Neuroendocrine tumors comprise a heterogeneous group of malignancies with a broad spectrum of clinical behavior. Poorly differentiated tumors follow an aggressive course with limited treatment options, and new approaches are needed. Oncogenic \( \text{BRAF}^{\text{V600E}} \) substitutions are observed primarily in melanoma, colon cancer, and non–small cell lung cancer, but have been identified in multiple tumor types. Here, we describe the first reported recurrent \( \text{BRAF}^{\text{V600E}} \) mutations in advanced high-grade colorectal neuroendocrine tumors and identify a \( \text{BRAF} \) alteration frequency of 9% in 108 cases. Among these \( \text{BRAF} \) alterations, 80% were \( \text{BRAF}^{\text{V600E}} \).

Dramatic response to BRAF–MEK combination therapy occurred in two cases of metastatic high-grade rectal neuroendocrine carcinoma refractory to standard therapy. Urinary \( \text{BRAF}^{\text{V600E}} \) circulating tumor DNA monitoring paralleled disease response. Our series represents the largest study of genomic profiling in colorectal neuroendocrine tumors and provides strong evidence that \( \text{BRAF}^{\text{V600E}} \) is an oncogenic driver responsive to BRAF–MEK combination therapy in this molecular subset.

**SIGNIFICANCE:** \( \text{BRAF}^{\text{V600E}} \) is an established oncogenic driver, but significant disparities in response exist among tumor types. Two patients with treatment-refractory high-grade colorectal neuroendocrine tumors harboring \( \text{BRAF}^{\text{V600E}} \) exhibited rapid and durable response to combined BRAF–MEK inhibition, providing the first clinical evidence of efficacy in this aggressive tumor type.

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on aggressive biologic behavior, and data from small retrospective series suggesting a multimodality approach with chemoradiation and surgery may be optimal for localized nonpancreatic high-grade NETs (2–4). The prognosis for advanced high-grade NETs remains poor, with median survival of less than 12 months (5, 6).

The molecular features of NETs are not comprehensively characterized and have focused on alterations in the tumor suppressor MEN1, occurring primarily in pancreatic neuroendocrine tumors (pNET), which differ from nonpancreatic NETs (7–9). Limited data exist on molecular features in nonpancreatic NETs, and alterations in SRC, SMAD family genes, AURKA, EGFR, HSP90, PDGFR, and amplification of AKT1 and AKT2 were observed in a limited series of low-grade, well-differentiated small intestinal NETs (10). Colorectal and high-grade NETs are even less well characterized, further limiting therapeutic options.

Here, we identify recurrent somatic BRAF alterations in high-grade colorectal NETs and describe two patients with treatment-refractory metastatic high-grade rectal NET harboring a BRAFV600E substitution who achieved a rapid and dramatic response to combination BRAF–MEK-directed therapy. This is the largest published colorectal NET series and the first reported oncogenic BRAF mutation in high-grade NET, demonstrating the ability of genomic profiling to identify therapeutically relevant alterations in this aggressive disease with no standard treatment approach.

RESULTS

Case 1

A 70-year-old woman presented with rectal bleeding from a fungating rectal mass, which on biopsy revealed a poorly differentiated high-grade (grade 3) rectal NET with a Ki67 proliferation index greater than 60% (Fig. 1A–D). Immunohistochemistry demonstrated microsatellite stability (MSI-stable). She received neoadjuvant chemoradiation with carboplatin and etoposide followed by low anterior resection and end colostomy. Histopathologic analysis confirmed residual NET, grade 2/3, Ki67 3%–4%, positive margins, and 4/22 positive lymph nodes. A PET-CT scan 1 month after resection demonstrated metastatic disease, confirmed on liver biopsy as high-grade NET (Figs. 1E–H and 2A). She was treated with temozolomide but developed rapid radiographic progression, left-leg paresthesia, and abdominal pain within 3 months of therapy (Fig. 2A). To investigate further therapeutic options, her liver biopsy was subjected to comprehensive genomic profiling as previously described (11). Genomic analysis revealed a BRAFV600E substitution at a mutant allele frequency (MAF) of 26%, with a median sample coverage depth of 529× (Table 1). The initial surgical specimen was retrospectively tested and also found to harbor a BRAFV600E mutation. In the absence of an available clinical trial, she was transitioned to dabrafenib 150 mg twice daily and trametinib 2 mg once daily, and her symptoms resolved within 10 days of treatment. Concurrent decrease in serum chromogranin A and urinary BRAFV600E circulating tumor DNA (ctDNA) using a clinical laboratory improvement amendment (CLIA)–approved assay correlated with rapid disease response (Fig. 2B and C). She obtained a dramatic radiographic response within 5 weeks that had continued for over 7 months at the time of manuscript submission (Fig. 2A). Serial chromogranin A remains decreased, and a quantitative algorithm applied to the CLIA test result indicated a nondetectable urinary BRAFV600E level from a peak ctDNA MAF of 0.6% (Fig. 2B and C).

