Original Article

Clinical Application of Targeted Deep Sequencing in Metastatic Colorectal Cancer Patients: Actionable Genomic Alteration in K-MASTER Project

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Purpose Next-generation sequencing (NGS) can facilitate precision medicine approaches in metastatic colorectal cancer (mCRC) patients. We investigated the molecular profiling of Korean mCRC patients under the K-MASTER project which was initiated in June 2017 as a nationwide precision medicine oncology clinical trial platform which used NGS assay to screen actionable mutations.

Materials and Methods As of 22 January 2020, total of 994 mCRC patients were registered in K-MASTER project. Targeted sequencing was performed using three platforms which were composed of the K-MASTER cancer panel v1.1 and the SNUH FIRST Cancer Panel v3.01. If tumor tissue was not available, cell-free DNA was extracted and the targeted sequencing was performed by Axen Cancer Panel as a liquid biopsy.

Results In 994 mCRC patients, we found 1,564 clinically meaningful pathogenic variants which mutated in 71 genes. Anti-EGFR therapy candidates were 467 patients (47.0%) and *BRAF* V600E mutation (n=47, 4.7%), deficient mismatch repair/microsatellite instability–high (n=15, 1.5%), *HER2* amplifications (n=10, 1.0%) could be incorporated with recently approved drugs. The patients with high tumor mutation burden (n=101, 12.7%) and DNA damaging response and repair defect pathway alteration (n=42, 4.2%) could be enrolled clinical trials with immune checkpoint inhibitors. There were more colorectal cancer molecular alterations such as *PIK3CA*, *KRAS* G12C, atypical *BRAF*, and *HER2* mutations and even rarer but actionable genes that approved or ongoing clinical trials in other solid tumors.

Conclusion K-MASTER project provides an intriguing background to investigate new clinical trials with biomarkers and give therapeutic opportunity for mCRC patients.

Key words Colorectal neoplasms, Genomics, High-throughput nucleotide sequencing

Introduction

In Korea, colorectal cancer (CRC) is the third most prevalent cancer and the most common cancer among women aged 65 years and older with estimated 54.9 new cases, per 100,000 men and women, per year and responsible for 10.7% of cancer deaths [1]. Even though ongoing efforts to establish an early preventive screening programs have reduced the incidence of advanced stage disease, and thus led to the improvement in 5-year overall survival rates up to 70.6% [2]. However, the progress in the development of therapeutic options for metastatic CRC (mCRC) was dismal until recent discoveries in molecular-based therapies.

Over the past decade, large-scale sequencing studies have advanced our understandings of molecular diversity among mCRC patients and uncovered potentially actionable genes across subgroups of patients with distinct molecular signatures including WNT, RAS-MAPK, PI3K, TGF-β, p53, and DNA mismatch repair pathway [3]. Next-generation sequencing (NGS) can facilitate precision medicine approaches by identifying actionable somatic events in tumor samples and reveal associations with sensitivity or resistance that can inform the development and implementation of targeted therapeutics and aid in the design of clinical trials to validate findings and actionability [2,4-6].

In June 2017, The K-MASTER cancer precision medicine diagnosis and treatment enterprise (K-MASTER project) was initiated as a nationwide precision medicine oncology clinical trial platform, which used NGS assay to screen the actionable mutations in 10,000 Korean patients with refractory solid tumors and assigned the patients to matched clinical trials. As of January 2020, 52 sites and 4,390 cancer patients have participated. Under the K-MASTER program, we investigate the current mCRC treatment landscape according to molecular

Table 1. Pathogenic gene list

Gene	No. of patients	Gene	No. of patients	Gene	No. of patients
KRAS	433	FLT3	5	MAP2K4	1
TP53	428	ATM	4	KIT	1
PIK3CA	126	PTCH1	4	GNAS	1
APC	80	POLE	4	DNMT3A	1
BRAF	66	SMO	4	CSF1R	1
FBXW7	38	HRAS	4	CHEK2	1
NRAS	32	MET	3	BRCA1	1
SMAD4	24	CDK4	3	ASXL1	1
ERBB2	22	AURKA	3	AR	1
STK11	21	TSHR	3	SPOP	1
BRCA2	19	RET	3	SETBP1	1
PTPN11	18	PTEN	3	RAD51C	1
MTOR	16	JAK2	3	NOTCH3	1
CTNNB1	13	MDM2	2	NF1	1
FANCA	13	STAT1	2	MYC	1
MSH6	12	SF3B1	2	ARID1A	1
AKT1	11	RB1	2	CCNE1	1
CDH1	10	NOTCH1	2	RICTOR	1
FGFR4	8	MLH1	2	RUNX1	1
MAP2K1	7	KMT2D	2	TSC2	1
ERBB3	6	JAK3	2	TP63	1
FGFR1	6	IDH1	2		
CCND1	6	MSH2	2		
CDKN2A	5	TERT	2		
EGFR	5	MAP3K1	1		

