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Colorectal carcinomas containing hypermethylated MLH1 promoter and wild type BRAF/KRAS are enriched for targetable kinase fusions

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Abstract

Kinase fusions are rare and poorly characterized in colorectal carcinoma (CRC), yet they present unique opportunities for targeted therapy. In this study, we characterized kinase fusions from patients with advanced CRC who had MSK-IMPACT testing of their tumors between January 2014 and June 2018. Patients were analyzed for the presence of fusions, microsatellite instability (MSI), and RAS/BRAF mutations. Mismatch repair (MMR) immunohistochemistry (IHC) and promoter hypermethylation status of MLH1 (MLH1ph) in MSI-H CRC with fusions were investigated. Fusion transcripts were confirmed using a targeted RNAseq panel assay. Of 2314 CRCs with MSK-IMPACT testing, 21 harbored kinase fusions. Overall 57% (12/21) of CRC fusions were MSI-H/MMR-D. Loss of MLH1 and MLH1ph was confirmed in all 12 and all 10

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cases with available material, respectively. Fusions were present in 5% of MSI-H/MMR-D CRC compared to 0.4% of MSS/MMR-P CRC (p<0.001), and 15% of MSI-H/MMR-D CRC with wild type RAS/BRAF. Of 24 total MLH1-deficient CRC with MLH1ph and wild type RAS/BRAF, 10 (42%) harbored kinase fusions. Kinase fusions in MSI-H CRC were associated with sporadic MLH1ph rather than with Lynch syndrome, and these patients may be eligible for kinase inhibitors, particularly following resistance or toxicity in response to immunotherapy. These findings identify a molecular subset of CRC with kinase fusions that may be responsive to kinase inhibitors.

PRECIS

A high frequency of targetable kinase fusions in BRAF/RAS wild type, microsatelltie instability-high colorectal carcinoma offers a rationale for routine screening to identify CRC patients with kinase fusions that may be responsive to kinase inhibitors.

Keywords

colorectal carcinoma; *MLH1* hypermethylation; microsatellite instability; mismatch repair deficiency; fusion; RET; ROS1; NTRK1; NTRK3; *BRAF*

INTRODUCTION

Approximately 15% of colorectal carcinomas (CRCs) demonstrate mismatch repair deficiency (MMR-D)/ microsatellite instability- high (MSI-H) status. The majority of these are MLH1/ PMS2 deficient due to *MLH1* promoter hypermethylation (*MLH1*ph). *BRAF* V600E mutations occur in approximately 50% of CRC with *MLH1*ph and have been shown to induce *MLH1*ph via upregulation of the transcriptional regulator MAFG (1). *KRAS* mutations occur in approximately 30% of MSI-H CRC *MLH1*ph (2), leaving 20% of CRC with *MLH1*ph without a known driver activating the MAPK signaling pathway. Isolated cases of MSI-H CRC with fusions have recently been reported (3–5), and we noted a similar trend in our clinical next generation sequencing (NGS) data. We provide a detailed delineation of this association, defining a previously unappreciated subset of CRC with important therapeutic implications.

MATERIALS AND METHODS

Written informed consent was obtained from patients, approval was obtained from our institutional review board, and this retrospective study was conducted in accordance with U.S. Common Rule. CRC accessioned for MSK-IMPACT (6) and/ or Archer NGS testing were assessed for kinase fusions. Patients with MSK-IMPACT testing had MSI status routinely assessed as a component of the assay (7). Archer fusion testing was clinically performed when sufficient remaining material was present for cases with WT *KRAS*, *NRAS*, and *BRAF* by MSK-IMPACT or a 95 gene Ampliseq-based assay, the latter performed when material was insufficient for MSK-IMPACT. Archer was also performed to confirm fusion transcripts in cases with novel DNA-level structural variants predicted to form kinase fusions. The custom Archer panel used covers fusions involving the kinase

domains of the following genes: *ALK*, *BRAF*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *KIT*, *MET*, *NTRK1*, *NTRK2*, *NTRK3*, *RET*, and *ROS1*. When tissue is available, MMR IHC is routinely clinically performed, and these data were recorded for patients with Ampliseq testing (which does not generate MSI status results).