Case 2

A 39-year-old woman presented with rectal bleeding and was found to have a large locally advanced high-grade rectal NET with a proliferative index of over 70% (Fig. 3A–D). Immunohistochemical stains are diffusely positive for synaptophysin (C) and chromogranin (not shown), with variable CDX2 staining (D). E–H highlight similar features from the liver biopsy with uniform cells (E and F), scattered mitotic figures (arrows), diffuse synaptophysin positivity (G), and Ki67 proliferative index of 40%–50% (H).
radiation but developed progressive disease with hepatic, pulmonary, and inguinal nodal metastases. She received carboplatin/docetaxel for 6 cycles and achieved initial stable disease followed by disease progression and worsening rectal pain. Comprehensive genomic profiling of the rectal mass revealed a \textit{BRAF}^{V600E} substitution at an MAF of 53% at a coverage depth of 734× (Table 1). An appropriate clinical trial was not available, and she was treated with vemurafenib 960 mg twice daily and trametinib 2 mg once daily and achieved a resolution of her pain within 3 days, followed by a dramatic radiographic response (Fig. 3E). She remains on combination BRAF–MEK therapy with an ongoing response lasting 9 months.

**BRAF Alterations Occur Frequently in Advanced Colorectal NET**

To identify additional neuroendocrine cases harboring \textit{BRAF} alterations, we retrospectively reviewed a database of over 55,000 clinical samples subjected to comprehensive genomic profiling as previously described (11). Including the incident case (case 1), we identified a total of 109 cases of colorectal NETs. Ten samples (9%) harbored alterations in \textit{BRAF} (80% \textit{BRAF}^{V600E}; Table 1). Two non-V600 alterations (G469A and R671Q) were found. \textit{BRAF}^{V600E} was mutually exclusive with oncogenic \textit{KRAS}/\textit{NRAS} alterations and other established driver alterations (Table 1). Among the 109 colorectal NETs, alterations in \textit{NRAS} were observed in 2 of 109 cases (1.8%), and \textit{KRAS} alterations occurred in a total of 35 of 109 cases (32%). The mean age was 61.4 years, and \textit{BRAF} alterations were split between male and female patients and colon and rectal locations. The majority of \textit{BRAF}-mutant samples (8/10) were of high-grade (grade 3) histologic appearance (Table 1). \textit{PIK3CA}E545K and N1068fs*3 alterations occurred in cases 5 and 9, respectively (Table 1). Other established oncogenic genomic alterations and alterations in \textit{MLH1}, \textit{MSH6}, and \textit{MSH2} were not identified.

**DISCUSSION**

Here, we identify recurrent somatic \textit{BRAF} alterations in high-grade colorectal NET and demonstrate rapid clinical...
Table 1. Clinicopathologic features of BRAF alterations in high-grade colorectal NETs

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor location</th>
<th>Stage, grade</th>
<th>KRAS, APC status</th>
<th>BRAF event</th>
<th>Tumor purity (%)</th>
<th>BRAF MAF (%)</th>
<th>BRAF coverage</th>
<th>Sample coverage</th>
<th>Response to BRAF-i</th>
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<tr>
<td>1*</td>
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<td>Rectum</td>
<td>IV, 3</td>
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<td>V600E</td>
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<td>V600E</td>
<td>19</td>
<td>9</td>
<td>552</td>
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Abbreviations: BRAF-i, BRAF inhibitor; F, female; M, male; WT, wild-type; N/A, data not available. Asterisk (*) represents the incident case, case 1.
MEK inhibition over traditional colorectal adenocarcinoma. Alterations were mutually exclusive, consistent with observations in BRAF V600E -mutant melanoma (16, 17). Recently, vemurafenib monotherapy demonstrated benefit across multiple tumor types, and the combination of BRAF–MEK inhibition in BRAFV600E -mutant high-grade rectal NET from case 2. Following initiation of therapy, there was a rapid decrease in urinary cctDNA, along with serum chromogranin.

