Comparative Genomic Analysis of Primary Versus Metastatic Colorectal Carcinomas

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See accompanying editorial on page 2937

ABSTRACT

Purpose
To compare the mutational and copy number profiles of primary and metastatic colorectal carcinomas (CRCs) using both unpaired and paired samples derived from primary and metastatic disease sites.

Patients and Methods
We performed a multiplatform genomic analysis of 736 fresh frozen CRC tumors from 613 patients. The cohort included 84 patients in whom tumor tissue from both primary and metastatic sites was available and 31 patients with pairs of metastases. Tumors were analyzed for mutations in the KRAS, NRAS, BRAF, PIK3CA, and TP53 genes, with discordant results between paired samples further investigated by analyzing formalin-fixed, paraffin-embedded tissue and/or by 454 sequencing. Copy number aberrations in primary tumors and matched metastases were analyzed by comparative genomic hybridization (CGH).

Results
TP53 mutations were more frequent in metastatic versus primary tumors (53.1% vs 30.3%, respectively; \( P < .001 \)), whereas BRAF mutations were significantly less frequent (1.9% vs 7.7%, respectively; \( P = .01 \)). The mutational status of the matched pairs was highly concordant (>90% concordance for all five genes). Clonality analysis of array CGH data suggested that multiple CRC primary tumors or treatment-associated effects were likely etiologies for mutational and/or copy number profile differences between primary tumors and metastases.

Conclusion
For determining RAS, BRAF, and PIK3CA mutational status, genotyping of the primary CRC is sufficient for most patients. Biopsy of a metastatic site should be considered in patients with a history of multiple primary carcinomas and in the case of TP53 for patients who have undergone interval treatment with radiation or cytotoxic chemotherapies.

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INTRODUCTION

Genetic testing of patients with advanced colorectal carcinoma (CRC) for somatic mutations in KRAS has become routine clinical practice,\(^1\)\(^-\)\(^5\) and epidermal growth factor receptor inhibitors are now recommended only for use in patients with CRC whose tumors are KRAS wild type.\(^6\) There is also emerging evidence that mutations in BRAF and PIK3CA are associated with resistance to epidermal growth factor receptor–targeted agents.\(^7\)\(^-\)\(^13\) Finally, it has been suggested that inactivation of the TP53 gene, which is observed in 40% to 50% of CRCs, may influence response to therapy,\(^14\)\(^,\)\(^15\) although this requires validation in prospective clinical studies. Despite the routine use of KRAS mutational status to guide treatment selection, questions remain as to the optimal tissue source for genomic testing.

In this study, we performed a multiplatform genomic analysis of clinically relevant biologic events in a large cohort of primary and metastatic CRC tumors. We found the mutational concordance for the KRAS, NRAS, BRAF, PIK3CA, and TP53 genes between primary and metastatic disease to be high. Discordant results, when identified, were associated with multiple CRC primary tumors and, in the case of TP53, with tissue sampling and interval treatment.

PATIENTS AND METHODS

Tumor Specimens
In all cases, tissue and clinical data were collected on patients under an institutional review board–approved...
protocol or waiver of authorization. For each tumor, a hematoxylin and eosin–stained section was reviewed by a GI pathologist (E.V. or J.S.). When indicated, tumors were macrodissected to maximize tumor content. For all tumor-tumor pairs, DNA was checked for mislabeling, contamination, and misidentification using a multiplexed polymerase chain reaction (PCR)/mass spectrometry–based genetic fingerprinting assay, as previously reported. Normal DNA was used in the case of TP53 to establish the somatic nature of the mutations.

**Genomic DNA Isolation**

For frozen tissues, genomic DNA was extracted from two 30-μm frozen slices using the Genfind kit (Beckman Coulter Genomics, Beverly, MA), in a 96-well format, following the manufacturer’s instructions. Tumor DNA was then whole genome amplified using the Repli-G Midi kit (Qiagen, Valencia, CA). The quality of whole genome–amplified DNA was verified by PCR reactions using two control amplicons.