Clinicopathologic characteristics of all CRC with kinase fusions were assessed. Primary site was classified as either proximal (cecum to transverse colon) or distal (splenic flexure to rectum). Differentiation and mucinous histology were scored based on World Health Organization criteria (8). Well differentiated CRC had >95% gland formation, moderately differentiated CRC had 50–95% gland formation, poorly differentiated CRC had 0–49% gland formation. Mucinous adenocarcinoma had an extracellular mucin component of >50% while CRC with a mucinous component had extracellular mucin pools comprising <50% of the lesion.

*MLH1*ph was detected via ebisulfite conversion followed by either pyrosequencing or methylation array depending on specimen availability. CRC with *MLH1*ph and wild type (WT) for *KRAS* or *NRAS* p. G12, G13, Q61, K117, A146 and *BRAF* p. V600 alleles were retrospectively screened with a custom Archer targeted RNAseq-based NGS assay used for fusion and alternative isoform testing (9). Confirmatory pan-Trk IHC was performed on CRC with *NTRK* fusions (10).

A subset of CRC with either *BRAF* V600E, kinase fusions, or *KRAS* mutations had genomic-wide methylation profiling performed using the Illumina methylationEPIC (850k) platform (11). After excluding CpG sites from the MLH1 gene and X/Y chromosomes from the datasets, unsupervised hierarchical clustering was performed on the 10,000 most variable CpG sites (by standard deviation) using Euclidean distance and Ward's method with R (version 3.4).

MSK-IMPACT including MSIsensor, Archer, Ampliseq MMR IHC, pan-Trk IHC, and *MLH1*ph assays are clinically validated assays that were performed in CLIA-accredited laboratories.

RESULTS

Prevalence and Spectrum of Kinase Fusions in CRC

We identified 2314 CRC accessioned for MSK-IMPACT and/ or Archer between January 2014 and June 2018. This dataset included 2309 CRC patients with MSK-IMPACT results of which 189 also underwent Archer targeted RNAseq testing, and 5 additional patients with insufficient material for MSK-IMPACT whose tumors underwent *RAS*/ *BRAF* testing by Ampliseq followed by Archer testing. Seventeen CRC were positive for kinase fusions via MSK-IMPACT. Four additional CRC with fusions were detected using Archer targeted RNAseq assay: 3 cases were negative by MSK-IMPACT due to lack of coverage of breakpoints (*EML4-NTRK3*, *FGFR3-STAB1*, and *FGFR2-MYH15*), while the fourth case (*TPM3-NTRK1*) identified by Archer testing alone had insufficient DNA for MSK-IMPACT and had WT *KRAS/NRAS/BRAF* by outside NGS testing, yielding a total of 21 CRC positive for kinase fusions.

The detected fusions included 8 *NTRK* fusions (6 *NTRK1* and 2 *NTRK3*), 5 *BRAF* fusions 4 *RET* fusions, 2 *FGFR* fusions (1 each of *FGFR2* and *FGFR3*), 1 *ROS1* fusion, and 1 *ALK* fusion(Table 1, Figure 1). All detected kinase fusions were predicted to be in frame, included the kinase domain of the 3' gene, and occurred in CRC that were *BRAF/RAS* WT. All 6 *NTRK1* fusions and 1 of the 2 *NTRK3* fusions were positive for pan-Trk IHC, with results as previously described (10).

Clinicopathologic Characteristics of Colorectal Carcinoma with Kinase Fusions

The age at diagnosis of these 21 CRC patients harboring kinase fusions ranged from 33–85 years with a median of 64 years. The majority (71%) of this cohort had CRC arising in the proximal colon. Poor differentiation (including medullary, n=2) was present in 57% of the fusion cases, while 16% of cases had a mucinous component. Looking further into the fusion cohort, 83% of MSI-H CRC had poor differentiation or were mucinous in histologic subtype while only 33% of MSS CRC with fusions had poor differentiation or a mucinous component. This data suggests that poor or mucinous differentiation may be associated with the MSI-H status rather than the presence of fusion. American Joint Committee on Cancer (AJCC) 8th edition stage at diagnosis included 6 stage II patients, 6 stage III patients, and 8 stage IV patients. Median follow up time since diagnosis was 18 months. Sixty-eight percent of patients had distant metastasis at end of follow up, and 76% of patients were alive at end of follow-up. These findings are summarized in Table 2.

