ORIGINAL RESEARCH

Recommendations for Specimen and Therapy Selection in Colorectal Cancer

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ABSTRACT

Introduction: Next-generation sequencing has emerged as a clinical tool for the identification of actionable mutations to triage advanced colorectal cancer patients for targeted therapies. The literature is conflicted as to whether primaries or their metastases should be selected for sequencing. Some authors suggest that either site may be sequenced, whereas others recommend sequencing the primary, the metastasis, or even both tumors. Here, we address this issue head on with a meta-analysis and provide for the first time a set of sensible recommendations to make this determination.

Methods: From our own series, we include 43 tumors from 13 patients including 14 primaries, 10 regional lymph node metastases, 17 distant metastases, and two anastomotic recurrences sequenced using the 50 gene Ion AmpliSeq cancer NGS panel v2.

Results: Based on our new cohort and a meta-analysis, we found that ∼ 77% of patient-matched primary-metastatic pairs have identical alterations in these 50 cancer-associated genes.

Conclusions: Low tumor cellularity, tumor heterogeneity, clonal evolution, treatment status, sample quality, and/or size of the sequencing panel accounted for a proportion of the differential detection of mutations at primary and metastatic sites. The therapeutic implications of the most frequently discordant alterations (*TP53*, *APC*, *PIK3CA*, and *SMAD4*) are discussed. Our meta-analysis indicates that a subset of patients who fail initial therapy may benefit from sequencing of additional sites to identify new actionable genomic abnormalities not present in the initial analysis. Evidence-based recommendations are proposed.

Keywords: Colorectal cancer; Next-generation sequencing; Paired primary and metastatic tumors; Patient-matched tumors; Specimen selection; Therapy selection; Tumor heterogeneity

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Key Summary Points

Why carry out this study?

Next-generation sequencing (NGS) is becoming the modality of choice for informing therapeutic decisions for patients with advanced-stage disease.

Previous studies sequencing primary and metastatic colorectal cancers have found differing levels of mutational concordance and have reached different conclusions regarding whether primaries or their metastases should be analyzed.

The study asked "what is the preferred sample for NGS, the primary or metastatic site?" Oncologists and pathologists need evidence-based guidelines to help with specimen selection for sequencing, as the decision may have significant impact on therapy, clinical trial enrollment, and possibly patient outcomes.

What was learned from the study?

Our data and meta-analysis show high mutational concordance for actionable mutations between primary and metastatic sites. However, over 20% are discordant and may identify new actionable mutations in resistant patients.

We present evidence-based recommendations that combine clinical considerations and pathologic parameters to help oncologists and pathologists arrive at a sensible approach to specimen selection.

DIGITAL FEATURES

This article is published with digital features, including a summary slide, to facilitate understanding of the article. To view digital features for this article, go to https://doi.org/10.6084/m9.figshare.14340374.

INTRODUCTION

Colorectal carcinoma (CRC) is the third leading cause of cancer and cancer mortality in both men and women in the United States with 149,500 new cases and 52,980 deaths projected in 2021 [1]. Approximately 20–25% of CRC patients have metastatic disease (mCRC) at the time of diagnosis, and the majority of these are incurable [2]. The treatment for mCRC may be resection of the primary CRC with or without metastasectomy followed by adjuvant chemotherapy [3, 4]; however, standard chemotherapy has limited benefit in prolonging survival.

Over the past decade, our improved understanding of CRC biology has resulted in the identification of novel molecular targets with corresponding targeted therapies which improve survival and have fewer side effects [5]. The most important of these is the proto-oncogene epidermal growth factor receptor (EGFR), which is often overexpressed in CRC [6, 7]. Two monoclonal antibodies, cetuximab and panitumumab, which target the extracellular ligand-binding domain of EGFR, are FDA-approved for treating mCRC lacking *KRAS*- and *NRAS*-activating mutations [8]. CRCs that harbor RAS-activating mutations, however, are resistant to anti-EGFR antibodies, and these therapies are contraindicated in this setting. Similarly, CRCs that harbor *BRAF*-activating mutations are typically resistant to anti-EGFR antibodies; however, combining anti-EGFR therapy with the BRAF V600E inhibitor encorafenib has demonstrated efficacy [9]. Given the therapeutic relevance of *KRAS*, *NRAS*, and *BRAF* mutations, testing for all three is recommended in the metastatic setting [3, 4, 10]. Many have found mutations in these genes to be highly concordant in paired primary and metastatic

colorectal cancers [11], leading to the NCCN guideline that either tumor is suitable for genotyping these markers [3, 4]. Conversely, others have found significant discordance in KRAS status between sites and after therapy [12–15].

Looking beyond RAS and BRAF mutations, about 15% of CRCs harbor DNA mismatch repair deficiency (dMMR), which results in microsatellite instability (MSI) [16]. The identification of this subset of CRCs is critical due to its prognostic and therapeutic implications [16]. First, patients with dMMR/MSI tumors tend to have significantly more favorable survival outcomes than those with MMR-proficient/microsatellite stable tumors. Second, dMMR/MSI tumors have poor response to standard CRC therapy with FOLFOX/FOLFIRI regimens but are exquisitely sensitive to immunotherapies targeting immune checkpoint molecules. Indeed, several immunotherapies are now FDA-approved for treating dMMR/MSI CRC [17]. Several studies have demonstrated a high degree of concordance in dMMR/MSI status between patient-matched primary and metastatic CRCs, but site-specific and treatment-induced differences were noted [18–22].

More recently, targeted next-generation sequencing (NGS) has entered the clinical arena as a cost-effective means of identifying additional actionable genetic abnormalities [23, 24]. Thus, for those CRC patients who are refractory to cytotoxic chemotherapy or are not candidates for anti-EGFR antibody therapy or checkpoint inhibitors, NGS may identify additional therapeutic targets beyond RAS, BRAF, and MSI and allow for placement in clinical trials. This raises the question of whether paired primary and metastatic CRCs will be concordant for these additional targets.