The rectum is the most common site of NET involvement, comprising of 29% of all gastroenteropancreatic (GEP) NETs (1). Outside of colorectal adenocarcinoma, BRAF mutations (5%-9%) have not been described in tubular gastrointestinal malignancies in The Cancer Genome Atlas (TCGA) or the Catalogue of Somatic Mutation in Cancer (COSMIC) datasets. However, NETs are significantly underrepresented, and true BRAF alteration frequency is likely underestimated. In our database including 109 colorectal NET samples, we identified BRAF mutations at a frequency of 9%, with the vast majority being BRAFV600E. Our homogeneous series suggests that BRAFV600E mutations are more common (9%) than previous limited studies of nonpancreatic NETs had indicated (10, 12). Recently, a subset of colorectal adenocarcinomas with large-cell neuroendocrine features and signet ring cells in which BRAF alterations may be enriched has been described, although the clinical impact is not known (13). A review of our cases did not identify signet ring features. Our series represents the largest dataset of colorectal NETs subjected to comprehensive genomic profiling and suggests that further clinical investigation with BRAF-directed therapies is warranted in high-grade colorectal NETs. Interestingly, alterations in KRAS/NRAS were 34%, significantly lower than the 45% to 53% incidence of KRAS/NRAS alterations reported from colorectal adenocarcinoma (14, 15). Whether or not these frequencies suggest a shared origin with early evolutionary divergence is beyond the scope of our study. In our samples, BRAF mutations and oncogenic KRAS alterations were mutually exclusive, consistent with observations in traditional colorectal adenocarcinoma.

The rationale for the superiority of combined BRAF and MEK inhibition over BRAF monotherapy is well established in BRAFV600E-mutant melanoma (16, 17). Recently, vemurafenib monotherapy demonstrated benefit across multiple tumor types, and the combination of BRAF–MEK inhibition in BRAFV600E -mutant advanced colorectal cancer confirmed a partial response rate of 12% (5/43) and one durable complete response (22). Whether or not neuroendocrine features were present in the complete response observed by Corcoran and colleagues is not reported. Although limited by small numbers, both patient responses in our series occurred in patients with rectal high-grade NETs wild-type for KRAS, APC, and PI3CA (Table 1). Although the BRAFG469A mutation is frequently observed in non–small cell lung cancer (NSCLC) and is known to activate downstream MAPK signaling, the responsiveness to BRAF and MEK inhibition is not well studied (23). Similarly, case 4 in our series harbors concurrent KRAST120S and BRAFV600E mutations at a low MAF (1%). Recently, the KRASG12S mutation was shown to be non-tumorigenic in a pancreatic cancer model (24). More data are needed to determine the impact of concurrent exon 2 KRAS mutations on BRAF-inhibitor sensitivity in BRAF-mutant colorectal adenocarcinomas and high-grade colorectal NETs (24, 25). Despite sharing anatomic locations within the colon, our results are suggestive of significant biologic differences between advanced colorectal adenocarcinomas and high-grade colorectal NETs. Functional validation in preclinical studies will be an important step in determining the behavior and genomic context of response in BRAF-mutant NET.

Figure 3. Histologic features and radiographic response to vemurafenib and trametinib in BRAFV600E-mutant high-grade rectal NET from case 2. Microscopic examination demonstrates organization of variably small cells (A) staining weakly positive for CD56 (B) and chromogranin (C) with a Ki67 proliferative index of over 70% (D). Dramatic radiographic response to therapy is shown in E.
A levels that paralleled clinical resolution of symptoms and preceded radiologic response. Although the serum chromogranin A never completely normalized, the correlation between serum chromogranin A, clinical improvement, and BRAF V600E urinary ctDNA detection (lower limit 0.03% MAF) provides early validation for the clinical utility of ctDNA testing methodologies in monitoring of tumor dynamics (26). Noninvasive methodologies allowing for molecular monitoring will play an increasing role in future clinical care, particularly in monitoring for drug resistance and early progression/recurrence (26).

Recent data suggest that in BRAF V600E colorectal cancer, resistance to combination of BRAF–MEK may be mediated by acquired MEK1 (MAP2K1) alterations (MEK1 F53L, ref. 27). Further work focusing on combination therapies and refining understanding of resistance will be essential to optimize outcomes in rare tumors harboring BRAF alterations, including our cases.

