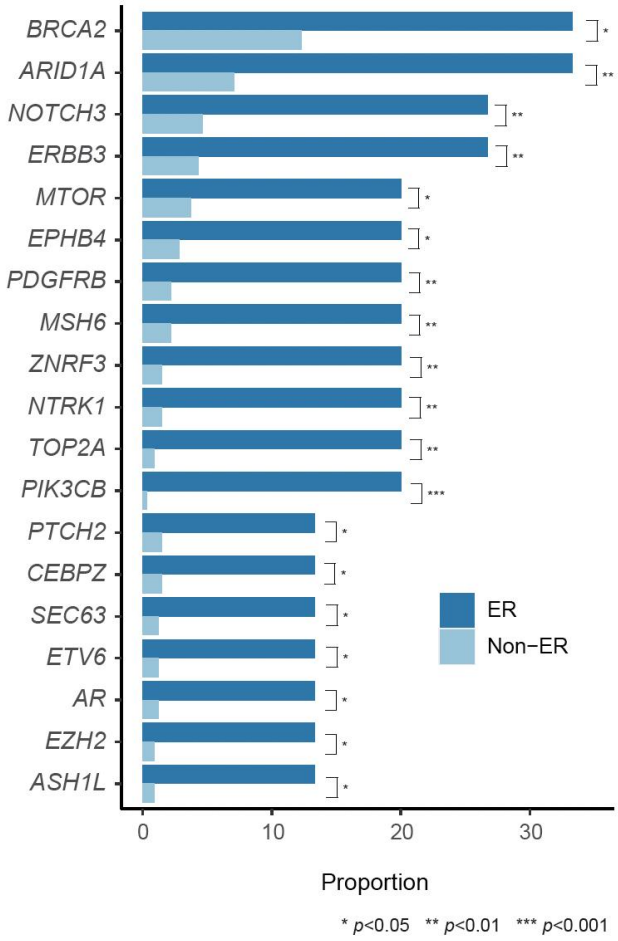


Figure 7. Differentially mutated genes

Abbreviations: ER, exceptional responders.

Among oncogenic signaling pathways (Sanchez-Vega et al., 2018), the most commonly mutated pathways were RTK-RAS (86.7% vs. 80.9% in the ER and non-ER group, p=0.745) and TP53 (86.7% vs. 85.2% in the ER and non-ER group, p=1.000) in both groups. Mutations in the cell cycle pathway were more frequent in the ER group (33.3% vs. 8.0%, p=0.007). Among genes comprising the cell cycle pathway, *CCND1/2/3, CDK4/6* oncogenes, and *CDKN2A/B/C, RB1* tumor suppressor genes were more commonly mutated in the ER group (Figure 8).

The proportion of patients with any copy number alterations was 46.7% (n=7) and 63.7% (n=207) in the ER and non-ER groups (p=0.273), respectively. In the ER and non-ER group, 20.0% (n=3) vs. 44.3% (n=144) had amplifications (p=0.107), and 40% (n=6) vs. 39.7% (n=129) had deletions (p=1.000). No significant differences in the proportion of copy number alterations in individual genes were observed between the groups.