**Sequence Analysis**

Mutations in KRAS (codons 12, 13, 22, 61, 117, and 146), NRAS (codons 12, 13, and 61), BRAF (codon 600), and PIK3CA (codons 345, 420, 542, 545, 546, 1043, and 1047) were detected using the iPLEX assay (Sequenom, San Diego, CA), as previously described. All mutations were confirmed either by a separate iPLEX assay or by Sanger sequencing. Mutations in TP53 were detected by Sanger sequencing of all coding exons, as previously reported. For 454 deep amplicon sequencing, PCR products for the desired targets were generated using primers designed with 5’ overhangs to facilitate emulsion PCR and sequencing. Primers for each sample were bar coded up to 10 per lane, followed by emulsion PCR and picotiter plate sequencing by-synthesis.

**Array Comparative Genomic Hybridization**

For comparative genomic hybridization (CGH) studies, labeled tumor DNA was cohybridized to Agilent 1M aCGH microarrays (Agilent, Santa Clara, CA) with a pool of reference normal. Raw copy number estimates were normalized and segmented with circular binary segmentation. Regions overlapping with copy number variations reported in the Database of Genomic Variants were excluded. Unsupervised hierarchical clustering was performed with one minus the Pearson correlation coefficient of the copy number profiles (segment means) as the distance measure and average linkage. Gains and losses were defined using a sample-specific threshold based on 2.2 median absolute deviations (approximately corresponding to 1.5 standard deviations) above and below the residual between the probe-level data and the segment means. The percent aberration was calculated per sample as the total number of gains and losses divided by the total number of probes. Patterns of gains and losses for primary-metastasis pairs were compared using a statistical methodology developed for testing clonality based on array CGH data using the Clonality R package.

**Statistical Analysis**

The Fisher’s exact test or χ² test was performed to evaluate differences between data sets consisting of categorical variables. For analysis of the distribution of mutations between primary tumors and metastases, an unmatched single patient cohort was used, where patients with matched pairs were excluded from the analysis.

**RESULTS**

**Clinical and Histologic Data**

To compare the genomic profiles of primary and metastatic CRC, we collected 736 frozen CRC tumor samples from 613 patients; clinical characteristics are listed in Appendix Table A1 (online only). Primary invasive carcinomas comprised 57% of the specimens (n = 406), whereas 291 specimens were collected from metastatic foci and 39 were adenomas with or without high-grade dysplasia/intramucosal carcinoma. The metastatic sites included liver (n = 227, 78%), lung (n = 34), soft tissue (n = 14), brain (n = 7), ovary (n = 5), and distant lymph nodes (n = 4).

The cohort included two matched data sets. Matched data set A consisted of 84 pairs of primary tumor and metastasis, whereas matched data set B was a set of 31 pairs of metastatic foci, with each pair derived from the same patient. The majority of metastases in the matched data sets were from the liver (n = 208, 91%); however, lung (n = 11), soft tissue, lymph node (n = 2), and brain (n = 1) metastases were also represented. For three of the primary tumors in matched data set A, the frozen tissue was from adenomas with high-grade dysplasia/intramucosal carcinoma.

**Mutational Profiling**

For all 613 patients, tumors were profiled for KRAS, NRAS, BRAF, PIK3CA, and TP53 mutations. To identify hotspot alterations in the KRAS, NRAS, BRAF, and PIK3CA genes, we used a mass spectrometry–based (Sequenom) assay. Because TP53 mutations are found scattered throughout the coding sequence, we performed Sanger sequencing of all TP53 coding exons. Mutations in KRAS or NRAS were identified in 277 patients (45.1%). Two hundred nineteen (35.7%) of the KRAS mutations were located at codon 12 or 13, whereas 58 patients (9.4%) had either NRAS mutation (2.9%) or mutations within exon 3 or 4 of KRAS (exon 3, 2%; exon 4, 4.6%). BRAF mutations were detected in 40 patients (6.5%), PIK3CA mutations were detected in 72 patients (11.7%), and TP53 mutations were detected in 247 patients (40.3%). The majority of PIK3CA mutations were in exon 9 (62.5%), whereas 29.2% of PIK3CA mutations were in exon 20. Mutations in KRAS, NRAS, and BRAF were all nonoverlapping in distribution. PIK3CA and TP53 mutations were found to co-occur with both RAS and BRAF mutations, although PIK3CA mutations were significantly more common in the KRAS mutant tumors than wild-type tumors (16.2% vs 8.1%, respectively; P = .001) and TP53 mutations were significantly more common in BRAF wild-type tumors than mutant tumors (9% vs 2.6%, respectively; P = .002). PIK3CA mutations were present both in TP53 mutant and wild-type tumors (34.7% vs 41%, respectively; P = .4). The distribution of mutations between adenomas, primary invasive carcinomas, and metastases in an unmatched single patient cohort is shown in Figure 1. KRAS mutations were more prevalent in adenomas compared with primary carcinomas (68.6% vs 42.7%, respectively; P = .004), whereas TP53 mutations were less common (8.6% vs 30.3%, respectively; P = .005). Significantly fewer BRAF