Relationship of MSI to the Presence of Kinase Fusions.

Of the 2314 total CRC, 230 were MSI-H/ MMR-D and 2084 were MSS/ MMR-P. The presence of kinase fusions was mutually exclusive with *BRAF* V600 and *RAS* hotspot mutations. The MSI-H/ MMR-D and MSS/MMR-P cohorts respectively harbored 74 (32%) vs 106 (5%) *BRAF* V600E mutations ($p<0.001$), 83 (36%) vs 912 (44%) *KRAS* hotspot mutations ($p=0.322$), 2 (1%) vs 86 (4%) *NRAS* hotspot mutations ($p=0.096$), and 12 (5%) vs 9 (0.4%) kinase fusions ($p<0.001$) (Figure 1). Fifteen percent of MSI-H/ MMR-D and 0.9% of MSS/ MMR-P CRC that were *RAS/BRAF* WT harbored kinase fusions.

MMR Deficiency and Relationship of MLH1 Hypermethylation Status to the Presence of Kinase Fusions

Twelve (57%) of 21 CRC with kinase fusions were MMR-D/ MSI-H. All MSI-H/MMR-D CRC with available material had MLH1/ PMS2 loss (n=12) and *MLH1*ph (n=10). Looking further into the 71 MSI-H CRC that were *RAS/ BRAF* WT, 47 were MLH1/ PMS2 deficient by IHC. Twenty-four of 37 of these MLH1/PMS2 deficient CRC with WT *RAS/BRAF* had *MLH1*ph data available were positive for *MLH1*ph. Of these 24 cases with *MLH1* promoter hypermethylation, 10 harbored kinase fusions. Therefore, the incidence of fusions in MLH1 deficient CRC with *MLH1*ph and WT *RAS*/ *BRAF* was 42% (Figure 1).

Methylation Array Results

Due to the similarity of our findings relating fusions and *MLH1*ph to those of *BRAF* V600E and *MLH1*ph (1), we performed unsupervised hierarchical clustering of Illumina 850k methylation array data on both MSS and MSI-H CRC samples with fusions, BRAF V600E,

and *KRAS* mutations after exclusion of *MLH1* loci. Clear separation of hypermethylated and hypomethylated groups was evident. The hypermethylated group was composed of 2 predominant sub-clusters, suggesting CIMP-H and CIMP-L subgroupings. Eight out of 11 (73%) fusion-driven and fourteen out of twenty (70%) of *BRAF* V600E CRCs localized to the hypermethylated group. All 19 (100%) *KRAS* mutants segregated to the hypomethylated group. Interestingly, 2 MSS CRC (1 fusion and 1 *BRAF* V600E) harbored *MLH1*ph.

DISCUSSION

In recent years, cancers bearing kinase fusions have shown some of the most dramatic and durable responses to kinase inhibitors (12–13). For instance, larotrectinib has shown a response rate of 75% in adult patients with *NTRK* fusions, with 71% of responses ongoing and 55% of patients being progression-free at 1 year of treatment (12). While such targetable fusions are rare in CRC overall, the present study shows that approximately 15% of advanced MSI-H/ MMR-D CRC which are WT for *BRAF*/ *KRAS*/ *NRAS* harbor kinase fusions, and that all of the detected kinase fusions in MSI-H CRC occurred specifically in non-Lynch Syndrome cases with MLH1 deficiency associated with *MLH1*ph. Further, fusions were present in almost half of MLH1 deficient CRC with WT *KRAS*/ *NRAS*/ *BRAF* with *MLH1*ph.

A mechanistic basis for the relationship between *BRAF* V600E, genome-wide hypermethylation, and MSI has been proposed by Fang et al, who showed that *BRAF* V600E mutations in CRC induce CpG island hypermethylation including *MLH1*ph via upregulation of ERK and MAFG, resulting in deficient MMR (1). The strong relationship between kinase fusions and *MLH1*ph suggests fusions may induce a similar phenomenon. Results from our methylation array studies show that *BRAF* p. V600E mutant and kinase fusion positive CRC have similar genomic CpG methylation patterns even after exclusion of data from the *MLH1* promoter CpG loci. Functional studies elucidating the mechanistic relationship between kinase fusions and *MLH1*ph are warranted.