genomic alterations and discuss potentially actionable genes currently being investigated in clinical trials that may provide further progress in this battle against this malicious disease. By analyzing the gap between potential theoretical possibilities and current treatment practice, we can try to find a way to realize the unmet need of mCRC patients.

Materials and Methods

1. Patients samples

Pathologically confirmed mCRC patients (n=994) have been referred from 54 institutions located all over South Korea between June 2017 and January 2020 under K-MASTER project (S1 Table). Specimens were prepared from formalin-fixed and paraffin-embedded tumor tissue (n=842) or blood if tumor tissue was not available (n=152). The most recent archival tissue, either from the primary tumor or a metastatic site, was reviewed for tumor cellularity. Details of the genomic DNA extraction are described in Supplementary Method.

2. Targeted sequencing and bioinformatics

Targeted sequencing was performed using three NGS platforms such as SNUH FIRST Cancer Panel [2], K-MASTER Cancer Panel [4], and Axen Cancer Panel 1. SNUH FIRST panel v3.01 was performed with exons of 183 cancer-related genes including microsatellite status with five microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26) and K-MASTER cancer panel v1.1 included the whole exomes of 409 cancer-related genes, the intronic regions of 23 genes and three fusion genes. Axen Cancer Panel 1, a liquid biopsy panel, was utilized which panel included the exomes of 88 cancer-related genes and the intronic regions of three genes (S2 Table). Detailed methods for targeted sequencing and variant annotation can be found in the Supplementary Method. Targeted sequencing generated approximately 100 Mb per sample with an average of 95% samples on target. Sequences of all samples achieved a mean depth was more than 650× of FIRST and K-MASTER panel and 2580x of Axen panel.

The molecular alterations as single nucleotide variants, insertions and deletions (indels), copy number alterations, or
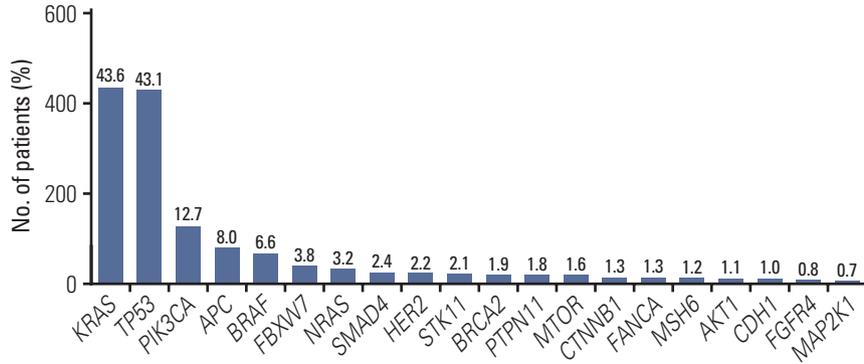
Fig. 1. Top 20 pathogenic gene list. Among 71 genes with pathogenic variants, listed top 20 frequent mutated genes.
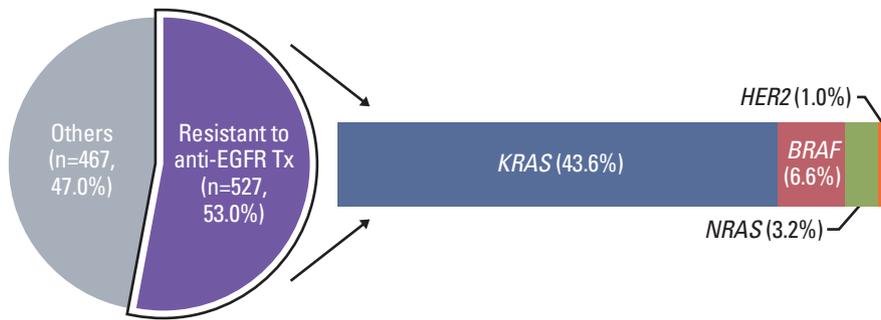