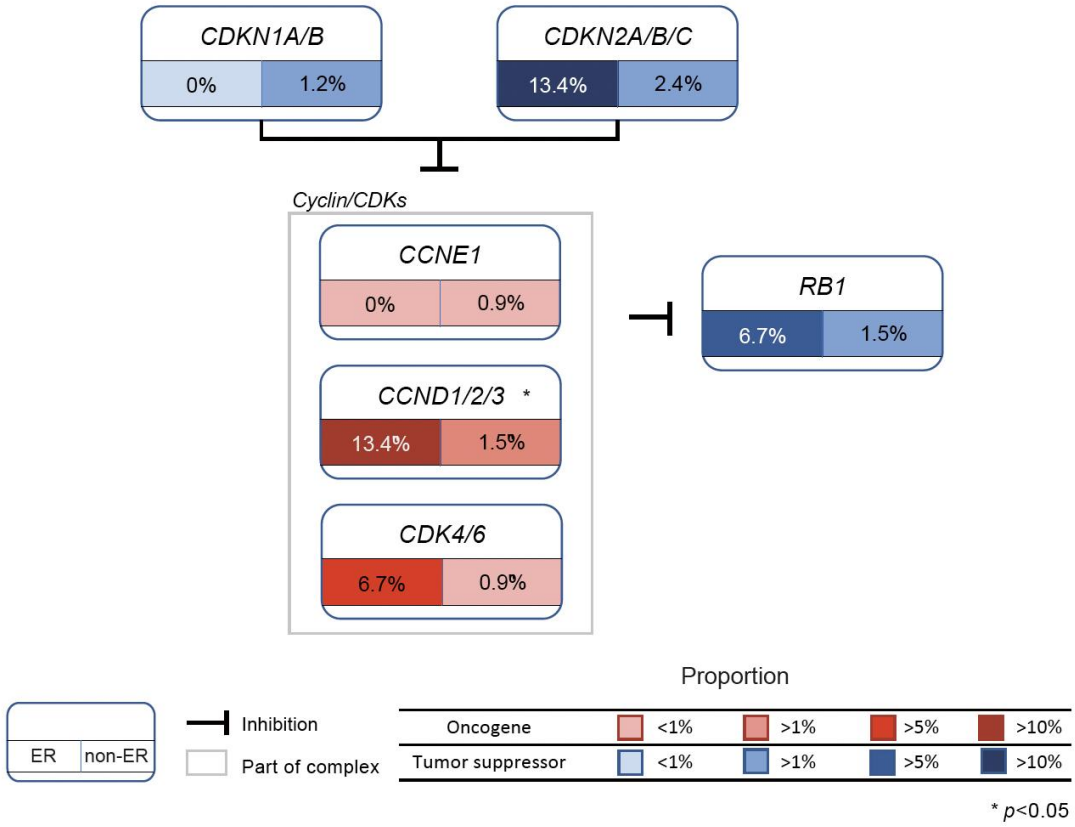


Figure 8. Cell cycle pathway mutations

Abbreviations: ER, exceptional responders.

DDR gene mutations

DDR genes were distinguished as germline and somatic variants using tumor tissue sequencing data as described above. The proportion of germline and somatic DDR mutations was compared between the groups (Figure 9). Germline DDR gene mutations were found in 26.7% (n=4) and 15.4% (n=50) of the ER and non-ER groups, respectively (p=0.272). Among the germline DDR mutations, *MSH6* (13.3% vs. 0.3%) and *GEN1* mutations (13.3% vs. 1.2%) were significantly more frequent in the ER group than in the non-ER group ($p<0.05$). Somatic DDR gene mutations were found in 40.0% (n=6) and 38.2% (n=124) of the ER and non-ER groups, respectively (p=1.000). Among the somatic DDR mutations, *MSH6* (20.0% vs. 2.2%), *BRCA2* (33.3% vs. 12.3%), and NBN mutations (6.7% vs. 0.0%) were significantly more frequent in the ER group ($p<0.05$).

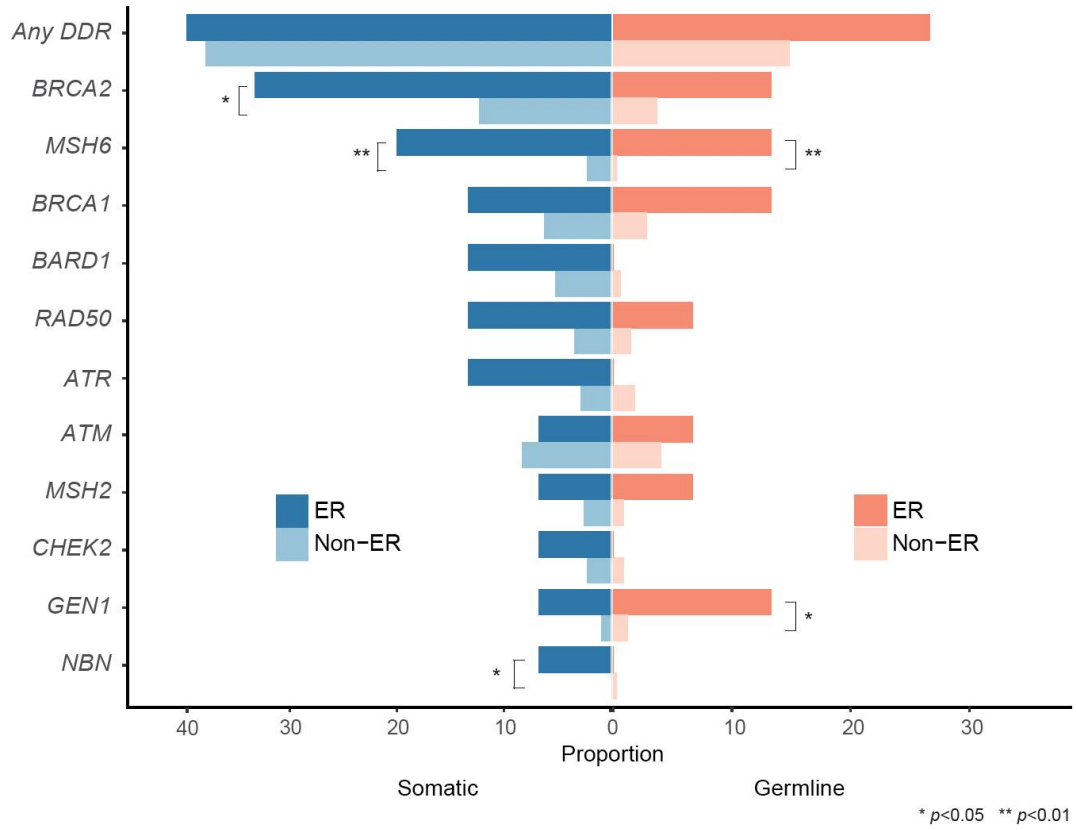


Figure 9. Germline and somatic DNA damage repair mutations.