Our study does have several limitations. These include the rarity of kinase fusions in CRC and resulting relatively small cohort, the fact that none of the MSI-H/ MMR-D CRC with fusions received a tyrosine kinase inhibitor and had available response data, and limited material on several of these cases, precluding MMR IHC, MSI testing, or *MLH1*ph.

To our knowledge, this study is the first to establish the relationship between kinase fusions and MSI-H CRC, specifically with *MLH1*ph. Given the rarity of fusions and the fact that fusion testing is not routinely performed on CRC, it is important to identify subtypes that are more likely to carry these fusions. Thus, testing for kinase fusions is warranted in advanced CRC with *MLH1*ph and WT *BRAF*/ *RAS* and the present findings inform an updated proposed molecular testing workflow for CRC (Figure 2). This proposed updated workflow begins with universal MSI or MMR IHC as recommended by the NCCN (14). CRC patients with MSS/ MMR-P tumors should undergo NGS testing if available or *KRAS*/ *NRAS* mutation analysis for eligibility for anti-EGFR therapy. Patients with MSI-H/ MLH1 deficient CRC should undergo *MLH1*ph testing as part of the work-up for Lynch Syndrome. If *MLH1*ph is not detected, *MLH1* germline testing to rule out Lynch Syndrome may be

performed. For CRC patients with deficiency of MSH2, MSH6, and/ or PMS2 but not MLH1, germline testing of the deficient MMR gene is recommended due to the potential presence of Lynch Syndrome. If *MLH1*ph is present, the patient has distant metastases, and the tumor is negative for *BRAF* p. V600E mutation, fusion testing may be performed due to the high likelihood of finding a kinase fusion with potential therapeutic implications.

Immune checkpoint inhibition produces response rates of 20%−50% of MSI-H CRC (15–16), and the presence of a kinase fusion would create a window of opportunity for treatment with kinase inhibitors when resistance or toxicity occurs after immune checkpoint inhibition therapy.

To conclude, while kinase fusions are rare in CRC overall (0.9%), 57% of kinase fusions in CRC occur in MMR-D/ MSI-H CRC. These cases have *MLH1*ph and WT *BRAF*/ *RAS*. Almost half of CRC with *MLH1*ph and WT *RAS*/ *BRAF* harbor kinase fusions. This subset of advanced CRC may benefit from screening for oncogenic kinase fusions.