One reason to expect otherwise is that tumors evolve over time and after branching, continue to evolve over time independently. Therefore, genetic differences within different areas of a tumor mass (intratumoral heterogeneity) and between paired primary and metastatic tumors (intertumoral heterogeneity) are to be expected. While this phenomenon of genetic heterogeneity of tumors is generally accepted, the qualitative and quantitative

extent of the heterogeneity between primary tumors (PTs) and their paired metastases to either distant organs (DMs) or regional lymph nodes (RLNs) is controversial. Metastases may potentially harbor a distinct spectrum of mutations compared to their PTs and therefore, may respond to a different set of drugs. Genetic discordance creates a challenge for the pathologist who must determine which formalin-fixed paraffin-embedded (FFPE) tissue block to select for NGS, a decision which may profoundly impact downstream therapeutics and ultimately patient outcomes.

Several groups have attempted to address this problem using NGS but have had conflicting results with some finding a high degree of concordance [25–28] and others finding a high degree of discordance [29]. Likewise, these groups have come to greatly differing conclusions regarding whether the decision of specimen selection is relevant at all and, if so, whether one or both sites should be analyzed. Herein, we compare the mutational spectrum of primary and metastatic CRCs using NGS with a 50 cancer-associated gene panel, present the first meta-analysis on the topic of mutational concordance in CRCs, and suggest some general guidelines to help with this dilemma of great clinical importance.

METHODS

Patient and Specimen Selection

Approval with a waiver for consent was obtained from the Cedars-Sinai Medical Center (CSMC) Institutional Review Board. To evaluate mutational concordance between PTs and metastases, CSMC pathology results between 2013 and 2014 were searched for patients with confirmed CRCs based on clinical, morphologic, and/or immunohistochemical features and who had archival tissue from both a PT and DM. Patients who had a history of a second cancer type were excluded to avoid confusion when assessing mutational concordance.

Hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists (J.R.L plus M.C.D., N.F., or S.B.P) to select the optimal FFPE

blocks from the PT and DM, as well as RLN and anastomotic recurrences when present, for NGS. Tumors with scant tissue or extensive necrosis were excluded. Tumors with < 20% tumor content (TC) were sequenced but excluded from pairwise mutational concordance analysis of the patient-matched primary tumor and its metastasis, as low TC is known to yield false negatives. When multiple sections of the tumor were available, the section with the greatest viable tumor cellularity was selected. When indicated, viable tumor on H&E-stained slides was outlined to enrich for TC. Two to four 10-μm-thick unstained tissue sections were prepared on uncharged slides.

DNA Extraction

The entire section or, when applicable, the outlined area was scraped from the unstained slides. Genomic DNA was extracted from the tissue using the QIAcube (9001882; Qiagen, Hilden, Germany) according to manufacturer's protocol. The purified DNA was subsequently quantitated using the Qubit 2.0 Fluorometer (Q32866; Thermo Fisher Scientific, Carlsbad, CA).

Next-Generation Sequencing

NGS was performed on the Ion Torrent Personal Genome Machine (4462921; Thermo Fisher Scientific) using the Ion AmpliSeq Cancer Hotspot Panel version 2 (4475346; Thermo Fisher Scientific) according to manufacturer's protocol. The 50 cancer-associated genes evaluated by this panel are listed in Table 1. The specific regions of the genes interrogated by the panel are available from the manufacturer. Briefly, a library of 207 amplicons was generated in a multiplex PCR using 10 ng of purified genomic DNA. Unique Ion Xpress barcodes (4474517; Thermo Fisher Scientific) were ligated to each sample. Barcoded libraries were purified, equalized, and combined for templating on ion sphere particles (4474517; Thermo Fisher Scientific). Templated ion sphere particles were enriched, loaded onto the 314 chip, and sequenced on the Personal Genome Machine.

Table 1 Fifty genes interrogated by the AmpliSeq Cancer Hotspot Panel v2

ABL1	AKT1	ALK	**APC**	**ATM**	**BRAF**	CDH1
CDKN2A	CSF1R	**CTNNB1**	EGFR	ERBB2	ERBB4	EZH2
FBXW7	FGFR1	FGFR2	FGFR3	FLT3	GNA11	GNAQ
GNAS	HNF1A	HRAS	IDH1	IDH2	JAK2	JAK3
KDR	KIT	**KRAS**	MET	**MLH1**	MPL	NOTCH1
NPM1	**NRAS**	PDGFRA	**PIK3CA**	PTEN	PTPN11	RB1
RET	**SMAD4**	SMARCB1	SMO	SRC	STK11	**TP53**
VHL						

The panel includes tumor suppressor genes and oncogenes recurrently mutated in cancer. In bold are the recurrently mutated genes in colorectal cancer. APC (81%), TP53 (60%), KRAS (43%), PIK3CA (18%), FBXW7 (11%), SMAD4 (10%), NRAS (9%), and CTNNB1 (5%) were the most highly mutated genes in non-hypermutated colorectal cancers, whereas APC (51%), BRAF (46%), FBXW7 (46%) ATM (40%), PIK3CA (34%), KRAS (31%), and MLH1 (14%) were among the most commonly mutated genes in hypermutated colorectal cancers (The Cancer Genome Atlas database [30]).

Bioinformatics Pipeline

Signal processing, base calling, and alignment were performed using Torrent Suite software version 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). To pass quality control requirements, samples must have > 250X coverage for at least 203 of the 207 amplicons on the panel. Variant calling was performed using NextGENe version 2.3.3 (SoftGenetics, State College, PA, USA) and Torrent Suite version 2.0. Manual review of alignments was performed using the NextGENe version 2.3.3 viewer (SoftGenetics). Only non-synonymous variants with quality scores > 25, coverage > 250 reads, similar forward/reverse ratios, and variant allele frequencies (VAFs) > 5% were evaluated. Calls in homopolymer regions longer than 3nt were excluded. Polymorphic variants identified in the 1000 Genomes database were excluded if the variant was present in > 10% of the population, which makes it highly unlikely to be pathogenic, or if literature supported it being benign.