Fig. 2. Resistant to anti-EGFR therapy. Out of 994 patients, 527 patients (53.0%) were resistant to anti-EGFR therapy; having pathogenic variants in *KRAS* (n=433, 43.6%), *BRAF* (n=66, 6.6%), *NRAS* (n=32, 3.2%) or *HER2* amplification (n=10, 1.0%). Others, 467 patients (47.0%) could be the candidate of anti-EGFR therapy with wild-type *RAS*.

structural variants with the potential to affect clinical decisions or impact the way patients are enrolled in clinical trials. Detected DNA alterations were annotated as pathogenic variants as the case of "likely-pathogenic" or "pathogenic" in the COSMIC and ClinVar and "likely-oncogenic" or "oncogenic" in the OncoKB database.

Results

1. Pathogenic variants in mCRC

This analysis included 994 mCRC patients, with 1,564 clinically meaningful pathogenic variants in 71 mutated genes (Table 1, S3 Table). On average, there are 1.5 clinically meaningful pathogenic variants per patient.

The top 20 frequent genes are as follows: *KRAS* (n=443, 43.6%), *TP53* (n=428, 43.1%), *PIK3CA* (n=126, 12.7%), *APC* (n=80, 8.0%), *BRAF* (n=66, 6.6%), *FBXW7* (n=38, 3.8%), *NRAS* (n=32, 3.2%), *SMAD4* (n=24, 2.4%), *HER2* (n=22, 2.2%), *STK11* (n=21, 2.1%), *BRCA2* (n=19, 1.9%), *PTPN11* (n=18, 1.8%), *MTOR* (n=16, 0.6%), *CTNNB1* (n=13, 1.3%), *FANCA* (n=13, 1.3%), *MSH6* (n=12, 1.2%), *AKT1* (n=11, 1.1%), *CDH1*

(n=10, 1.0%), *FGFR4* (n=8, 0.8%), and *MAP2K1* (n=7, 0.7%) (Fig. 1, S4 Fig.).

