Priority Report

Cancer Research

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, yet they present unique opportunities for targeted therapy. In this study, we characterized kinase fusions from patients with advanced colorectal carcinoma 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), IHC, and promoter hypermethylation status of MLH1 (MLH1ph) in microsatellite instability-high (MSI-H) colorectal carcinoma with fusions were investigated. Fusion transcripts were confirmed using a targeted RNA-seq panel assay. Of 2,314 colorectal carcinomas with MSK-IMPACT testing, 21 harbored kinase fusions. Overall 57% (12/21) of colorectal carcinoma fusions were MSI-H/MMR-D. Loss of MLH1 and MLH1ph was confirmed in all 12 and all 10 cases with available material, respectively. Fusions were present in 5% of MSI-H/MMR-D colorectal carcinoma compared with 0.4% of MSS/MMR-P colorectal carcinoma ($P < 0.001$) and 15% of MSI-H/MMR-D colorectal carcinoma with wild-type *RAS/BRAF*. Of 24 total MLH1-deficient colorectal carcinomas with MLH1ph and wild-type *RAS/BRAF*, 10 (42%) harbored kinase fusions. Kinase fusions in MSI-H colorectal carcinoma 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 colorectal carcinoma with kinase fusions that may be responsive to kinase inhibitors.

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

See related commentary by Valeri, p. 1041

Introduction

Approximately 15% of colorectal carcinomas 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 colorectal carcinomas 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 colorectal carcinoma *MLH1*ph (2), leaving 20% of colorectal carcinomas with *MLH1*ph without a known driver activating the MAPK signaling pathway. Isolated cases of MSI-H colorectal carcinoma 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 colorectal carcinomas 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. Colorectal carcinomas accessioned for MSK-IMPACT (6) and/or Archer NGS testing were assessed for kinase fusions. Reagents and primers for Archer NGS testing were obtained from ArcherDx. 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*,

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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 colorectal carcinomas 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 on the basis of World Health Organization criteria (8). Well-differentiated colorectal carcinomas had >95% gland formation, moderately differentiated colorectal carcinomas had 50%–95% gland formation, poorly differentiated colorectal carcinomas had 0%–49% gland formation. Mucinous adenocarcinoma had an extracellular mucin component of >50%, whereas colorectal carcinoma with a mucinous component had extracellular mucin pools comprising <50% of the lesion.

*MLH1*ph was detected via bisulfite conversion followed by either pyrosequencing or methylation array depending on specimen availability. Colorectal carcinomas 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 RNA-seq-based NGS assay used for fusion and alternative isoform testing (9). Confirmatory pan-Trk IHC was performed on colorectal carcinomas with *NTRK* fusions (10).

A subset of colorectal carcinomas with either *BRAF* V600E, kinase fusions, or *KRAS* mutations had genome-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 method with R (version 3.4).

All of the above assays were clinically validated assays that were performed in CLIA-accredited laboratories.

Results

Prevalence and spectrum of kinase fusions in colorectal carcinoma

We identified 2,314 colorectal carcinomas accessioned for MSK-IMPACT and/or Archer between January 2014 and June 2018. This dataset included 2,309 patients with colorectal carcinomas with MSK-IMPACT results, of which 189 also underwent Archer-targeted RNA-seq testing, and 5 additional patients with insufficient material for MSK-IMPACT whose tumors underwent *RAS*/*BRAF* testing by Ampliseq, followed by Archer testing. Seventeen colorectal carcinomas were positive for kinase fusions via MSK-IMPACT. Four additional colorectal carcinomas with fusions were detected using Archer-targeted RNA-seq assay: 3 cases were negative by MSK-IMPACT due to lack of coverage of breakpoints (*EML4-NTRK3*, *FGFR3-STAB1*, and *FGFR2-MYH15*), whereas 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 colorectal carcinomas 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 and Fig. 1). All detected kinase fusions were predicted to be in frame, included the kinase domain of the 3′ gene, and occurred in colorectal carcinomas 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 described previously (10).