Meta-Analysis

PubMed was interrogated with the following search criteria: (colon OR rectal OR colorectal) AND (cancer OR neoplasm OR tumor) AND (primary AND metastatic) AND ("next generation sequencing" OR "next-generation sequencing" OR "massively parallel sequencing" OR "targeted sequencing" OR "exome sequencing" OR "genome sequencing" OR "molecular profiling") AND (comparison OR paired OR pairwise OR "patient-matched" OR matched OR "primary-metastatic" OR concordance OR discordance). Search results were individually reviewed. Studies with insufficient methods for teasing out CRCs from other tumors or insufficient NGS panel information were excluded. Five studies that compared results of NGS in primary and metastatic CRC were included. Factors that potentially influence concordance rates, including number of genes on the panel, tumor purity cutoffs, mean coverage, and VAF cutoffs, were also extracted. The total number of primary-metastatic pairs and concordant pairs were extracted from each publication. The

Table 2 Clinical and specimen summary

Clinical	
Patients, *n*	13
Age, mean ± SD (years)	60 ± 18
Sex, male/female	8:5
Pathologic stage at diagnosis, *n*	
I	1
II	3
III	1
IV	8
Histologic differentiation, *n*	
Well–moderate	2
Moderate	6
Moderate–poor	3
Treated, N/A	2
Chemo/radiotherapy, *n*	
Neoadjuvant	3
Adjuvant	10/10*
Specimens	
Primary site, *n*	
Cecum	1
Ascending colon	2
Transverse colon	2
Descending colon	1
Sigmoid colon	2
Rectosigmoid	3
Rectum	3
Total	14[#]
Distant metastatic site, *n*	
Liver	8
Lung	2
Distant lymph node	3
Peritoneum	2
Small bowel	1
Presacral soft tissue	1

Table 2 continued

Clinical	
Total	17
Regional lymph node metastases, *n*	10
Anastomotic recurrences, *n*	2
Surgery	
Biopsy	3
Resection	31

*Three patients could not be verified as having received adjuvant therapy, so denominators were adjusted accordingly
One patient had two primaries

number of concordant pairs were redetermined after considering only the 50 genes on the Ion AmpliSeq Cancer Hotspot Panel version 2 to allow for a fair comparison across all studies.

Statistical Analysis

The two-tailed Fisher's exact test with α = 0.05 was used to compare proportions. For multiple pairwise comparisons, a Bonferroni correction was applied to control the type I error rate. Statistical analyses were performed by S.B.P., N.D, and Z.L. The meta (Version 4.15–1) and metafor (Version 2.4–0) packages for R programming language (Version 4.0.2) were used for the meta-analysis and for generating diagnostic plots and forest plots. To identify outlier studies, leave-one-out analysis and Baujat plots were used to evaluate for disproportionate influence of any given study. No
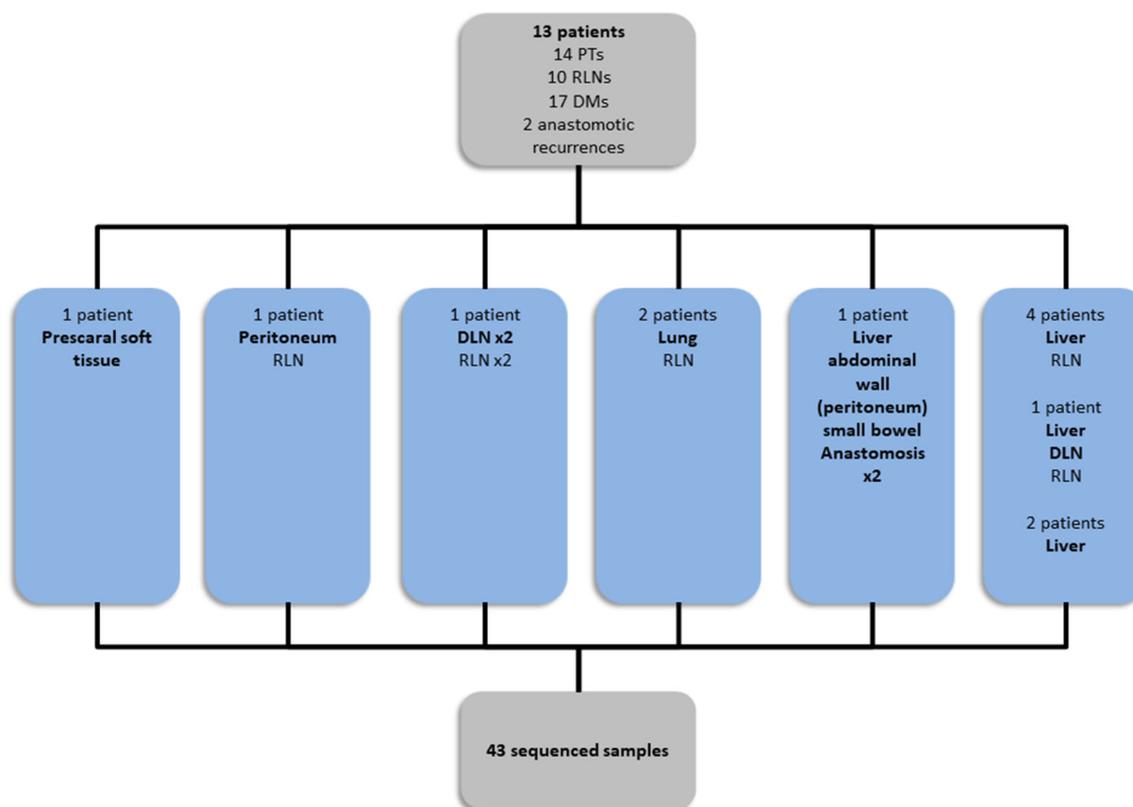
Fig. 1 Distribution of samples. *PTs* primary tumors, *RLNs* regional lymph node metastases, *DLNs* distant lymph node metastases, *DMs* distant organ metastases. Note that one patient had two synchronous primaries

transformations were applied to the data, a random effects model was selected for calculating effect sizes, and the DerSimonian–Laird method was used to estimate τ.