![Fig 1. Frequency of RAS (KRAS, NRAS), BRAF, PIK3CA, and TP53 mutations in an unmatched, single-patient cohort of colon adenomas (n = 36), primary invasive colorectal adenocarcinomas (n = 323), and metastases (n = 160).](chart)
mutations were found in the metastases compared with the primary tumors (1.9% vs 7.7%, respectively; \( P = .01 \)), whereas TP53 mutations were significantly more prevalent (53.1% vs 30.3%, respectively; \( P < .001 \)).

**Concordance of Mutations in Matched Pairs of Primary Carcinomas and Metastases**

To determine whether differences in the distribution of mutations between primary tumors and metastases were a reflection of their variable prognostic impact and not a result of differences in the genetic profiles of the predominant cell populations within the tumor-metastasis pairs, we examined the mutational concordance of a cohort of 84 pairs of primary and metastatic tumors. To minimize DNA quality as a confounding variable, we chose only patients for whom frozen tumor tissue from both the primary tumor and at least one metastasis was available. The mutational concordance rates for RAS/BRAF, PIK3CA, and TP53 after genotyping the frozen tissue were 92.8%, 96.4%, and 90.5%, respectively. Discrepant results were observed in 14 pairs affecting all genes studied (Appendix Table A2, online only). To exclude the possibility that the discordant results were attributable to tissue necrosis or low tumor content within the frozen samples, we analyzed formalin-fixed, paraffin-embedded (FFPE) material from all discordant pairs. After analysis of FFPE tissue from initially discordant pairs, the concordance rates increased to 97.6%, 98.8%, and 92.8% for RAS/BRAF, PIK3CA, and TP53, respectively, with discrepant results observed in only eight pairs (Fig 2).

After analysis of both frozen and FFPE tissue, two of the 84 pairs had discordant KRAS mutational results (patients 20 and 42). In both patients, there was suspicion of a second primary adenocarcinoma. The first KRAS discordant pair was from a patient who underwent synchronous resection of a KRAS/TP53 wild-type, T3N0 cecal adenocarcinoma and a liver metastasis, the latter showing KRAS G12D and TP53 R248Q mutations (Fig 3A). Retrospective review of the patient’s clinical record revealed that 6 months earlier, the patient had a T3N0 adenocarcinoma of the sigmoid colon resected, raising the possibility that the liver metastasis was derived from the preceding sigmoid colon carcinoma rather than the cecal adenocarcinoma resected at the time of her liver resection. Because the preceding procedure was performed at an outside institution, tissue from the sigmoid colon adenocarcinoma was not available for analysis.

The second KRAS discordant pair was from a patient who had a synchronous resection of a T3N0 mucinous adenocarcinoma harboring a KRAS A146T mutation and a nonmucinous liver metastasis expressing a KRAS G12D mutation (Fig 3B). Although, no clinical history was available, the different tumor morphologies suggested that the liver metastasis may have been derived from a second primary lesion. Of note, our data set included another patient who had two primary invasive adenocarcinomas synchronously resected, a T3N2 cecal adenocarcinoma and a T3N1 rectal adenocarcinoma with mucinous features showing distinct mutational profiles (KRAS G12V, TP53 wild type vs KRAS A146T, TP53 R306*, respectively). A liver metastasis removed during the same procedure was an adenocarcinoma with mucinous features whose mutational profile was identical to that seen in the rectal adenocarcinoma (KRAS A146T, TP53 R306*).