2. Current treatment landscape of mCRC
1) Targeting anti-EGFR pathway

Anti-epidermal growth factor receptor–directed monoclonal antibodies (anti-EGFR mAbs) were the first targeted therapies for mCRC which were active both as single agents and in combination with cytotoxic chemotherapy [5,6]. Out of 994 patients, 467 patients (47.0%) were able to get anti-EGFR therapy with wild-type RAS. The other 527 patients (53.0%) were having pathogenic variants in *KRAS* (n=433, 43.6%), *BRAF* (n=66, 6.6%), *NRAS* (n=32, 3.2%), or *HER2* amplification (n=10, 1.0%) which were known to be resistant to anti-EGFR therapy and avoided as an ineffective drug for treatment decision (Fig. 2) [7,8] . Fourteen patients have concurrent mutations related with anti-EGFR therapy resistant genes; seven patients with *KRAS* plus *BRAF* mutation, three patients with *KRAS* plus NRAS mutation, two patients with *NRAS* plus *BRAF* mutation, and two patients with *KRAS* mutation plus *HER2* amplification.
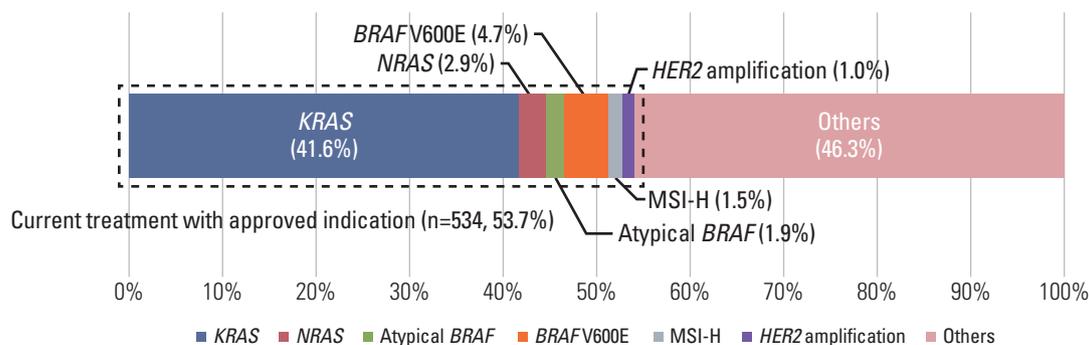
Fig. 3. Current treatment landscape of metastatic colorectal cancer (mCRC). The current treatment landscape (n=534, 53.7%) will be *KRAS*/*NRAS*/*BRAF* mutants (resistant to anti-EGFR therapy) and several biomarkers that can help using targeted therapy or immunotherapy. For patients with *BRAF* V600E mutated mCRC, the combination of encorafenib and cetuximab or panitumumab was approved. The atypical *BRAF* mutated patients have pathogenic variants in *BRAF* other than V600E. Pembrolizumab and nivolumab (or ipilimumab combination) were approved for patients with microsatellite instability–high (MSI-H) mutations. Finally, dual blockade of anti-agents could be incorporated in *HER2*-amplified mCRC patients. Others (n=460, 46.3%), the remaining patients have unmet need of precision medicine approach.
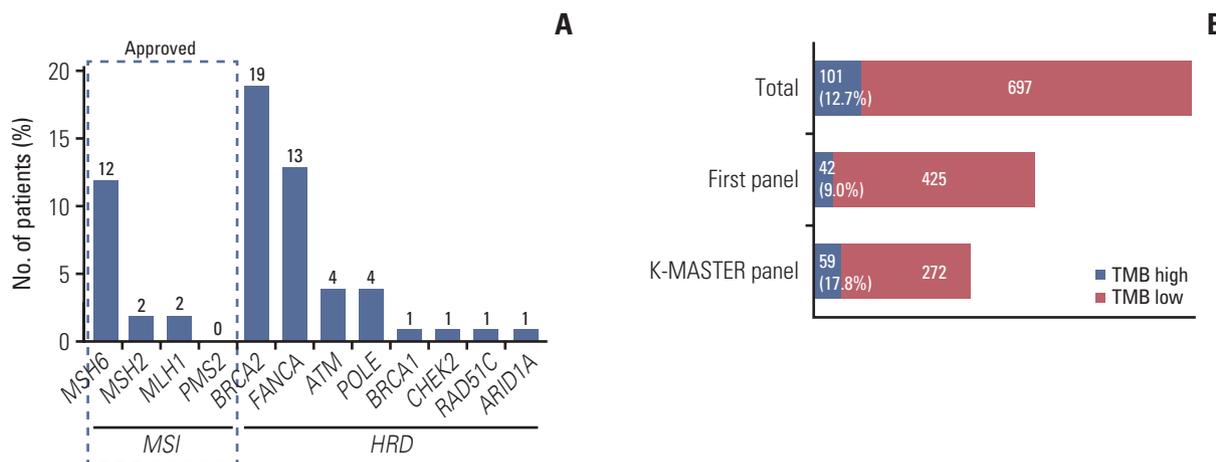
Fig. 4. (A) Candidate for immune checkpoint inhibitors. Microsatellite instability–high (MSI-H) genes are *MSH6*, *MSH2*, *MLH1*, *PMS2*, and *EPCAM* (However, *EPCAM* was included only in FIRST Panel and not in Axen or K-MASTER panels. There was no pathogenic variant of *EPCAM* within FIRST Panel). Patients who have MSI-H pathogenic variants were approved for immune checkpoint inhibitors (n=15, 1.5%). One patient had two pathogenic variants with *MSH2* deletion and *MSH6* T1102fs simultaneously. Total 42 patients (4.2%) have pathogenic variants in defected DNA damage and repair pathway genes (homologous recombination deficiency genes, HRD genes) such as *BRCA1/2*, *CHEK1/2*, *FANCA*, *ATM*, *POLE*, *RAD51B/C/D*, *RAD54L*, *ARID1A*, *BARD1*, *BRIP1*, *CDK1/2*, *PALB2*, etc. (B) Tumor mutation burden. In 994 metastatic colorectal patients, 798 patients analyzed tumor mutation burden (TMB) with K-MASTER Panel (n=331) or FIRST Panel (n=467). Tumor mutational burden cut-off value defined as > 16 per 10^6 base pair in K-MASTER panel and as > 13 per 10^6 base pair in FIRST panel which were 59 patients (17.8%) and 42 patients (9.0%) irrespectively.