RESULTS

Clinical and Pathologic Characteristics

To compare mutational differences between primary and metastatic CRCs, 13 patients with available clinical data and paired tumors were included in the study (Table 2, Supplemental Table S1). Eight had pathologic stage IV disease at diagnosis (synchronous), and five developed metastases subsequently (metachronous). The time interval from colectomy to first metastasectomy averaged 1.7 years (0.5–4.8 years). Forty-three tumors were sequenced, including 14 PTs, 10 RLNs, 17 DMs, and two anastomotic recurrences (Fig. 1, Table 2). One patient had two synchronous PTs, three had no RLNs (pN0), and one had only a microscopic RLN, which was not suitable for NGS.

None had clinical evidence of other malignancies, colorectal polyposis, or Lynch syndrome/hereditary non-polyposis colorectal cancer. Two patients had PTs with immunohistochemical evidence of loss of mismatch repair protein expression (Supplemental Table S1). Both tumors were right-sided and had morphologic features of microsatellite instable tumors. One had a BRAF V600E mutation (patient 10), which effectively excludes Lynch syndrome. While the other (patient 6) did not have a BRAF mutation, only about half of sporadic microsatellite instable tumors harbor these [23].

Two tumors (PT in patient 8 and liver DM in patient 4) had TC less than 20%, our clinical laboratory-established threshold for adequacy. While these samples were sequenced for completeness, we excluded them from concordance calculations.

Table 3 Mutation rates and discordance rates in primary-metastatic tumor pairs

	PT: DM ($n = 12$ pairs[#])	PT: RLN ($n = 9$ pairs)
Total mutations*, n: n	21: 17	16: 14
Mutation rate, mutations/tumor	1.8: 1.4	1.8: 1.6
Mutations		
Concordant, n	17 (83%)	14 (82%)
Discordant, n	4 (17%)	3 (18%)
Tumor pairs		
Concordant, n	10 (83%)	7 (80%)
Overlapping, n	1 (8%)	1 (10%)
Discordant, n	1 (8%)	1 (10%)
Number of mutations, n: n: n	Both: PT only: DM only	Both: PT only: RLN only
KRAS	2: 0: 0	2: 0: 0
NRAS	0: **1**: 0	–
BRAF	1: 0: 0	1: 0: 0
TP53	7: **1**: 0	5: **1**: 0
APC	3: **1**: 0	3: 0: 0
PIK3CA	1: **1**: 0	1: **1**: 1
ATM	1: 0: 0	1: 0: 0
PTEN	1: 0: 0	1: 0: 0
FBXW7	1: 0: 0	1: 0: 0

PTs primary tumors, *DMs* distant metastases, *RLNs* regional lymph node metastases

[#] For patients 4 and 8, the liver metastasis and PT, respectively, were excluded from analysis. In a clinical setting, these tumors which had inadequate tumor cellularity (< 20%) would not be sequenced when another sample with adequate tumor cellularity was concurrently available

*To avoid falsely inflated contribution from patients with multiple metastases, they were counted as a single metastasis
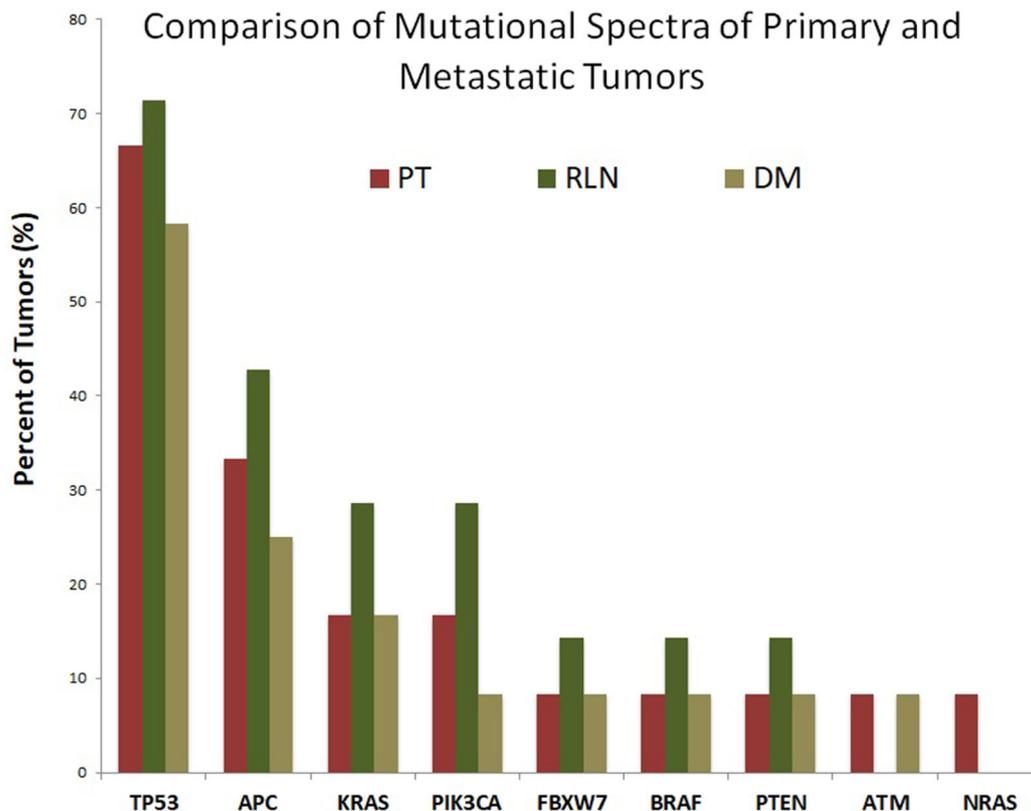
Fig. 2 Prevalence of mutations across primary and metastatic tumors. *PTs* primary tumors, RLNs = regional lymph node metastases, *DLNs* distant lymph node metastases, *DMs* distant organ metastases

Gross Comparison of Mutational Burden and Spectra of Primaries and Metastases

To obtain a broad view of whether there are dramatic differences between primary-metastatic pairs, we compared the overall and gene-specific mutational burden in PTs, RLNs, and DMs. Interestingly, metastatic tumors had mutational burdens that were generally similar to PTs (Table 3), and the spectrum of genes mutated and their prevalence were also comparable (Fig. 2). In descending order, *TP53*, *APC*, and *KRAS* were the three most frequently mutated genes, which is somewhat different from the relative frequencies in the Cancer Genome Atlas database [30], possibly related to more advanced disease in our cohort (Table 1).