Overall, our series underscores the oncogenic potential and therapeutic implications of BRAF-directed therapies in high-grade colorectal NETs and identifies a new molecularly defined disease subset. Although prospective validation through clinical trials is the optimal approach to confirm our findings, this was not available to either of our patients, and important issues surrounding off-label treatment costs are beyond the scope of this report.

METHODS

Next-Generation Sequencing Analysis of Tumor Biopsies

DNA and RNA were extracted, and adaptor ligated sequencing libraries were captured by solution hybridization using custom bait-sets targeting 315 cancer-related genes and 28 frequently rearranged genes by DNA sequencing (FoundationOne, Foundation Medicine). All captured libraries were sequenced to high depth (Illumina HiSeq) in a CLIA-certified CAP-accredited laboratory (Foundation Medicine), averaging >500x for DNA. Sequence data from gDNA and cDNA were mapped to the reference human genome (hg19) and analyzed through a computational analysis pipeline to call genomic alterations present in the sample, including substitutions, short insertions and deletions, rearrangements, and copy-number variants.

Identification of BRAF Altered NET

Following the identification of BRAF V600E in the incident case (case 1), a large database containing comprehensive genomic profiling sequencing data for over 55,000 clinical samples was retrospectively interrogated to identify additional neuroendocrine patient samples. DNA sequences associated with clinical samples carrying a colorectal neuroendocrine diagnosis were analyzed for base substitutions, short insertions, deletions, gene copy-number alterations (focal amplifications and homozygous deletions), and gene fusions. Basic clinicopathologic characteristics were captured; however, treatment and disease response data were not available in all cases.

Detection of BRAF V600E in Urinary ctDNA

Urinary ctDNA BRAF V600E test for qualitative detection of BRAF V600E was performed in a CLIA-certified CAP-accredited laboratory (Trovagene) as previously described (28). Following urinary ctDNA extraction and quantitation, a two-step PCR assay targeting a very short (31 bp) amplicon was used to enhance detection of rare mutant alleles in ctDNA. The first-step amplification was done with two primers flanking the BRAF V600E locus and a complementary blocking oligonucleotide, which suppressed wild-type BRAF amplification, achieving enrichment of the mutant BRAF V600E sequence. The second step entailed a duplex ddPCR reaction using FAM (BRAF V600E) and VIC (wild-type BRAF) TaqMan probes to enable quantification of mutant versus wild-type fragments, respectively. The RainDrop ddPCR platform (RainDance) was used for PCR droplet separation, fluorescent reading, and counting droplets containing mutant sequence, wild-type sequence, or unreacted probe. For each patient sample, the assay identified BRAF V600E–mutant fragments detected as a percentage of detected wild-type BRAF. Thresholds for BRAF V600E detection were developed by evaluating a training set of urinary ctDNA from patients with BRAF V600E metastatic cancer (positives) and healthy volunteers (negatives) by applying a classification tree algorithm that maximizes the true-positive and true-negative rates, yielding two threshold values (<0.05 not detected; 0.05–0.107 indeterminate; >0.107 detected; ref. 25). A quantitative algorithm was applied to the CLIA test result to obtain the values for BRAF V600E allele frequencies.

Patient Studies

A review of available treatment options and detailed risk–benefit discussion was undertaken, and informed consent was obtained from both patients prior to treatment initiation. Patient care was conducted in accordance with the Declaration of Helsinki. Study medications were obtained through insurance and/or the drug manufacturer and monitored in accordance with the approved label. Treatment was conducted in the absence of Institutional Review Board approval.

Disclosure of Potential Conflicts of Interest

S.J. Klempner has received speakers bureau honoraria from Foundation Medicine. K. Gowen has ownership interest (including patents) in Foundation Medicine. D. Morosini has ownership interest (including patents) in Foundation Medicine. J.S. Ross reports receiving a commercial research grant from and has ownership interest (including patents) in Foundation Medicine. V.A. Miller has ownership interest (including patents) in Foundation Medicine. P.J. Stephens has ownership interest (including patents) in Foundation Medicine. S.M. Ali has ownership interest (including patents) in Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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REFERENCES


BRAF<sup>V600E</sup> Mutations in High-Grade Colorectal Neuroendocrine Tumors May Predict Responsiveness to BRAF–MEK Combination Therapy

Samuel J. Klempner, Bruce Gershenhorn, Phu Tran, et al.


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