2) Targetable genes with approved indications

Beyond anti-EGFR therapy with wild-type RAS, there are several biomarkers that can help using targeted therapy or immunotherapy. For patients with *BRAF* V600E mutated mCRC, the combination of encorafenib and cetuximab or panitumumab was recommended to second- or third-line mCRC treatment [8]. Dual blockade of anti-HER2 agents such as trastuzumab plus pertuzumab [9] or trastuzumab plus lapatinib [10] could be incorporated in *HER2*-amplified mCRC after progression of second-line cytotoxic chemotherapy. Pembrolizumab [11] and nivolumab [12] (or ipilimumab combination) [13] were approved for subsequent-line treatment of mismatch repair genes (MMR includes *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) deficient mismatch repair (dMMR)/microsatellite instability (MSI)–high cancers based on high response rates and encouraging clinical out-

comes. In our study, there were mCRC patients with *BRAF* V600E mutation (n=47, 4.7%), dMMR/MSI-high (n=15, 1.5%, one patient had two pathogenic variants with *MSH2* deletion and *MSH6* T1102fs simultaneously), *HER2* amplification (n=10, 1.0%) which meant additional 72 patients (7.2%) could have had an opportunity to treat with promising drugs (Fig. 3). While 53.7% of all patients with mCRC can receive the customized treatment based on the type of pathogenic mutations, the remaining 46.3% of patients have unmet need of precision medicine approach.

3) Candidates for immune checkpoint inhibitors

A small subset of the microsatellite stable tumors exhibited high tumor mutational burden (TMB) and DNA damage and repair (DDR) defected cancer patients responded immune checkpoint inhibitors [14]. In our study, 42 patients (4.2%) have pathogenic variants in defected DDR pathway genes (homologous recombination deficiency genes, HRD genes) such as *BRCA1/2, CHEK1/2, FANCA, ATM, POLE, RAD51B/C/D, RAD54L, ARID1A, BARD1, BRIP1, CDK1/2, PALB2*, etc. (Fig. 4A). Each patient had one pathogenic variant except one who had three pathogenic variants at the same time (*BRCA2, FANCA, ATM*).

Out of 798 patients analyzed TMB with K-MASTER panel (n=331) or FIRST panel (n=467). TMB cut-off value defined as > 16 per 10^6 base pair in K-MASTER panel [4] and as > 13 per 10^6 base pair in FIRST panel [15] which were 59 patients (17.8%) and 42 patients (9.0%) irrespectively. Although the definition of TMB is different for both panel, a number of patients (n=101, 12.7%) might be treated with immune checkpoint inhibitor approximately (Fig. 4B) [16].

4) Actionable genes under ongoing clinical trials

There are several ongoing trials targeting *PIK3CA* with *RAS/RAF/PTEN/PIK3CA* blockade, *KRAS* G12C with AMG 510, atypical *BRAF* with *BRAF/MEK* blockade, and *HER2* mutation with anti-HER2 agents for mCRC patients. If the patients can participate in these clinical trials, total 180 patients (18.1%) who have *PIK3CA* (n=126, 12.7%), *KRAS* G12C (n=30, 3.0%), atypical *BRAF* (n=17, 1.7%), and *HER2* mutations (n=12, 1.2%, one patient has concurrent mutations with *HER2* amplification) can get an opportunity to treat with promising drugs.

If we extend these pathogenic variants to all solid tumors beyond mCRC, clinical trials with high probability of success are underway with actionable genes such as *NRAS* (n=32, 3.2%), *MTOR* (n=16, 1.6%), *AKT1* E17K (n=9, 0.9%), *MAP2K1* (n=7, 0.7%), *FGFR1* amplification (n=6, 0,6%), *CDKN2A* (n=5, 0.5%), *FLT3* amplification (n=5, 0.5%), *EGFR* amplification (n=5, 0.5%), *HRAS* (n=4, 0.4%), *CDK4* amplification (n=3, 0.3%), *MET* amplification (n=3, 0.3%), *PTEN* (n=3, 0.3%),