After analysis of frozen and FFPE tissues, six patients showed discrepancies in TP53 mutational status. In five of the six patients, the primary tumor was TP53 wild type, whereas the liver metastasis was TP53 mutant. In one patient (patient 65), a TP53 mutation was identified in the primary tumor but not in the liver metastasis. This patient was clinically notable because the patient had received neoadjuvant chemoradiotherapy treatment for a rectal primary tumor after resection of the liver metastasis. This clinical history suggests that the TP53 mutation found in the primary tumor may have been induced by the preceding chemoradiotherapy.

To further investigate the TP53 discordant pairs, we performed 454 deep amplicon sequencing of six primary-metastasis pairs (patients 8, 29, 39, 43, 65, and 81). In two of these pairs, the primary lesion contained invasive adenocarcinoma (patients 29 and 81), and in both
pairs, we detected by 454 sequencing the mutant allele identified by Sanger sequencing in the metastasis at low allele frequency in the CRC primary tumor. A third pair that was discordant after analysis of the frozen samples (patient 8) was attributed to the absence of invasive adenocarcinoma in the original frozen section. When the invasive carcinoma and noninvasive components of this sample were analyzed separately using FFPE tissue, the \( TP53 \) mutation (R306*) found in the metastatic sample was detected in the invasive component but was absent in the noninvasive component (Fig 4). In two additional patients (patients 39 and 43), the primary frozen lesion was composed predominantly of adenoma with high-grade dysplasia/intramucosal carcinoma. In both of these latter patients, \( TP53 \) was wild type in the primary lesion by 454 sequencing, as was the liver metastasis of patient 65. In summary, the data suggest that \( TP53 \) status is highly concordant between primary and metastatic CRC tumors but that false-negative results may occur as a result of clonal/tissue heterogeneity and/or interval treatment with radiation or chemotherapy.

**Concordance of Mutations in Matched Pairs of Metastases**

To examine the genetic heterogeneity of different metastatic foci, we compared the mutational profiles of 31 pairs of metastases. After analysis of the frozen material and then FFPE in any initially discordant pairs, the concordance rates were 100%, 100%, and 96.7% for \( RAS/BRAF, PIK3CA \), and \( TP53 \), respectively (Appendix Table A3, A).
online only). The single discordant pair was from a 57-year-old woman who developed a solitary lung metastasis 2 years after resection of a stage III colon adenocarcinoma. This lung metastasis was TP53 wild type, whereas a second lung metastasis resected 2 years later, after treatment with several cytotoxic chemotherapeutic regimens, was TP53 mutant (R196*); 454 deep sequencing of the initial metastatic site failed to identify this TP53 mutant allele.

Analysis of Copy Number Aberrations in Primary and Metastatic CRCs

Whole genome copy number profiling has previously been shown to be of utility in distinguishing between metastatic and second primary carcinomas in patients with breast and lung cancers.25,26 Given the suspicion that the mutational discordance between several of the primary and metastatic samples was attributable to either multiple primary tumors or intervening chemotherapy or radiotherapy, we performed whole genome copy number analysis on 25 of the tumor-metastasis pairs, including three of the mutationally discordant pairs (patients 20, 42, and 65). Seventeen of the paired samples were derived from synchronous resections, whereas the remaining eight pairs were obtained from metachronous resections. Twenty samples (40%) were from patients who had received prior treatment. Overall, the liver metastases showed more chromosomal aberrations compared with the primary tumors (9.6% v 7.5%, respectively). An analysis of the copy number data revealed no recurrent focal areas of copy number change in the metastatic samples that were not present in the primary lesions. However, focal aberrations in well-characterized cancer-associated genes were present in individual metastases but not in the primary lesions. For example, the metastasis from patient 38 had a focal deletion in the PARK2 gene, whereas the primary tumor from this patient was copy neutral at the PARK2 locus.