Abbreviations: ER, exceptional responders.

MSI status and TMB

MSI status was assessed by PCR, IHC, and NGS on a total of 340 patients in our study. In 173 individuals, PCR findings were obtained, and in 211 patients, IHC data were obtained. There were 142 persons who had findings from both the PCR and the IHC tests. When the results of PCR and NGS were compared, there was 100% agreement. There was a disagreement in 3 cases between IHC and NGS. However, PCR was also performed for those three cases and the results of both PCR and NGS were the same. Therefore, it is judged that there is no problem in determining the MSI state based on the NGS result.

MSI status and TMB detected by targeted sequencing were compared between the groups. Patients with MSI-H comprised 13.3% (n=2) of the ER group and 2.5% (n=8) of the non-ER group (Figure 10A). The median TMB value was 12.5 mutations/Mb (range: 7.8–167.2) vs. 12.5 mutations/Mb (range: 1.6–178.1) in the ER and non-ER group, respectively (p=0.335) (Figure 10B). In the microsatellite stable (MSS) subset of patients, the median TMB was 12.5 mutations/Mb (range: 7.8–167.2) vs. 12.5 mutations/Mb (range: 1.6–75.0) in the ER (n=13) and non-ER group (n=317), respectively (p=0.806) (Figure 10C). Tumors

with higher TMB (TMB-H) were more enriched in the ER group than in the non-ER group (cutoff ≥20 mutations/Mb; 26.7% vs. 9.8%; p=0.335). In the MSS subset of patients, those with TMB-H comprised 15.4% (n=2/13) of the ER group and 7.6% (24/317) of the non-ER group (p=0.806).

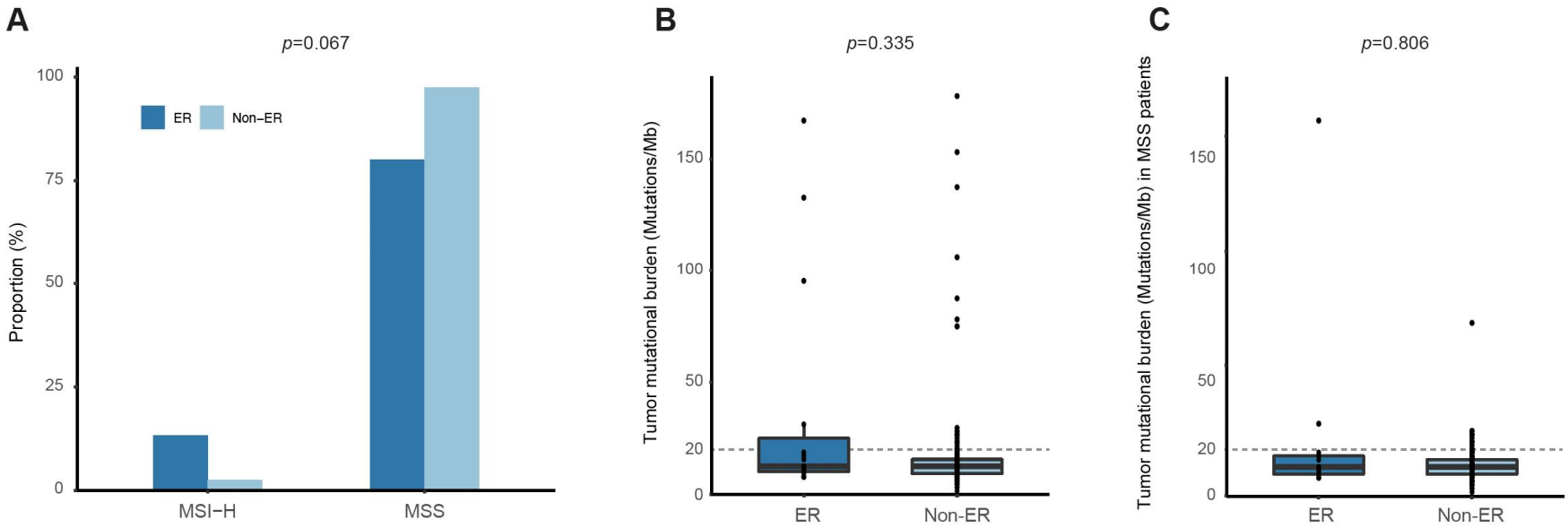


Figure 10. Microsatellite instability status and tumor mutational burden by responder groups. (A) Microsatellite instability status, and tumor mutational burden (B) in the entire study population, and (C) in the microsatellite stable colorectal cancers.