Table 1. Spectrum and molecular characteristics of kinase fusions in colorectal carcinoma

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)

Abbreviation: N/A, testing was not performed.

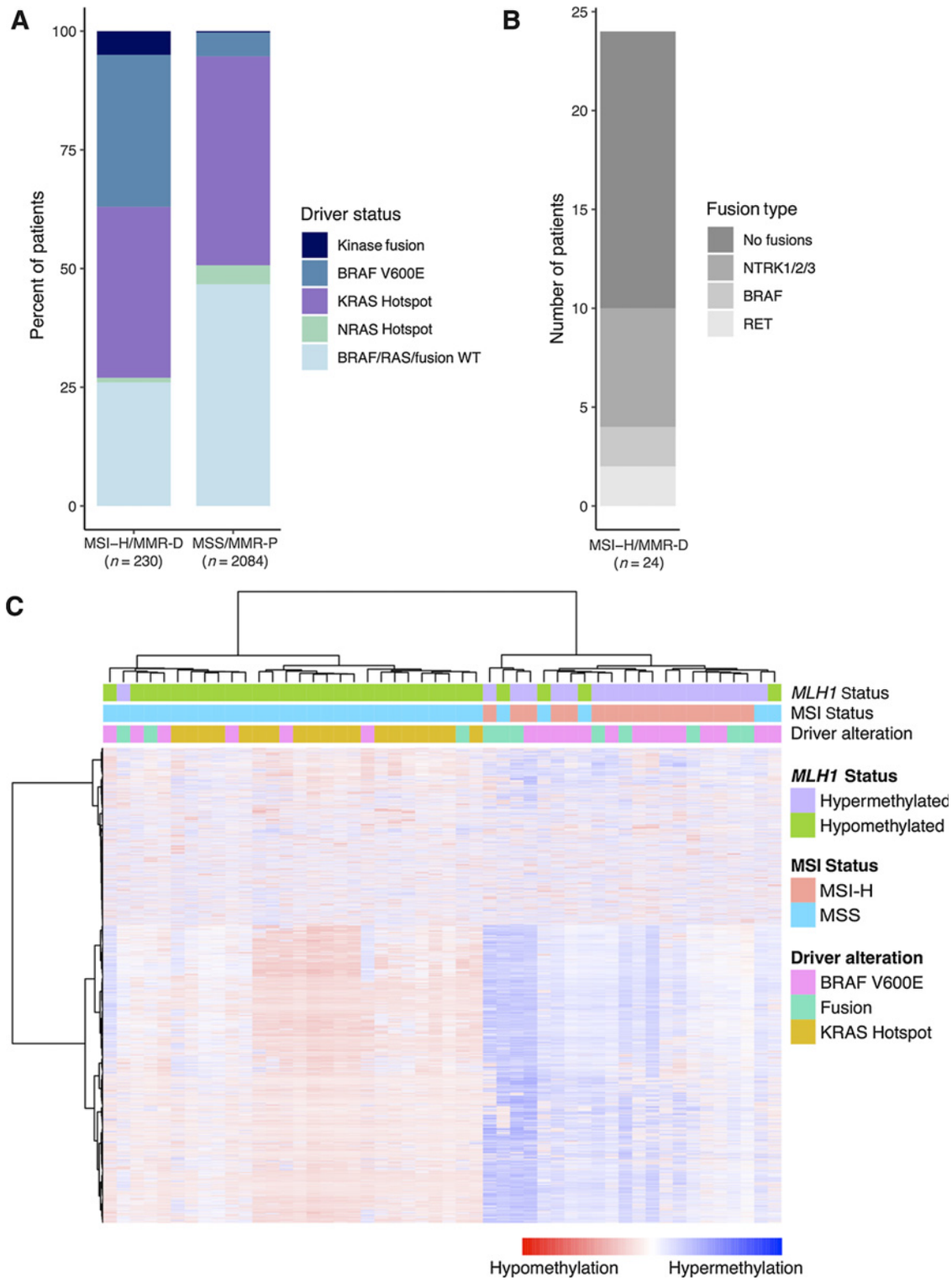

Figure 1.