Pairwise Genetic Comparison of Primaries and Metastases

Next, we address the crux of this study, the extent to which metastases differ genetically from the primary from which they originated. In our cohort, metastases generally had similar mutations as their PTs (Tables 3, 4). Patient 8 was excluded from pairwise comparison between PT and DM due to low TC (15%) of its PT. The liver DM from patient 4 had a low TC (10%) and therefore, this site was excluded from analysis. However, the patient was included in the concordance analysis, as he had multiple other DMs with adequate TC. Mutational spectra in PT-DM pairs were identical in 10 of 12 (83%) patients but only partially overlapping in patient 5 (8%) and entirely discordant in patient 6 (8%). Among the PT-DM pairs, a total of 21 mutations were detected, 17 (83%) of

Table 4 Mutations in primary, metastatic, and recurrent colorectal cancer

Patient	Primary tumor	VAF (%)	Regional lymph node	VAF (%)	Distant metastasis/recurrence	VAF (%)
1	Rectosigmoid				Lung	
	PIK3CA c.1616C > G	28	PIK3CA c.1616C > G	13	PIK3CA c.1616C > G	20
	TP53 c.734G > A	43	TP53 c.734G > A	39	TP53 c.734G > A	27
2	Rectosigmoid				Lung	
	APC c.4037C > A	31	APC c.4037C > A	18	APC c.4037C > A	21
3	Rectum		RLN1		Inguinal LN1	
	APC c.4033_4034del	15	APC c.4033_4034del	29	APC c.4033_4034del	39
	TP53 c.743G > A	20	TP53 c.743G > A	28	TP53 c.743G > A	33
	KRAS c.35G > A	21	KRAS c.35G > A	34	KRAS c.35G > A	15
	FBXW7 c.1745C > T	16	FBXW7 c.1745C > T	28	FBXW7 c.1745C > T	39
			RLN2		Inguinal LN2	
			APC c.4033_4034del	52	APC c.4033_4034del	39
			TP53 c.743G > A	25	TP53 c.743G > A	41
			KRAS c.35G > A	21	KRAS c.35G > A	42
			FBXW7 c.1745C > T	38	FBXW7 c.1745C > T	40
4	Distal transverse		No lymph node metastases		Liver **10%**[#]	None detected
	ATM c.1009C > T	33			Abdominal wall	
	TP53 c.818G > A	52			ATM c.1009C > T	34
					TP53 c.818G > A	41
					Small bowel	
					ATM c.1009C > T	34
					TP53 c.818G > A	41
					Recurrence 1	
					ATM c.1009C > T	48
					TP53 c.818G > A	57
					Recurrence 2	
					ATM c.1009C > T	23
					TP53 c.818G > A	23

Table 4 continued

Patient	Primary tumor	VAF (%)	Regional lymph node	VAF (%)	Distant metastasis/recurrence	VAF (%)
5	Distal descending 40%[#]		No lymph node metastases		Presacral soft tissue 80%[#]	
	TP53 c.404G > T	23			TP53 c.404G > T	21
	APC c.3916G > T	**13**				
	NRAS c.181C > A	**6**				
6	Proximal transverse 80%[#]		None detected 40%[#]		Peritoneum 30%[#]	None detected
	PIK3CA c.322C > T	15				
	TP53 c.844C > T	13				
7	Ascending		KRAS c.38G > A	12	Pyloric LN	
	KRAS c.38G > A	36	TP53 c.707A > G	37	KRAS c.38G > A	30
	TP53 c.707A > G	45			TP53 c.707A > G	40
					Liver	
					KRAS c.38G > A	64
					TP53 c.707A > G	74
8	Rectosigmoid **15%**[#]		Micrometastases		Liver 70%[#]	
	KRAS c.35G > T	8			KRAS c.35G > T	25
	TP53 c.404G > T	9			TP53 c.404G > T	19
					PIK3CA c.1633G > A	11
					APC c.2626C > T	16
9	Rectum		TP53 c.1024C > T	5	Liver	
	TP53 c.1024C > T	60			TP53 c.1024C > T	46
10	Cecum		BRAF c.1799 T > A	11	Liver	
	BRAF c.1799 T > A	10			BRAF c.1799 T > A	20
11	Ascending 80%[#]		PTEN c.737C > T	13	Liver 90%[#]	
	PTEN c.737C > T	9	APC c.3871C > T	43	PTEN c.737C > T	22
	APC c.3871C > T	45	**PIK3CA c.1633G > A**	84	APC c.3871C > T	49

Table 4 continued

Patient	Primary tumor	VAF (%)	Regional lymph node	VAF (%)	Distant metastasis/recurrence	VAF (%)
12	Sigmoid TP53 c.262_272del	25	TP53 c.262_272del	51	Liver TP53 c.262_272del	45
	APC c.3340C > T	29				
	Rectum TP53 c.512A > G	21				
	SMAD4	30				
	c.1082G > A					
13*	Distal sigmoid 80%# None detected		No lymph node metastases		Liver None detected	

VAF variant allele frequency

*Regional lymph node and liver metastases have identical mutations as the sigmoid primary and completely different mutations as the rectal primary, indicating that they originated from the sigmoid tumor

Histologically estimated tumor content is indicated for tumors pairs with discordant mutations, if H&E was retrievable

which were shared. Importantly, this pattern held true for PT-RLN pairs as well.