Table 2. Pathogenic variants in critical signaling pathway genes

Pathway	Gene	No. of patients
RAS	*KRAS*	433
	NRAS	32
	HRAS	4
	NF1	1
PI3K/mTOR	*PIK3CA*	126
	MTOR	16
	AKT1	11
	PTEN	3
	TSC2	1
RAF/MAPK	*BRAF*	66
	MAP2K1	7
Cell cycling	*CCND1*	6
	CDKN2A	5
	CDK4	3
	CCNE1	1
Receptor tyrosine kinase /growth factors	*FGFR4*	8
	FGFR1	6
	EGFR amplification	5
	RET	3
	MET amplification	3
	KIT	1
p53	*TP53*	428
	MDM2	2

Classified pathogenic variants by critical signaling pathways. There are patients with co-variant mutations, total patient number different from the total sum of each genes.

RET (n=3, 0.3%), *TP53* E285K (n=3, 0.3%) , *IDH1* (n=2, 0.2%), *MDM2* amplification (n=2, 0.2%), *KRAS* G13V (n=1, 0.1%), *KIT* (n=1, 0.1%), *NF1* R1276Q (n=1, 0.1%), *TSC2* (n=1, 0.1%), and HRD genes (n=42). Putting all these rare mutations together, these 137 patients (13.8%) might be able to challenge to get therapeutic opportunities.

We classified pathogenic variants by critical signaling pathways (Table 2). Total 465 patients (46.8%) have pathogenic variants in RAS pathway, 430 patients (43.3%) in p53 pathway, 151 patients (15.2%) in PI3K/mTOR pathway, 72 patients (7.2%) in RAF/MAPK pathway, 26 patients (26.1%) in receptor tyrosine kinase/growth factor pathway, and 14 patients (14.1%) in cell cycling pathway (Fig. 5).

There are several fusion mutations that indeterminant significance in CRC but significant in other solid tumors; *ALK-EML4* fusion genes (n=2, 0.2%) and *RET-NCOA4* fusion genes (n=4, 0.4%) (S5 Fig.) are significant in non–small cell lung cancer. *NTRK* fusion genes are very difficult to find the right patients in the daily practice setting but it is known that
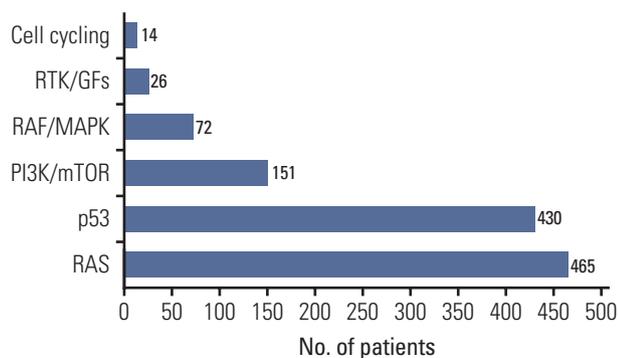
Fig. 5. Critical signaling pathways beyond metastatic colorectal cancer. Classifying pathogenic variants by critical signaling pathways: cell cycling pathway (*CCND1, CDKN2A, CKD4,* and *CCNE1*), receptor tyrosine kinase/growth factors (RTK/GFs) pathway (*FGFR1/4, EGFR* amplification, *MET* amplification, *RET,* and *KIT*), RAF/MAPK pathway (*BRAF* and *MAP2K1*), PI3K/mTOR pathway (*PIK3CA, MTOR, AKT1, PTEN,* and *TSC2*), p53 pathway (*TP53* and *MDM2*), and RAS pathway (*KRAS, NRAS, HRAS,* and *NF1*).
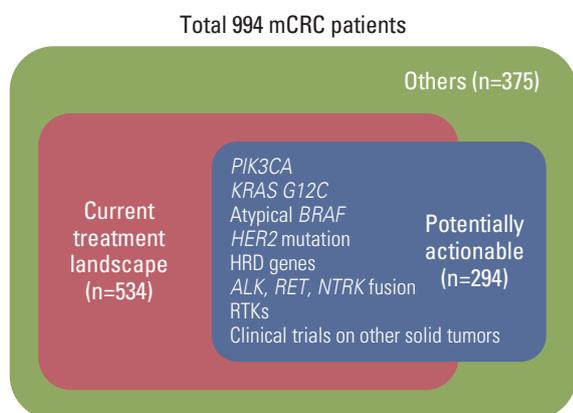
Fig. 6. Maximize the therapeutic opportunity for metastatic colorectal cancer (mCRC) in precision medicine era. In this study, 534 patients (53.7%) have therapeutic options by current treatment landscape (Fig. 3). If all mCRC patients with 'Potentially actionable' variants would have challenged to get therapeutic opportunities, 294 patients (29.6%) would have tried to enroll the clinical trial. This group contains 180 (18.1%) patients with *KRAS* G12C/atypical *BRAF/PIK3CA, HER2* mutations who could be participated in ongoing trials within mCRC and 137 patients (13.8%) who could take part in clinical trials with all solid tumors with novel variants. HRD, homologous recombination deficiency; RTK, receptor tyrosine kinase.