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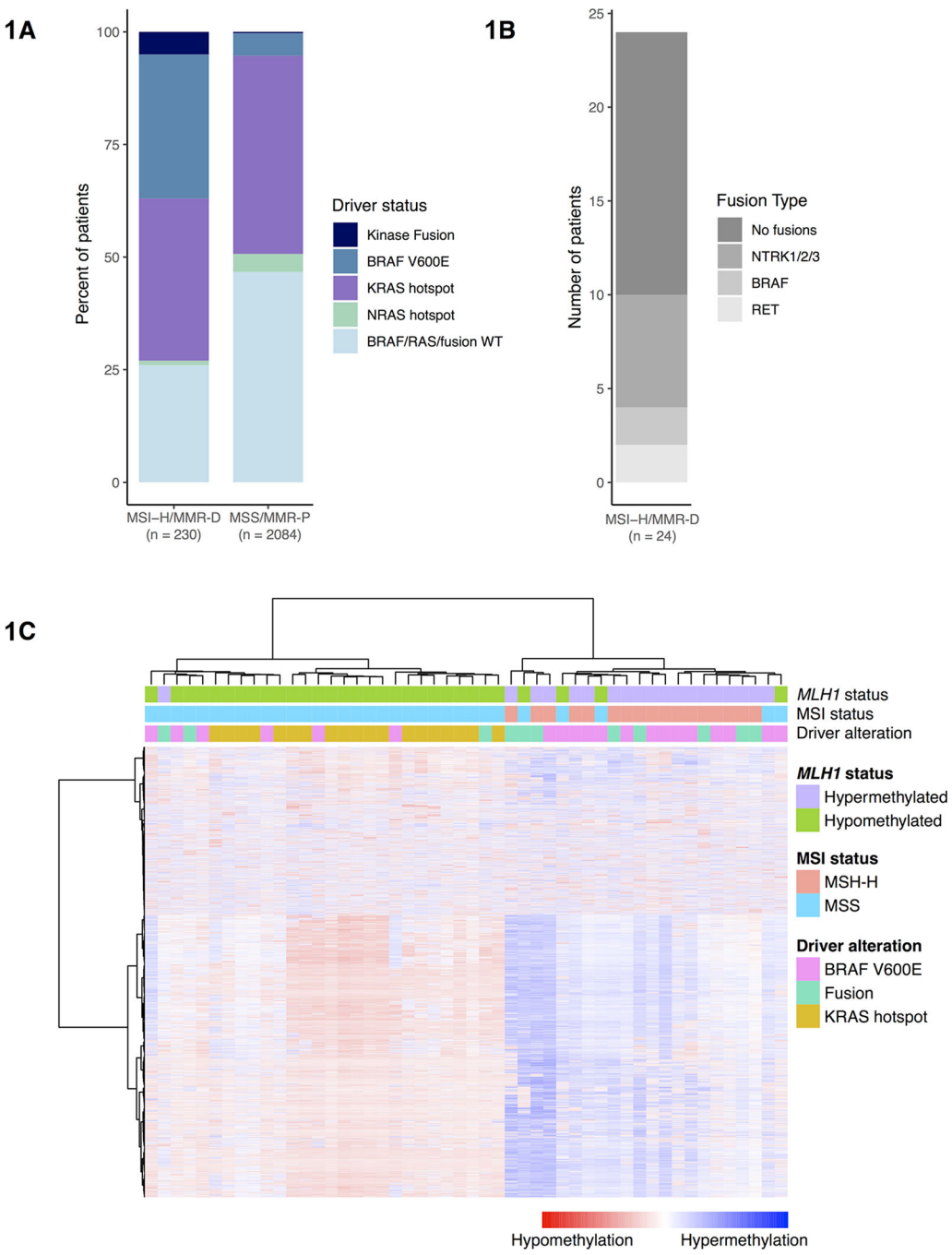

Figure 1.

Prevalence of major MAPK driver alterations in molecular subgroups of CRC and methylation patterns. A) MSI-H (n=230) vs MSS CRC (n=2084) respectively harbored 74 (32%) vs 106 (5%) *BRAF* p. V600E mutations (p<0.0001), 83 (36%) vs 912 (44%) *KRAS* hotspot mutations (p=0.322), 2 (1%) vs 86 (4%) *NRAS* hotspot mutations (p=0.096), and 12 (5%) vs 9 (0.4%) kinase fusions (p<0.001). B) Of the 10 fusions detected in the group of 24 CRC with *MLH1* promoter hypermethylation and WT *RAS/ BRAF*; there were 6 *NTRK* fusions, 2 *BRAF* fusions, and 2 *RET* fusions. C) Unsupervised hierarchical clustering of

methylation array data using the most variable 10,000 CpG sites (excluding *MLH1* loci) in a subset of *BRAF* p. V600E, *KRAS* mutant, and fusion positive CRC shows that MSI-H (*MLH1* hypermethylated) *BRAF* p. V600E and fusion positive CRC predominantly colocalized to the hypermethylated cluster. *KRAS*-mutated CRCs all localized to the hypomethylated cluster.