Prevalence of major MAPK driver alterations in molecular subgroups of colorectal carcinoma and methylation patterns. **A,** MSI-H ($n = 230$) versus MSS colorectal carcinoma ($n = 2,084$), respectively, harbored 74 (32%) versus 106 (5%) *BRAF* p. V600E mutations ($P < 0.0001$), 83 (36%) versus 912 (44%) *KRAS* hotspot mutations ($P = 0.322$), 2 (1%) versus 86 (4%) *NRAS* hotspot mutations ($P = 0.096$), and 12 (5%) versus 9 (0.4%) kinase fusions ($P < 0.001$). **B,** Of the 10 fusions detected in the group of 24 colorectal carcinoma 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 colorectal carcinoma shows that MSI-H (*MLH1* hypermethylated) *BRAF* p. V600E and fusion-positive colorectal carcinoma predominantly colocalized to the hypermethylated cluster. *KRAS*-mutated colorectal carcinomas all localized to the hypomethylated cluster.

Clinicopathologic characteristics of colorectal carcinoma with kinase fusions

The age at diagnosis of these 21 patients with colorectal carcinoma harboring kinase fusions ranged from 33–85 years with a median of 64 years. The majority (71%) of this cohort had colorectal carcinoma 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 colorectal carcinoma had poor differentiation or were mucinous in histologic subtype, whereas only 33% of MSS colorectal carcinoma with fusions had poor differentiation or a mucinous component. This data suggest that poor or mucinous differentiation may be associated with the MSI-H status rather than the presence of fusion. American Joint Committee on Cancer 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 2,314 total colorectal carcinomas, 230 were MSI-H/MMR-D and 2,084 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%) versus 106 (5%) *BRAF* V600E mutations ($P < 0.001$), 83 (36%) versus 912 (44%) *KRAS* hotspot mutations ($P = 0.322$), 2 (1%) versus 86 (4%) *NRAS* hotspot mutations ($P = 0.096$), and 12 (5%) versus 9 (0.4%) kinase fusions ($P < 0.001$; Fig. 1). Fifteen percent of MSI-H/MMR-D and 0.9% of MSS/MMR-P colorectal carcinoma 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 colorectal carcinoma with kinase fusions were MMR-D/MSI-H. All MSI-H/MMR-D colorectal carcinoma with available material had MLH1/PMS2 loss ($n = 12$) and *MLH1*ph ($n = 10$). Looking further into the 71 MSI-H colorectal carcinomas that were *RAS*/*BRAF* WT, 47 were MLH1/PMS2 deficient by IHC. Twenty four of 37 of these MLH1/PMS2–deficient colorectal carcinomas 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 colorectal carcinoma with *MLH1*ph and WT *RAS*/*BRAF* was 42% (Fig. 1).

Methylation array results

Because of 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 colorectal carcinoma 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 two predominant subclusters, suggesting CIMP-H and CIMP-L subgroupings. Eight of 11 (73%) fusion driven and 14 of 20 (70%) of *BRAF* V600E colorectal carcinomas localized to the hypermethylated group. All 19 (100%) *KRAS* mutants segregated to the hypomethylated group. Interestingly, 2 MSS colorectal carcinomas (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

Table 2. Clinicopathologic features of patients with colorectal carcinoma 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 ×2 (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