Abbreviations: ER, exceptional responders; MSI-H, microsatellite instability-high; MSS, microsatellite stable.

NTRK1-TPM3 fusion

Chromosomal rearrangements of the *NTRK1* gene, encoding the high affinity nerve growth factor receptor (tropomyosin related kinase, TRKA), have been observed in several epithelial cancers, such as colon cancer, papillary thyroid carcinoma or non-small cell lung cancer. The various *NTRK1* fusions described so far lead to constitutive activation of TRKA kinase activity and are oncogenic (Créancier et al., 2015). Regardless of tumor histotype or cell of origin, inhibitors of tropomyosin receptor kinase (TRK) have demonstrated promising activity against neurotrophic TRK (*NTRK*) fusion-driven cancers. In CRCs less than 1% of, *NTRK* gene fusions are found (Svrcek et al., 2021). In this study we found one fusion in the exceptional responders. Breakpoints occurred at the Chr1:154139990 genomic coordinate of the *TPM3* gene and the Chr1: 156844095 location of the *NTRK1* gene (Figure 11). This patient had MSI-H and TMB-high.

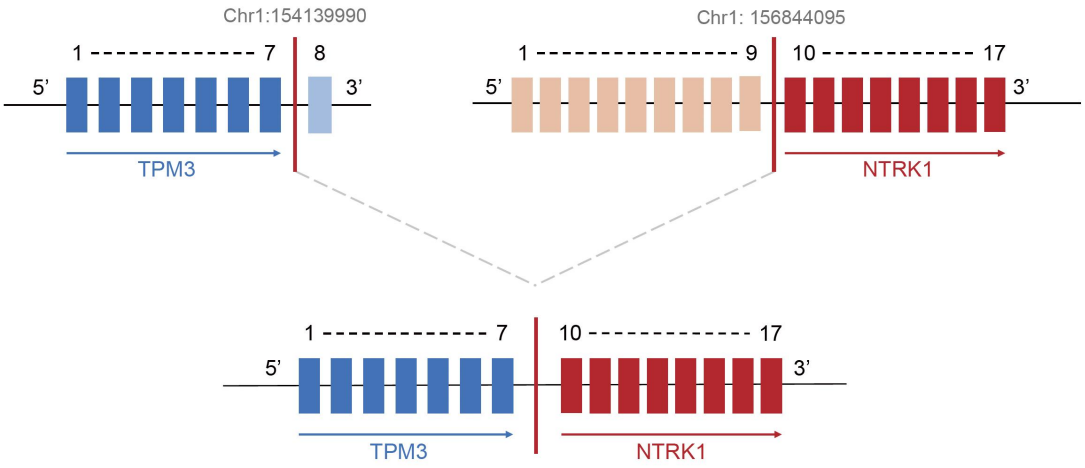


Figure 11. NTRK1-TPM3 gene fusion (case1)

Fusion of TPM3_ENST00000368533 and NTRK1_ENST00000392302.

(COSMIC Fusion, GRCh37/hg19)

Correlative analysis for survival

Among the more frequently mutated genes in the ER group, no gene mutation except *ZNRF3* (hazard ratio [HR] 0.24 [95% CI, 0.06-0.98], p=0.047) was significantly associated with OS in our study cohort. We also checked whether survival was associated with paired gene sets, but no significant association was found. Amplification (CN ≥ 5) and deletion (CN ≤ 0) were not significantly associated with survival in CNV. For additional correlative survival analyses, an independent public cohort of mCRC patients from MSKCC who did not receive metastasectomy was selected (n=521) (Figure 12) (Yaeger et al., 2018). In the MSKCC cohort, no gene mutation except for *PIK3CB* (HR 0.11 [95% CI, 0.02-0.81], p=0.030), *ARID1A* (HR 0.58 [95% CI, 0.35-0.95], p=0.030), and *AR* (HR 0.41 [95% CI, 0.18-0.93], p=0.034) was significantly associated with improved OS. Cell cycle pathway mutation was not associated with OS in our study population nor the MSKCC cohort (Figure 13). MSI-H tumors showed marginal association with improved OS in our study cohort (n=10/340, HR 0.48 [95% CI, 0.18-1.29], p=0.143) although not statistically significant; they

were associated with improved OS in the MSKCC cohort (n=29/369, HR 0.46 [95% CI, 0.24-0.89], p=0.020).