Since testing for activating mutations in *KRAS*, *NRAS*, and *BRAF* is recommended to predict response to FDA-approved therapies, we specifically evaluated concordance for mutations in this gene trio. For 12 of 13 (92%) patients, their mutational status was concordant, and the choice of sample for biomarker testing would not have impacted eligibility for FDA-approved therapies (Tables 3, 4). *KRAS* and *BRAF* mutations were detected in three and one patients, respectively, and were completely concordant. However, patient 5 had an *NRAS* mutation, which was detected only in the PT. Therefore, while there is generally a high degree of concordance between primary and metastatic tumor pairs for mutations in the gene trio, important actionable differences may occasionally exist.

Because NGS is also used to identify biomarkers for eligibility into clinical trials, we also examined discordances in other genes in the 50-gene panel (Table 3). Three of 12 (25%) patients had at least one mutation detected in the PT but not in the DM (patients 5 and 6) or, conversely, in the RLN but not in the PT or DM (patient 11; Tables 3, 4). *APC* mutations which are considered early events in colorectal tumorigenesis were detected in four patients, and *TP53* mutations which are considered late events were detected in eight patients. One patient was discordant for an *APC* mutation and another for a *TP53* mutation. *PIK3CA* mutations were detected in three patients, and two had discordances.

One unique feature of our study is that we analyzed multiple metastatic sites, when available and suitable for sequencing, to address spatial intertumoral heterogeneity. Eleven of 13 patients had at least two metastatic and/or recurrent tumors sequenced, including patients 3, 4, and 7 who had three to five tumors. We found that mutational concordance was maintained spatially across multiple RLNs and DMs and temporally across synchronous and metachronous tumors (Table 4, Supplemental Table S1).

	Current Study	Goswami et al (2015)	Crumley et al (2015)	Brannon et al (2014)	Kim et al (2015)	Lee et al (2014)
Patients, n	12	86	16	69	18	15
Panel size	50 genes	46 genes	50 genes	230 genes/WGS	1321 genes	WES
Samples	Any	Any	Only Rectal PTs	Only MSS	DM:DM pairs, included	Only MSS PTs & Synchronous liver DM
Treatment	Any	Any	Any	Any	Any	Naïve
Sequencing	Ion Torrent	Ion Torrent	Ion Torrent	Illumina	Illumina	Illumina
TC QC	20%	20%	5%	50%	80%	70%
VAF QC	5%	5%	4.5%	5%	10%	N/A
Coverage QC	250x	250x	100x	692x, avg	144x, avg	52x, avg
Conclusion	Either initially	Both	Either initially	Either	Primary initially, if chemonaive	Evaluate metastasis

Fig. 3 Mutational concordance between tumor pairs across six studies. **a** Studies examining genetic differences in primary and metastatic CRCs were compared for variables that potentially explain differences in their results. Brannon et al. also performed WGS on four of their 69 patients. Kim et al. had one patient with two primary-metastatic pairs and two patients with only metastatic-metastatic pairs, all of which were included in the analysis below. **b** Most studies find that patient-matched tumor pairs are highly concordant for mutations in common cancer genes. Numbers in bars represent the number of pairs in the respective category. *WGS* whole genome sequencing, *WES* whole exome sequencing, *Kim et al. and Lee et al. studies reanalyzed after including only 50 genes interrogated in the current study. Compared studies are boxed in

Technical, Biological, and Clinical Factors Influence Mutational Discordance

To explore potential causes of discordance, we first confirmed that each tumor in a pair was in fact derived from the same patient. All samples from the same patient but not samples from other patients in our cohort had an identical set of putative germline variants, thus excluding the possibility of a sample mix-up contributing to discordance (Supplement Figure S1). Secondly, all of our samples exceeded the laboratory-established minimum requirements to pass quality control, excluding poor sequencing quality as a significant cause of discordance. Even though the read depth cutoff was 250X, the average across samples was much higher at

2700X, and the sample with the lowest coverage had a mean of 1700X coverage per amplicon. All of our reported variants also passed all quality metrics, and all were confirmed by Sanger sequencing or other method.

Next, we sought to determine whether sample inadequacy may have contributed to any of the discordances. Therefore, we revisited the H&E slides, when possible, from our discordant cases to re-evaluate the TC (Table 4). The liver metastasis in patient 4 had a TC of 10%, which is below our laboratory's established QC limit of 20% TC, and likely accounts for the absence of *ATM* and *TP53* mutations in liver metastasis. The possibility of two driver mutations being lost due to clonal evolution in the DM is highly

unlikely, especially when three other metastases and both recurrences had the mutations.

In some instances, true biological factors may account for discordances. In patient 11, for instance, the spatial intertumoral heterogeneity for the *PIK3CA* mutation may reflect branched evolution with the emergence of a novel variant in the RLN that is subsequently amplified. An *APC* mutation is present in the PT, RLN, and DM at a VAF of $\sim 45\%$ in all three samples, suggesting it is a clonal heterozygous variant. The *PIK3CA* variant, however, was present at an even higher VAF of 84%, suggesting that the mutant allele is amplified (loss of the wild-type allele is also explanatory but is less likely for oncogenes). The presence of a very minor subclone harboring the *PIK3CA* mutation in the PT that subsequently expanded in the RLN remains a formal possibility.

Intratumoral heterogeneity of the PT may also account for discordance between primaries and metastases. In patient 5, the low VAF of the *APC* (13%) and *NRAS* (6%) mutations relative to the adequate TC (40%) are suggestive of their presence in a small subclone within the PT. Regardless of whether this subclone arose before or after the metastatic event, it clearly did not contribute to the DM (80% TC). A similar situation is found in patient 6 with low VAF for *PIK3CA* (15%) and *TP53* (13%) relative to high TC (80%) in the PT. However, these mutations were not detected in either the RLN (40% TC) or DM (30% TC), suggesting that these small clones did not contribute to the metastases.