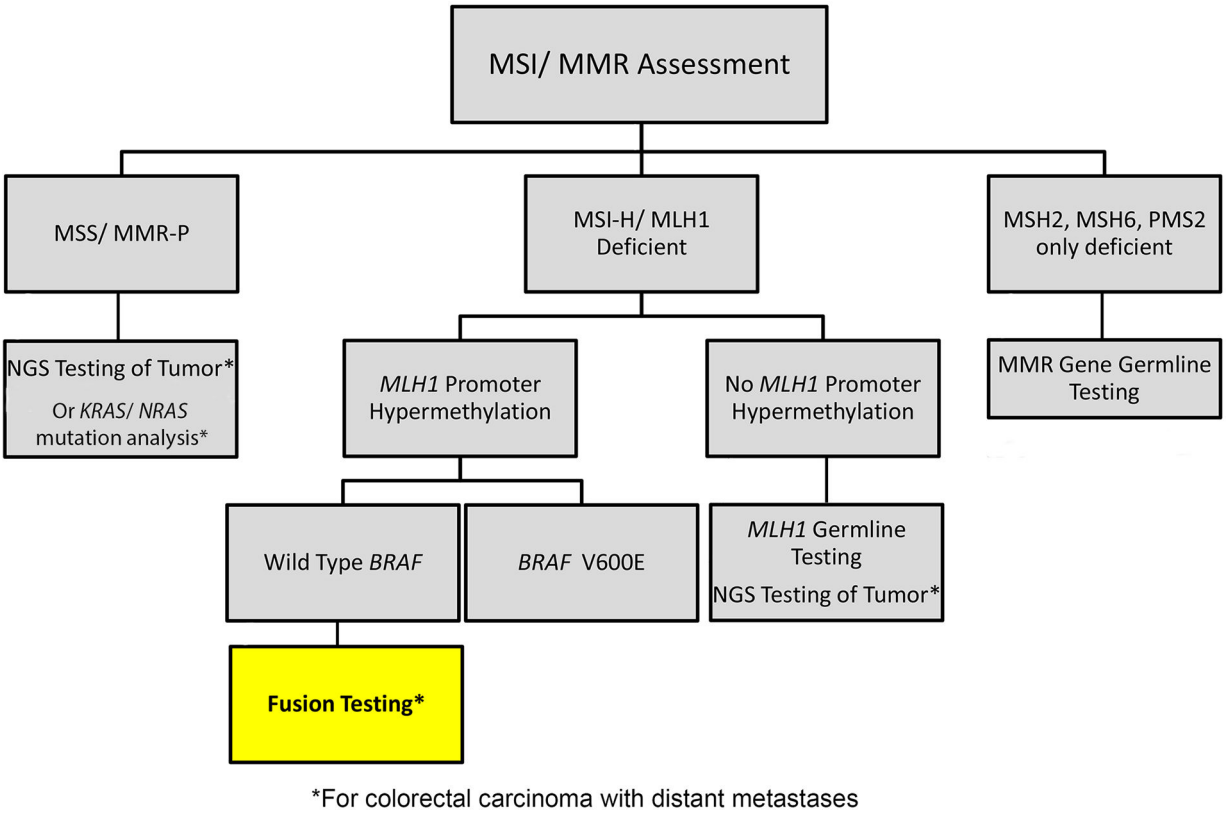

Figure 2.

Workflow for molecular testing in colorectal carcinoma. Testing for MSI/ MMR status should be performed universally in CRC. Patients with metastatic MSS/ MMR-P CRC should undergo next generation sequencing or *RAS*/ *BRAF* mutation testing. Patients with MLH1 deficiency of MSI-H results without available MMR IHC should undergo *MLH1* promoter hypermethylation testing. If *MLH1* promoter hypermethylation is detected in metastatic CRC and the tumor is negative for *BRAF* p. V600E, fusion testing should be performed. Patients with MMR-D of MSH2, MSH6, or PMS2 should receive germline testing.

Table 1.

Spectrum and molecular characteristics of kinase fusions in CRC.