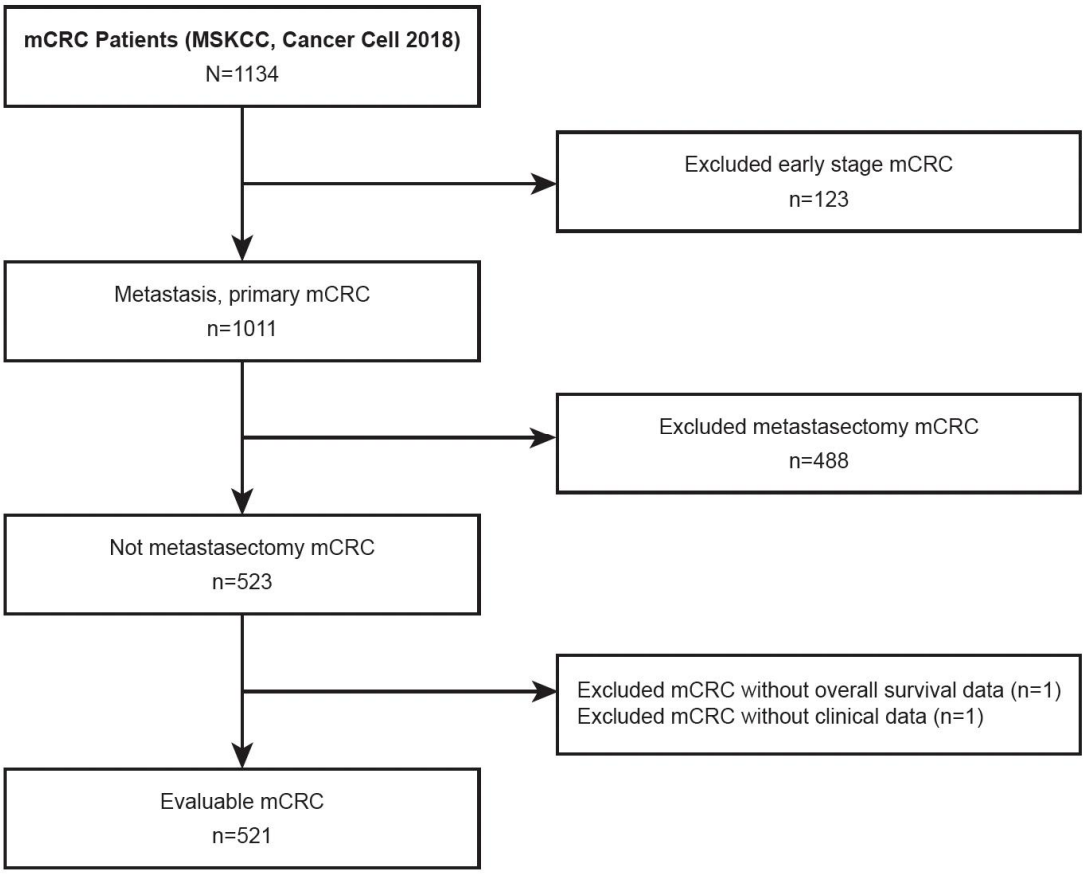
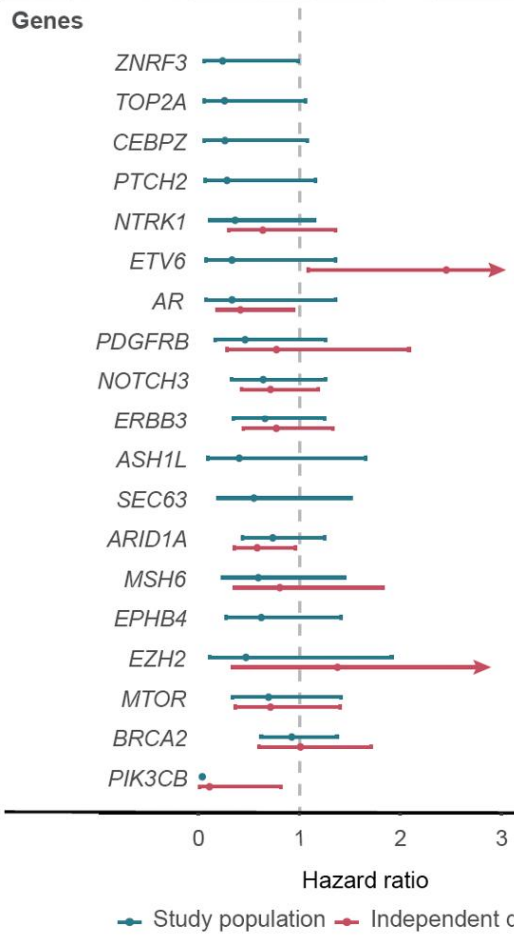