the treatment response rate is very high if drugs targeting pathogenic variants are incorporated [17-20].

Discussion

Cancer treatment is moving to a new paradigm where the molecular characteristics of the tumor are used to inform treatment decision. In recent decades, people investigated the genetic basis of CRC and large-scale sequencing studies discovered key pathways such as WNT, RAS/MAPK, PI3K, TGF-β, p53, and dMMR. Although our understanding of genomic alteration of mCRC has rapidly improved, we still do not fully understand the heterogenous properties of CRC and do not provide the personalized treatments based on individual molecular characteristics.

In this study, we investigated mutations within 409 selected oncogenes and tumor suppressor genes and found 71 genes with 1,564 pathogenic variants from 994 mCRC patient samples. Overall, our results showed that our population had a similar mutation profiles to populations that reported in other studies [21-25], with alterations in driver genes, such as *KRAS, TP53, PIK3CA, APC, BRAF,* and *FBXW7*.

In order to overcome a resistance mechanism to anti-EGFR therapy, the innovative challenge has been initiated in mCRC harboring identified acquired targets such as *BRAF* mutation, *HER2* amplification, and c-*MET* amplification by means of NGS [8,26]. Nonetheless, we are currently only able to apply anti-EGFR therapy (n=527, 53.0%) as a negative predictive biomarker under the conditions of Korean regulatory licenses. The rare mutations such as *BRAF* V600E (n=47, 4.7%), MSI-high (n=15, 1.5%), *HER2* amplification (n=10, 1.0%) are rising targets with novel agents which neither approved nor reimbursed in Korea so these population could not be helped determining treatment at this moment.

Although there is a difference in the cut-off value between two panels, a significant number of TMB high mCRC patients should be considered as a target population with immune checkpoint inhibitors. Even rarer genetic alterations are also considered as actionable targets. For example, 30 patients with *KRAS* G12C could be targeted with AMG 510 as a first-in-class *KRAS* G12C inhibitor. *EML4-ALK* fusion (n=2) and *RET-NCOA4* fusion (n=4) occurred in less than 1% of cases which were not classified as a pathogenic variant due to no evidence in CRC but could be the targets. Clinical trials should be conducted with various promising drugs for mCRC patients in order to expand therapeutic opportunities. For example, 180 patients (18.1%) with *KRAS* G12C/atypical *BRAF/PIK3CA, HER2* mutations could be participated in ongoing trials within mCRC and 137 patients (13.8%) could take part in clinical trials in all solid tumors with novel variants. If all mCRC patients with potentially actionable variants would have challenged to get therapeutic opportunities, additional 294 patients (29.6%) would have tried to enroll the clinical trial (Fig. 6).

Under K-MASTER project, a nationwide cancer genome screening project in Korea, this study aimed to identify key alterations in mCRC that may represent important targets for novel drugs using customized cancer panels at the nationwide level. K-MASTER project with customized cancer panel can be accomplished in a realistic timeframe for use in day-to-day clinical care and could be used to expand treatment options for mCRC patients. Using efficient genomic screening systems, relatively minor cancer genome alterations are necessary for the successful development of targeted therapies under clinical trials.

This mCRC study assessing the feasibility of precision oncology for mCRC patients in real-world setting found that about 52.8% of patients will able to treat with targeted therapies and 29.6% of patients could be candidate of clinical trials with innovative drugs. It is believed that a precise medical approach can contribute to broadening the treatment opportunities for mCRC patients.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

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