To determine whether pairs with discordant mutational profiles represented distinct primary cancers, we performed unsupervised, hierarchical clustering and clonality analyses of the array CGH data. The majority of pairs (20 of 25 pairs) clustered together (Appendix Fig A1, online only), and all 22 pairs with concordant mutational profiles were classified as clonal. Both pairs with discordant KRAS mutational results (patients 20 and 42) were deemed to be distinct primary tumors by this analysis (Table 1; Appendix Fig A2, online only). Furthermore, patient 65, who had received chemoradiotherapy to the primary

<table>
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<th>Patient Pair</th>
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Abbreviations: P, primary tumor; M, metastasis.
With the development of cancer therapies that specifically target molecular alterations that mediate cancer progression, genotyping of patients with advanced CRC has become a component of routine clinical practice. Specifically, KRAS mutational testing has now been incorporated into several clinical practice guidelines for the treatment of patients with metastatic CRC. There is, however, no consensus as to how such testing should be performed, and it remains unknown whether testing of the primary lesion is sufficient. The National Comprehensive Cancer Network recommends testing either the primary tumor or a site of metastasis. This recommendation is based on several studies that have found a high (> 95%) KRAS mutational concordance between primary CRCs and metastases. In contrast to these results, several studies have reported a significant rate of discordance between the mutational profile of primary tumors and their corresponding metastatic lesions. These latter studies have led some to suggest testing of metastatic tumors when available. However, material from metastases is not routinely collected, and furthermore, its utility is often limited by low tumor content secondary to necrosis or prior treatment effect.

In this study, we sought to determine the incidence of clinically relevant mutations in primary CRC and metastases and their concordance in paired primary and metastatic samples. We genotyped tumors for mutations in the KRAS, NRAS, BRAF, PIK3CA, and TP53 genes using a multiplex approach, which included mass spectrometry–based genotyping and Sanger sequencing. We found that the frequency of KRAS and PIK3CA mutations did not differ significantly between primary tumors and metastases. In contrast, TP53 mutations were significantly more frequent in metastases, whereas BRAF mutations were less frequent. Despite these differences, analysis of matched primary tumors and metastases showed a high concordance rate (> 90%) for the five genes examined. These results suggest that differences in the distribution of mutations are a reflection of their variable prognostic impact.

Consistent with the model that alterations in RAS and BRAF occur early in CRC pathogenesis, only two (2.4%) of 84 tumor-metastasis pairs were discordant for KRAS. Clinicopathologic assessment and a clonality analysis based on array CGH data suggested that both KRAS discordant pairs were not clonal. Thus, discordance in KRAS status between primary and metastatic lesions when it occurs may be attributable to the presence of more than one independent primary cancer, although larger studies will be needed to confirm this hypothesis. Synchronous colorectal adenocarcinomas are estimated to occur in 3.4% to 6.2% of patients with CRC. Although they remain a poorly studied group of tumors, one study found that nine of 13 pairs of synchronous primary colorectal neoplasms had discordant KRAS mutational profiles. Given these results and our results, it is prudent to recommend that, in this clinical context, genotyping should be performed on tissue obtained from a site of metastasis.

In the case of TP53, we found several reasons for discordant results, including the absence of invasive carcinoma in the frozen primary tumor and treatment effect. The former highlights the limitations of frozen tissue. Although current genotyping methods do not require the use of frozen tissue, it is considered by some to be the gold standard for molecular analyses, because it yields higher quality nucleic acids. However, only a small portion of a tumor is typically frozen, and the sample collected may not include the invasive component. This latter concern is of particular relevance in CRC because there is often an exophytic preinvasive component. Our data are thus consistent with the model that TP53 mutations occur later than KRAS mutations in CRC pathogenesis and suggest that TP53 genotyping should be performed using DNA derived from the invasive component of the primary lesion or from a metastatic site.

In summary, our results suggest that in most clinical scenarios, analysis of the primary colorectal tumor is sufficient for determining RAS, BRAF, PIK3CA, and TP53 mutational status. However, in patients with a history of more than one CRC primary tumor, a biopsy of a metastatic site should be considered if a treatment decision is being based on the mutational profiling results obtained.

REFERENCES