Figure 12. Flow diagram of MSKCC patients included in the study



	Study population (n=340)			Independent dataset (n=521)		
	Number of patients (%)	Hazard ratio (95% CI)	*p* value	Number of patients (%)	Hazard ratio (95% CI)	*p* value
ZNRF3	8 (2.4)	0.24 (0.06-0.98)	0.047	0 (0.0)	NA	NA
TOP2A	6 (1.8)	0.26 (0.06-1.05)	0.059	0 (0.0)	NA	NA
CEBPZ	7 (2.1)	0.26 (0.07-1.07)	0.061	0 (0.0)	NA	NA
PTCH2	7 (2.1)	0.28 (0.07-1.15)	0.077	0 (0.0)	NA	NA
NTRK1	8 (2.4)	0.36 (0.12-1.14)	0.082	14 (2.7)	0.63 (0.3-1.34)	0.232
ETV6	6 (1.8)	0.33 (0.08-1.34)	0.122	9 (1.7)	2.42 (1.07-5.45)	0.033
AR	6 (1.8)	0.33 (0.08-1.34)	0.122	22 (4.2)	0.41 (0.18-0.93)	0.034
PDGFRB	10 (2.9)	0.46 (0.17-1.24)	0.125	10 (1.9)	0.77 (0.28-2.06)	0.596
NOTCH3	19 (5.6)	0.64 (0.33-1.24)	0.185	35 (6.7)	0.71 (0.43-1.17)	0.181
ERBB3	18 (5.3)	0.65 (0.35-1.24)	0.192	26 (5)	0.76 (0.44-1.31)	0.331
ASH1L	5 (1.5)	0.40 (0.10-1.63)	0.203	0 (0.0)	NA	NA
SEC63	6 (1.8)	0.55 (0.20-1.50)	0.239	0 (0.0)	NA	NA
ARID1A	28 (8.2)	0.73 (0.43-1.23)	0.240	44 (8.4)	0.58 (0.35-0.95)	0.030
MSH6	10 (2.9)	0.59 (0.24-1.43)	0.242	12 (2.3)	0.80 (0.35-1.80)	0.587
EPHB4	12 (3.5)	0.62 (0.27-1.40)	0.247	0 (0.0)	NA	NA
EZH2	5 (1.5)	0.47 (0.12-1.89)	0.286	5 (1.0)	1.36 (0.34-5.48)	0.666
MTOR	15 (4.4)	0.69 (0.34-1.40)	0.301	22 (4.2)	0.71 (0.36-1.39)	0.314
BRCA2	45 (13.2)	0.91 (0.62-1.35)	0.655	24 (4.6)	1.00 (0.59-1.69)	0.998
PIK3CB	4 (1.2)	0 (0.0-Inf)	0.991	8 (1.5)	0.11 (0.02-0.81)	0.030