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). Although such targetable fusions are rare in colorectal carcinomas overall, this study shows that approximately 15% of advanced MSI-H/MMR-D colorectal carcinomas, which are WT for *BRAF*/*KRAS*/*NRAS*, harbor kinase fusions and that all of the detected kinase fusions in MSI-H colorectal carcinoma occurred specifically in non-Lynch syndrome cases with MLH1 deficiency associated with *MLH1*ph. Furthermore, fusions were present in almost half of MLH1-deficient colorectal carcinomas 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 and colleagues, who showed that *BRAF* V600E mutations in colorectal carcinoma 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 colorectal carcinomas 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 colorectal carcinoma and resulting relatively small cohort, the fact that none of the MSI-H/MMR-D colorectal carcinoma 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 colorectal carcinoma, specifically with *MLH1*ph. Given the rarity of fusions and the fact that fusion testing is not routinely performed on colorectal carcinoma, it is important to identify subtypes that are more likely to carry these fusions. Thus, testing for kinase fusions is warranted in advanced colorectal carcinoma with *MLH1*ph and WT *BRAF*/*RAS* and the current findings inform an updated proposed molecular testing workflow for colorectal carcinoma (Fig. 2). This proposed updated workflow begins with universal MSI or MMR IHC as recommended by the NCCN (14). Patients with colorectal carcinoma 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 colorectal carcinoma should undergo *MLH1*ph testing as part of the