The cause of the discordance may be multifactorial and may be elusive. In patient 8, there are at least two possible causes for the absence of the *PIK3CA* and *APC* mutations in the PT. First, the low TC (15%) of the PT suggests that these mutations may have been missed. Alternatively, spatial intertumoral heterogeneity may be explanatory. Since both mutations are subclonal (VAF of 11% for *PIK3CA* and 16% for *APC*) in the DM (70% TC), they may be emerging mutations entirely novel to the DM. Enrichment of tumor cells in the PT with laser capture micro-dissection would prove useful in teasing out these possibilities.

Finally, clinical factors are paramount when considering discordance between primaries and metastases. For patient 12, we initially only analyzed the rectal primary which is clearly genetically different from the RLN and DM. This apparent discordance was not resolved until careful review of the patient's records revealed a history of a second primary in the sigmoid colon. Sequencing the latter tumor revealed a *TP53* mutation identical to the one found in the RLN and DM. In a clinical setting, excluding metastasis from a second primary, including a primary from another organ or cryptic primary, is critical.

Meta-Analysis

A total of five studies were included in the final meta-analysis. From each study, the total number of patients, the size of the NGS panel, sequencing parameters, and final conclusion were extracted (Fig. 3).

DISCUSSION

Pathologists and oncologists are often faced with difficult decisions of which sample to select and whether to collect a new sample for biomarker testing. These decisions can potentially influence downstream clinical management and patient outcomes. The NCCN guidelines state that either the PT or the DM may be tested for RAS and *BRAF* mutations due to the high concordance rate. Consistently, we find that *KRAS* and *BRAF* mutations were always concordant in our study. One patient, however, had discordant *NRAS* status, indicating that important potentially actionable differences may be discoverable by sequencing the other paired tumor.

The current NCCN guidelines make no specific recommendations for NGS, yet it has become common practice to comprehensively profile tumors using NGS. With a 50 cancer-related gene panel, we demonstrated that the majority of patient-matched primary and metastatic CRCs show a high degree of concordance. PT and metastatic tumors were concordant in 83%, partially overlapping in 8%, and entirely discordant in another 8% of patients. Our findings are consistent with those reported

by Crumley et al. [26] and Goswami et al. [25] who used identical and similar NGS panels, respectively, and with Kim et al. [27] and Brannon et al. [28] after we re-evaluated their data by considering only the 50 genes on our panel (Fig. 3, Supplemental Figure S2). The proportion of patients whose tumor pairs had identical (64–85%), overlapping (8–36%), or completely discordant (0–11%) mutations among the studies was variable, although tending towards concordance. Variability between these studies may be attributable to sample selection criteria, sequencing methodology (including gene panel size), and bioinformatic pipelines (Fig. 3). In contrast, Lee et al. [29] found a markedly high degree of discordance, even after we re-evaluated their data by considering only the 50 genes, compared to the above studies, and was a clear outlier for reasons that are unclear (Supplemental Figure S2). Based on all other studies, about 77% [68–86%] (95% CI) of all tumor pairs are expected to have identical mutations when analyzed on a panel of about 50 common cancer genes (Supplemental Figure S3). In our study, we excluded from comparison samples with low TC, consistent with other studies, due to potential for false negatives. Expectedly, inclusion of these samples would have significantly increased the number of partially overlapping and entirely discordant PT-DM pairs from 8% each to 15% each. It confirms the importance of ensuring adequate sampling and TC in any sequenced sample to draw reliable results to genomically guide therapy. Based on our meta-analysis, we propose a set of sensible guidelines to aid pathologists and oncologists in specimen selection:

Clinical Recommendations

Situation 1: Only one sample is available and there is no plan for a second collection.

Recommendation: Attempt analyzing the available sample.

Considerations: While it is understood that any inadequate/low-quality samples carry the risk of false negatives, the cost and risk to the patient of resampling generally outweigh that of testing the available sample. Therefore, testing on the available sample should be attempted unless there is a strong reason to defer. If applicable, the final report should indicate that the sample was insufficient (low nucleic acid yield, noisy sequencing results, etc.) and suggest that a second collection be performed if clinically warranted.

Situation 2: Multiple samples are available contemporaneously at the time sequencing is ordered, even if they were collected extemporaneously.

Recommendation: Decide based on clinical indication and sample adequacy. Simultaneous sequencing of more than one sample is not generally recommended due to low yield and cost and resource considerations.

Considerations: Clinical factors should dominate decision making.

Initial therapy: Any sample may be considered for testing the primary resistance markers, KRAS, NRAS, and BRAF, because of high concordance. Therefore, specimen selection should be based on the possibility that the tumor may be negative for these markers. In this setting, specimen adequacy and/or clinical considerations should be the primary determinant for specimen selection. Whether metastasis is synchronous or metachronous, the time interval between the diagnosis of the primary and metastatic tumors, whether there was neoadjuvant therapy, whether the primary or metastasis was resected or simply biopsied, and whether the resection was complete or incomplete are potential considerations. However, the impact of these factors on sample selection remains to be elucidated on a case-by-case basis.

Acquired resistance: The resistant focus/tumor should be prioritized. If a sample is suboptimal due to factors related to sample processing (heavy-metal fixatives, harsh decalcifying agents, extended cold ischemic times, extended fixation times) or due to the sample itself (age of sample, cellularity, tumor purity), attempt testing and report if sample passed quality controls.

Reflex testing of an alternate sample, however, may be considered if the initial analysis failed quality control, an actionable marker was not detected, or a resistant focus develops subsequently.

Situation 3: Only one sample is available, but a second collection is planned before therapy initiation.

Recommendation: Wait for pathology to review both samples.

Considerations: Unless immediate testing is clinically indicated, waiting on the availability of both samples for review is advised. Reflex to situation 2.