CASE	PARTNER GENE	EXON	KINASE GENE	EXON	MMR IHC	MSI STATUS	MLH1 PROMOTER HYPERMETHYLATION	FUSION DETECTED BY
1	*LMNA*	8	*NTRK1*	12	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT
2	*CCDC*	8	*RET*	12	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT + ARCHER
3	*TPM3*	10	*NTRK1*	9	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT
4	*LMNA*	2	*NTRK1*	11	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT
5	*ETV6*	6	*NTRK3*	15	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT
6	*SPTBN1*	7	*ALK*	20	MMR-D (MLH1/ PMS2)	MSI-H	*N/A**	IMPACT
7	*GEMIN5*	24	*RET*	12	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT + ARCHER
8	*TPM3*	8	*NTRK1*	10	MMR-D (MLH1/ PMS2)	*N/A**	POSITIVE	IMPACT + ARCHER
9	*AGAP3*	10	*BRAF*	9	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT
10	*EML4*	2	*NTRK3*	14	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	ARCHER (IMPACT NEGATIVE)
11	*TPM3*	8	*NTRK1*	10	MMR-D (MLH1/ PMS2)	*N/A**	*N/A**	ARCHER (IMPACT INSUFFICIENT)
12	*TRIM24*	14	*BRAF*	9	MMR-D (MLH1/ PMS2)	MSI-H	POSITIVE	IMPACT + ARCHER
13	*NCOA4*	10	*RET*	12	MMR-P	MSS	POSITIVE	IMPACT
14	*LMNA*	12	*NTRK1*	12	MMR-P	MSS	NEGATIVE	IMPACT + ARCHER
15	*GOPC*	4	*ROS1*	36	MMR-P	MSS	NEGATIVE	IMPACT + ARCHER
16	*NCOA4*	8	*RET*	12	MMR-P	MSS	NEGATIVE	IMPACT
17	*CUL1*	7	*BRAF*	9	MMR-P	MSS	*N/A**	IMPACT
18	*MKRN1*	3	*BRAF*	10	*N/A**	MSS	*N/A**	IMPACT + ARCHER
19	*AGAP3*	9	*BRAF*	9	MMR-P	MSS	*N/A**	IMPACT
20	*FGFR3*	17	*STAB1*	51	MMR-P	MSS	*N/A**	ARCHER (IMPACT NEGATIVE)
21	*FGFR2*	14	*MYH15*	31	MMR-P	MSS	*N/A**	ARCHER (IMPACT NEGATIVE)

*N/A**: Testing was not performed.

Table 2.

Clinicopathologic features of colorectal carcinoma patients harboring kinase fusions.

CASE	AGE AT DIAGNOSIS	SEX	PRIMARY SITE	SPECIMEN TESTED	HISTOLOGY/ DIFFERENTIATION	STAGE AT DIAGNOSIS	DISTANT METASTASES (AT END OF FOLLOW-UP)	FOLLOW-UP (MONTHS)	VITAL STATUS
1	33	F	Distal	Primary	Poor, Mucinous Component	IV	Liver	195	Alive
2	85	M	Proximal	Primary	Poor	III	Liver	9	Deceased
3	60	F	Proximal	Primary	Poor	II	None	120	Alive
4	72	M	Proximal	Metastasis (Adrenal)	Poor	III	Adrenal	71	Alive
5	61	F	Proximal	Primary	Poor	IV	Liver, Lungs	6	Deceased
6	57	M	Distal	Primary	Poor	II	None	18	Alive
7	70	F	Proximal	Primary	Moderate	III	None	15	Alive
8	58	F	Proximal	Primary	Poor (medullary)	II	None	11	Alive
9	83	M	Proximal	Primary	Mucinous adenocarcinoma	II	None	18	Alive
10	69	F	Distal	Primary	Poor, Mucinous Component	IV	Liver, Stomach	30	Alive
11	70	F	Proximal	Metastasis (Neck)	Moderate	IV	Liver, Lung, Retroperitoneum, Neck	15	Alive
12	83	F	Proximal	Primary	Poor (medullary)	II	None	6	Alive
13	66	F	Proximal	Primary	Poor, Mucinous Component	IV	Apical Lymph Node	26	Deceased
14	52	F	Proximal	Primary, Metastasis x2 (Right abdomen, Liver)	Moderate	II	Abdominal wall, Liver	58	Alive
15	36	F	Distal	Primary	Moderate	III	None	16	Alive
16	65	M	Distal	Primary	Poor	III	Lung	18	Alive
17	64	F	Proximal	Primary	Poor	IV	Omentum, Peritoneum	8	Deceased
18	64	M	Proximal	Metastasis (Cerebullum)	Moderate	IV	Cerebellum, Lung	80	Alive
19	63	F	Proximal	Primary	Moderate	IV	Liver, Retroperitoneum, Lung, Spleen, Adrenals	18	Deceased
20	52	M	Distal	Primary	Moderate	4	Liver	17	Alive
21	58	F	Proximal	Primary	Moderate	3	Liver	24	Alive