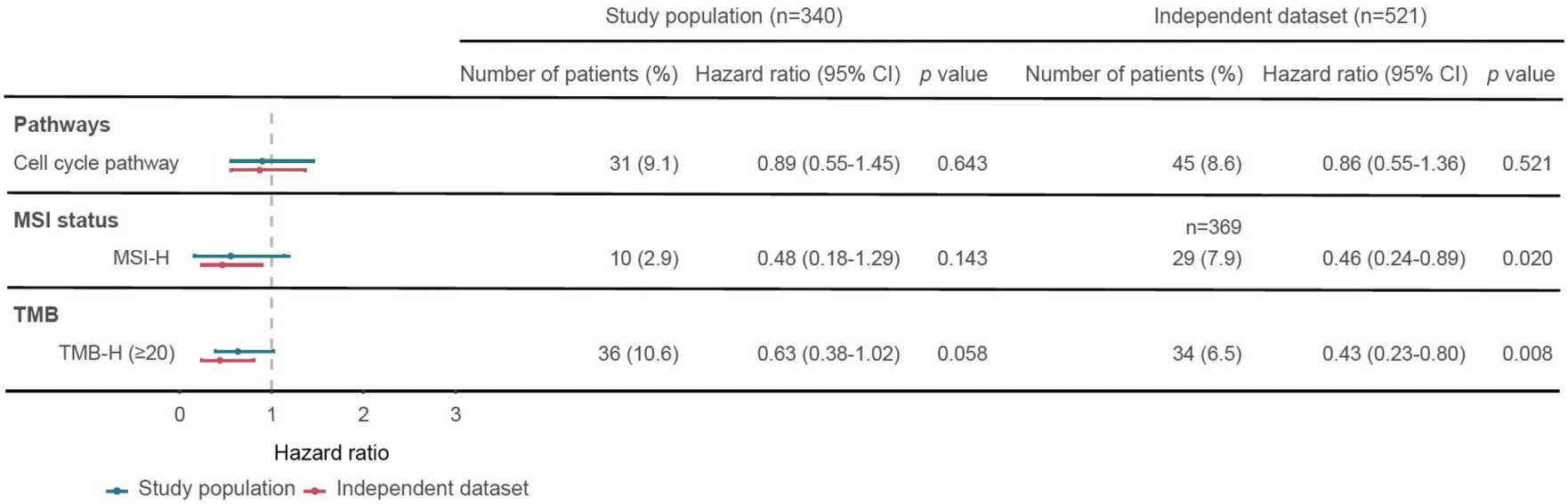


Figure 13. Correlative analysis for overall survival.

Abbreviations: MSI, microsatellite instability; TMB, tumor mutational burden.

Discussion

This study is one of the first studies that evaluated the clinical and genomic characteristics of ERs in a palliative chemotherapy-treated mCRC cohort. ERs comprised 4.4% of our study population, consistent with previous studies on mCRC that reported CR rates of 1-3% with chemotherapy (Zhang et al., 2015). The clinical characteristics were similar between ERs and non-ERs, and no differences in the frequency of major gene mutations including *TP53, APC*, or *RAS* were observed between then groups. However, more patients in the ER group had a tendency of having MSI-H tumors, without any patients having *BRAF* V600 mutations. We were also able to identify genes and pathways that were more frequently mutated in the ER group. However, these mutations were not associated with improved survival outcomes individually and did not provide mechanistic explanations for the exceptional responses.

The proportion of MSI-H patients in this study was 2.9%, similar with previous data (Koopman et al., 2009). However, it was significantly higher in the ER group than in

the non-ER group, with 13.3% of ERs having MSI-H tumors. In early CRCs, MSI-H has been recognized as a favorable prognostic factor (Roth et al., 2012). However, the prognostic implications of the MSI-H status in mCRC are conflicting (Koopman et al., 2009; Taieb et al., 2019). Some recent studies have suggested that the poor prognosis of mismatch repair-deficient tumors was driven by the *BRAF* mutants (Venderbosch et al., 2014). In terms of tumor response, MSI-H was known to be a negative predictor for 5-fluorouracil response (Ribic et al., 2003). However, given the effect of concurrent chemotherapeutic agents and targeted therapies, as well as the heterogeneity among MSI-H CRCs (Fallik et al., 2003; Innocenti et al., 2019), the predictive value of the MSI status for chemotherapy response is yet to be determined.

One of the characteristic findings in the ER group of our study is the absence of *BRAF* V600 mutants despite the higher incidence of MSI-H disease. It is known that CMS1 (microsatellite instability immune type) CRCs comprised both *BRAF* mutants and non-*BRAF* mutants, and different prognoses between these two subgroups were previously reported (Guinney et al., 2015; Smeby et al., 2018), similar to the cases of MSI-H CRCs (Taieb et al.,

2019). Further studies are needed to confirm which subsets of mCRC, possibly including non-*BRAF* MSI-H CRC, are prone to show exceptional responses to chemotherapy. Despite the higher incidence tendency of MSI-H tumors in the ER group, we did not observe significant differences in TMB by the responder status, possibly ascribing to the small number of MSI-H patients.

Approximately 10% of CRCs were reported to have cell cycle pathway alterations, which is less frequent compared with that in other solid cancers (Helsten et al., 2016). We observed that cell cycle pathway mutation was more frequent in the ER group (33% vs. 8% in the ER- vs. non-ER group, respectively). However, inconsistent with these findings, the poor prognostic implication of cell cycle pathway alteration was also suggested in the literature (Kato et al., 2015; Schwaederlé et al., 2014). Nonetheless, due to the limited number of patients, the prognostic implication of cell cycle pathway mutations cannot be determined in this study.

In this study, more than half of the patients received platinum (oxaliplatin)-containing chemotherapy as palliative first-line chemotherapy. However, the frequency of

both germline and somatic DDR mutations did not differ between the groups, nor did the frequency of each DDR gene mutations. These findings are consistent with those of a previous study that showed no clinical benefit with oxaliplatin use by the presence of DNA damage immune response signature positivity (Malla et al., 2021). The lack of an association between the DDR status and exceptional response might also be attributed to the limitations of targeted sequencing in discriminating germline or somatic variants of DDR genes, the heterogeneous composition of DDR genes among different panels, or the different modes of actions between platinum compounds (Mauri, Arena, Siena, Bardelli, & Sartore-Bianchi, 2020).