A small sample size reflects the most important limitation of our work. However, as we incorporate our data into a meta-analysis, we are able to provide a robust estimate of the genetic similarity of patient-matched tumors. Furthermore, our comprehensive analysis of every patient, including multiple metastases and recurrences, further substantiates our conclusion that primaries and metastases are generally genetically similar. While our study only focused on 50 genes, we do not consider this to be a limitation of significant importance given that the objective of our study was to determine differences in actionable gene mutations in primary and metastatic CRCs. At this time, the number of therapeutically actionable genes is small, and the vast majority are reflected in the 50-gene panel, and those that are not, such as NTRK, are exceptionally rare in CRC. While this study was conducted at a single institution, we do not believe this to be a major shortcoming to the generalizability of our study due to the diversity of our patient population.

Bridging our technical findings to the clinical arena, the advent of deep NGS profiling with large gene panels has illuminated previously unknown cancer complexity such as intratumoral heterogeneity, heterogeneity between primaries and metastases, tumor evolution over time and with treatment, and genetic heterogeneity across tumor types. The ultimate arbiter of significance of these testing activities is impact on care and outcomes. This study's use of a panel of genes frequently altered in solid tumors suggests that these discordances may be of clinical significance, especially when the RLN and/or DM sites display entirely discordant actionable mutations compared to their PT.

The clinical utility of KRAS, NRAS, and BRAF is certainly clear. In our meta-analysis, there were only rare RAS discordances and no BRAF discordances. However, rare differences in the mutational status of these genes could have a significant impact on treatment. For instance, patient 5 had an NRAS mutation in the PT but not in the DM and, therefore, would have potentially qualified for anti-EGFR therapy. Among significantly altered CRC genes, TP53, APC, PIK3CA, and SMAD4 were the most frequently discordant in our meta-analysis (Supplemental Table S2). Always a leader in frequency, mutated TP53 is a target of extremely high unmet need without well-established therapeutic options, but some inroads are in progress, including a phase 2 study of the antiviral agent lamivudine in patients with p53-mutant mCRC (NCT03144804).

PIK3CA and the PI3K/AKT/MTOR pathway are also emerging therapeutic targets; there are already many FDA-approved indications for some tumor types and promising late-phase clinical trial therapies in advanced and/or refractory cancer patients. At the time of this writing, three trials evaluating drugs that target activating PIK3CA mutations in mCRC were in progress (NCT03006172, NCT02465060, NCT03711058). In our study, sequencing of the metastatic sites in patients 8 and 11 revealed a PIK3CA mutation not present in their PT. These PIK3CA mutations would have likely qualified them for enrollment, which would not have been possible if only the PT had been sequenced. Conversely, the lack of a detectable PIK3CA variant in either metastasis in patient 6, although detected in the PT, would have potentially excluded this patient. Currently, there are no trials targeting APC or SMAD4. However, Zhang et al. [31] described a preclinical small molecule selective for killing cells carrying common truncating mutations of this gene (as are all APC variants shown in Table 4). Inactivation of APC results in the dysregulation of the Wnt signaling through beta-catenin, and several Wnt pathway

inhibitors are in clinical trials for solid tumors [32]. In addition, beta-catenin activation confers synthetic lethality when tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is upregulated by agonists, which are also currently in clinical trials in some solid tumor types [33].

Finally, as it is an area of intense activity, we briefly discuss the utility of liquid biopsies in addressing the issue of sample selection. The analysis of liquid biopsies may provide a comprehensive view of the genomic landscape of multifocal disease [34]. Liquid biopsies include circulating tumor cells, exosomes, and circulating tumor DNA (ctDNA) and RNA. Of these, ctDNA has achieved the greatest advancement to the clinical arena [35]. The analysis of ctDNA offers several advantages over the analysis of tissue. One that is directly relevant to this manuscript pertains to its potential to capture intra- and intertumoral genetic heterogeneity. Because ctDNA reflects the released DNA from tumor cells throughout a given tumor mass (intratumoral heterogeneity) and across multiple tumor masses (spatial intertumoral heterogeneity) in a patient, its analysis in theory will reflect the total genetic composition of all tumor masses, which is in contrast to analysis of tissue [36, 37]. While in our manuscript, we analyzed up to six tumor masses from a single patient, this is not feasible on a routine clinical basis. With ctDNA, such analyses of multiple sites may not be necessary. However, ctDNA has important drawbacks that has limited its use as a biospecimen, including higher failure rates due to insufficient quantity of ctDNA for analysis and limited analytical sensitivity. At this time, there are no FDA-approved ctDNA-based companion diagnostic tests for CRCs. In lung, breast, ovarian, and prostate cancer, however, FDA approval has been granted for select well-established biomarkers, but negative test results must be followed up with tissue-based tests due to the aforementioned limitations of ctDNA-based testing [35].

CONCLUSIONS

In summary, our own findings and our meta-analysis support that the initial analysis can be made on either the primary or metastatic tumor depending on availability, as there is a high degree of mutational concordance and because the occasional novel mutation may not always be actionable. Therefore, unless a biopsy of the mCRC is deemed necessary in the setting of resistance, the majority of patients will not need to be re-biopsied exclusively for identifying actionable novel mutations. We also showed that mutations are similar across various metastatic sites, including regional lymph nodes, and, therefore, the latter may be suitable alternative specimens for sequencing. However, we demonstrated that systematic evaluation of tissue and sequencing QC metrics, especially TC and VAF, is paramount, as these are common causes of discordance. Therefore, if the initial analysis failed QC, additional samples, if and when available, should be considered for sequencing. For about a quarter of patients, additional mutations may be detected due to clonal evolution or tumor heterogeneity by sequencing additional samples, and for a smaller subset of these patients, findings may offer new opportunities to enroll in clinical trials.

Authors' Contributions. All authors provided review of the manuscript. S.B.P. and J.L. wrote the manuscript. S.B.P., R.B., N.F., M.C-D., and J.R.L. performed pathologic review of samples, analyzed genomic results, participated in experimental design, and provided various aspects of the manuscript text or figures. J.C.H. analyzed genomic results, helped with experimental design, and created tables. A.A., C.R., and A.P. performed wet bench analysis of samples. S.B.P., Z.L., and N.D. performed the statistical analysis.

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Data Availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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