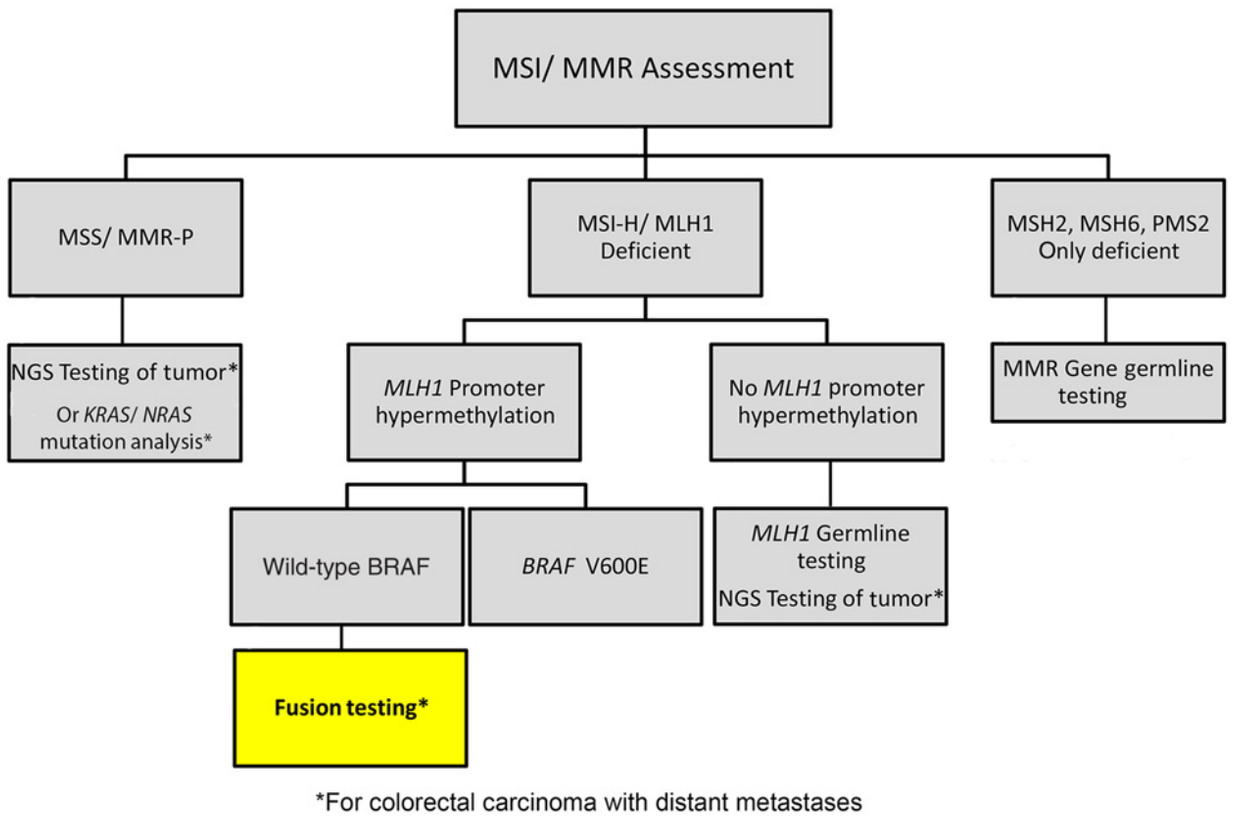

Figure 2.
Workflow for molecular testing in colorectal carcinoma. Testing for MSI/MMR status should be performed universally in colorectal carcinoma. Patients with metastatic MSS/MMR-P colorectal carcinoma should undergo NGS or *RAS*/*BRAF* mutation testing. Patients with MLH1 deficiency of MSI-H results without available MMR IHC should undergo *MLH1* promoter hypermethylation testing. If *MLH1* promoter hypermethylation is detected in metastatic colorectal carcinoma 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.

work-up for Lynch syndrome. If *MLH1*ph is not detected, *MLH1* germline testing to rule out Lynch syndrome may be performed. For patients with colorectal carcinoma with deficiency of MSH2, MSH6, and/or PMS2 but not MLH1, germline testing of the deficient *MMR* gene is recommended because of 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 because of the high likelihood of finding a kinase fusion with potential therapeutic implications.

Immune checkpoint inhibition produces response rates of 20% to 50% of MSI-H colorectal carcinoma (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 colorectal carcinomas overall (0.9%), 57% of kinase fusions in colorectal carcinomas occur in MMR-D/MSI-H colorectal carcinoma. These cases have *MLH1*ph and WT *BRAF*/*RAS*. Almost half of colorectal carcinomas with *MLH1*ph and WT *RAS*/*BRAF* harbor kinase fusions. This subset of advanced colorectal carcinomas may benefit from screening for oncogenic kinase fusions.

Disclosure of Potential Conflicts of Interest

R. Yaeger reports receiving commercial research grant from Array BioPharma, Novartis Pharmaceuticals, and GlaxoSmithKline. M. Scaltriti reports receiving commercial research grant from Puma Biotechnology, Menarini Ricerche, Immunomedics, Daiichi Sankio, Targimmune and has ownership interest (including stock, patents, etc.) in Medendi Medical Travel. He is consultant/advisory board member for Menarini Ricerche, ADC Pharma, and Biocience Institute. A. Drilon reports receiving commercial research grant from Pfizer, GlaxoSmithKline, Teva, and Taiho; is a consultant/advisory board member for Ignyta, Loxo Oncology, BergenBio, Hengrui Therapeutics, Exelixis, Bayer, Tyra Biosciences, TP Therapeutics, AstraZeneca, Pfizer, Blueprint Medicines, Genentech/Roche, Takeda/Ariad/Millenium, Helsinn, and Beigene; and has provided expert testimony for Foundation Medicine, Wolters Kluwer, Merck, Medscape, OncLive, PeerVoice, PER, Targeted Oncology, and RTP. Z.K. Stadler is a consultant/advisory board member for Allergan, Genentech/Roche, Regenxbio, Regeneron, Optos, Adverum, Biomarin, Alimera Sciences, Novartis, Spark, and Fortress. D.M. Hyman reports receiving commercial research grant from Loxo Oncology, Puma Biotechnology, and AstraZeneca and is a consultant/advisory board member for Atara Biotherapeutics, Chugai Pharma, CytomX Therapeutics, Boehringer Ingelheim, AstraZeneca, Pfizer, Bayer, and Genentech. M. Ladanyi reports receiving commercial research grant from Loxo Oncology and Helsinn Therapeutics and is a consultant/advisory board member for Bayer. J.F. Hechtman reports receiving commercial research grant from Bayer, has received Speakers Bureau Honoraria from Medscape and Cor2Ed, and is a consultant/advisory board member for Axiom Biotechnologies. No other potential conflicts of interest were disclosed by the other authors.

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Others (anything pertaining to iScan Methylation sample processing): L. Villafania

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