Although we identified several differentially mutated genes in the ERs, they did not provide a mechanistic explanation for the favorable responses in these patients. In addition, the correlative analysis for overall survival did not show significant associations between these individual gene mutations and OS both in the study population and in the public mCRC cohort. These findings suggest that exceptional responses are unlikely to be explained by tumor genomics only. Previous studies that attempted to assess the underlying

tumor DNA mutations in ERs or long-term survivors of metastatic solid cancers also did not provide plausible explanations for these exceptional responses (Bilusic et al., 2021; Datta et al., 2020; Hoppenot, Eckert, Tienda, & Lengyel, 2018). Moreover, they often included a heterogeneous population or patients who underwent surgical resection, which could strongly impact clinical outcomes irrespective of chemotherapy responses.

Recently, the National Cancer Institute ER initiative reported the genomic profiling results of ERs in a nationwide cohort by conducting multi-platform analyses covering not only tumor somatic mutations, but also tumor microenvironments, epigenetic features, and germline polymorphisms (Wheeler et al., 2021). They suggested potential mechanisms including oncogene addiction, synthetic lethality, and the tumor microenvironment in a quarter of patients included. Consistent with these observations, other recent studies suggested the association between immune signature profiling and survival outcomes in mCRC patients (Innocenti et al., 2021), or the association between the gut microbiome and tumor response (Jang et al., 2020; McQuade, Daniel, Helmink, & Wargo, 2019). Taken

together, multiple factors including genetic, epigenetic, and environmental factors are likely to contribute to the exceptional responses, rather than tumor DNA alteration alone.

This study is limited by its single-centered retrospective nature and the small number of ERs and MSIH patients. However, it is one of the first attempts to assess the molecular characteristics of ERs using targeted sequencing data in a relatively homogeneous mCRC patient population that received palliative chemotherapy only, and reported observations that warrant further study including the higher incidence of BRAF wild-type MSI-H patients in ERs.

We conclude that tumor genomics alone is insufficient to predict exceptional treatment response in mCRC patients. Future studies that include multi-factorial analyses are needed to define the association between exceptional response to chemotherapy and possible factors beyond tumor genomics, as they should offer further insights into the pathophysiology of these fascinating patient populations.

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국문 요약

전이성 대장암 (mCRC)에서 화학 요법에 대해 지속적인 반응을 달성한 예외적으로 좋은 반응을 보인 환자들 (Exceptional Responder)이 가끔씩 존재하는 것으로 보고되었다. 이 연구는 표적 염기서열분석 (Targeted sequencing) 데이터를 사용하여 ER 의 유전체 특성과 예후의 의미를 확인하는 것을 목표로 하였다.

2016 년 1 월부터 2018 년 12 월 사이에 완화 화학 요법으로 치료를 받고 종양 조직에서 표적 염기 서열 분석 결과를 얻은 전이성 또는 재발성 대장암 환자를 확인하여 본 연구에 포함했다. 치료적 또는 전이 절제술을 받은 환자는 제외되었다. 예외적으로 좋은 반응을 보인 환자들 (Exceptional Responder)은 완화 1 차 또는 2 차 화학 요법에 대해 28 개월 이후 동안 전체 반응이 가장 우수하거나 부분 반응이 지속되는 경우로 정의되었다.

포함된 340 명의 환자 중 15 명 (4.4 %)이 ER 로 분류되었다. 연령, 성별, 종양 등급 또는 측면을 포함한 임상적 특성은 그룹간에 차이가 없었다. ER 그룹의 환자는 BRAF V600 돌연변이가 없었다. DDR 손상 반응 및 복구 유전자 돌연변이를 가진 환자의 비율은 그룹간 차이가 없었다 (생식 세포 돌연변이; 26.7 % 대 15.4 %, p =

0.272; 체세포 돌연변이, 40.0 % 대 38.2 %, p = 1.000). 우리는 또한 세포주기 경로 돌연변이를 포함하여 ER 그룹에서 더 빈번한 차등 돌연변이 유전자 및 경로를 확인했지만 개별적으로 유리한 생존 결과와는 관련이 없었다. 우리는 ER 에서 더 빈번한 19 개의 유전자 및 1 개의 경로 돌연변이를 확인했지만 개별적으로 유리한 생존 결과와 연관되지 않았으며 관찰된 예외적인 반응에 대한 그럴듯한 메커니즘을 제공하지 않았다.

완화적 화학요법으로 치료받은 mCRC 환자로부터 수집된 표적 시퀀싱 데이터는 ER 이 BRAF 야생형 MSI-H 환자의 비율이 더 높은 것으로 나타났다. 그러나 종양 유전체학만으로는 치료 반응을 예측하기에는 충분하지 않다. 종양 유전체학 이상의 요인을 확인하려면 다인자 분석이 필요할 수 있다.