**Human Cancer Biology**

**Somatic Profiling of the Epidermal Growth Factor Receptor Pathway in Tumors from Patients with Advanced Colorectal Cancer Treated with Chemotherapy ± Cetuximab**

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**Abstract**

**Purpose:** To study the somatic molecular profile of the EGF receptor (EGFR) pathway in advanced colorectal cancer, its relationship to prognosis, the site of the primary and metastases, and response to cetuximab.

**Experimental Design:** We used Sequenom and Pyrosequencing for high-throughput somatic profiling of the EGFR pathway in 1,976 tumors from patients with advanced colorectal cancer from the COIN trial (oxaliplatin and fluoropyrimidine chemotherapy ± cetuximab). Correlations between mutations, clinicopathologic, response, and survival data were carried out.

**Results:** Sequenom and Pyrosequencing had 99.0% (9,961/10,063) genotype concordance. We identified 13 different KRAS mutations in 42.3% of advanced colorectal cancers, 2 BRAF mutations in 9.0%, 4 NRAS mutations in 3.6%, and 5 PIK3CA mutations in 12.7%. 4.2% of advanced colorectal cancers had microsatellite instability (MSI). KRAS and PIK3CA exon 9, but not exon 20, mutations cooccurred (P = 8.9 × 10-4) as did MSI and BRAF mutations (P = 5.3 × 10-10). KRAS mutations were associated with right colon cancers (P = 5.2 × 10-5) and BRAF mutations with right (P = 7.2 × 10-5) and transverse colon (P = 9.8 × 10-6) cancers. KRAS mutations were associated with lung-only metastases (P = 2.3 × 10-4), BRAF mutations with peritoneal (P = 9.2 × 10-4) and nodal-only (P = 3.7 × 10-5) metastases, and MSI (BRAFWT) with nodal-only metastases (P = 2.9 × 10-4). MSI (BRAFWT) was associated with worse survival (HR = 1.89, 95% CI 1.30–2.76, P = 8.5 × 10-4). No mutations, subsets of mutations, or MSI status were associated with response to cetuximab.

**Conclusions:** Our data support a functional cooperation between KRAS and PIK3CA in colorectal tumorigenesis and link somatic profiles to the sites of metastases. MSI was associated with poor prognosis in advanced disease, and no individual somatic profile was associated with response to cetuximab in COIN.

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**Introduction**

Worldwide, more than a million people are diagnosed with colorectal cancer each year. Colorectal cancer is among the cancers showing the greatest improvement in survival (1) and this is due, in part, to advances in drug therapy. Approximately two-thirds of patients now receive chemotherapy either as a component of curative treatment or to extend survival with incurable disease, and there is good randomized controlled trial evidence of effectiveness in both of these settings (2–4). Patients with advanced colorectal cancer have shown further benefits with monoclonal therapies targeting the EGF receptor (EGFR). For example, cetuximab increased median survival by 4.7 months compared with best supportive care alone in patients with KRAS wild-type tumors who had exhausted standard cytotoxic therapy options (5).

EGFR acts as the gateway for multiple downstream intracellular signaling pathways including the RAS-RAF-MAP...
Translational Relevance

Cetuximab, a monoclonal antibody against the EGFR receptor (EGFR), improves overall survival in patients with advanced colorectal cancer in whom other treatments have failed. Efficacy is thought to be dependent upon an absence of somatic mutations in members of the EGFR signaling cascade. We profiled this pathway in 1,976 tumors from patients with advanced colorectal cancer that had been treated with oxaliplatin and fluoropyrimidine chemotherapy ± cetuximab (from the MRC COIN trial). KRAS and PIK3CA exon 9 mutations significantly cooccurred as did microsatellite instability (MSI) and BRAF mutations, supporting their functional cooperation in colorectal tumorigenesis. KRAS mutations were associated with lung-only metastases, BRAF mutations with peritoneal and nodal-only metastases, and MSI (BRAFmut) with nodal-only metastases, suggesting that different biologic patterns influence tumor behavior. MSI was associated with worse survival, independent of somatic mutation status. No individual mutation, subsets of mutations, or MSI status were associated with response to cetuximab in the COIN trial.

Furthermore, mutations in KRAS and BRAF, which are more prevalent in colorectal cancers from the right/transverse colon (19, 20), appear to be associated with particular sites of metastases. KRAS mutations have been associated with lung (21, 22), brain (21) but not liver metastases, and BRAF mutations have been associated with peritoneal (20, 23) and distant lymph node metastases (20). Further studies in large independent series are necessary to validate these observations and to help unravel the underlying mechanisms.

Here, we used 2 mutation detection platforms, Pyrosequencing and Sequenom, for high-throughput somatic profiling of the EGFR pathway in 1,976 tumors from patients with advanced colorectal cancer from the MRC COIN trial who received oxaliplatin and fluoropyrimidine chemotherapy with and without cetuximab. We studied the inter-relationships between the somatic mutations, together with their correlations to the sites of the primary and the metastases, and response to cetuximab.

Materials and Methods

Patient samples

COIN (ISRCTN27286448) is a MRC sponsored, Cancer Research-UK funded, fully accrued 2,445 patient UK national trial, in which patients were randomized in a 1:1:1 ratio to receive continuous oxaliplatin-based chemotherapy (Arm A), continuous chemotherapy plus cetuximab (Arm B), or intermittent chemotherapy (Arm C), in first-line treatment of advanced colorectal cancer. All patients chose between oral capecitabine, a 5-fluorouracil (5-FU) prodrug, (two-thirds of patients) or infusional 5-FU (one-third) as the partner for oxaliplatin before randomization. All patients had measurable metastatic or locally advanced colorectal adenocarcinoma and received no previous chemotherapy for advanced disease. All patients had unresectable disease, whose only treatment option was palliative chemotherapy. Thus, nodal-only metastases included those with distant nodal metastases, such as para-aortic, mediastinal, or supraclavicular fossa nodes as well as unresectable nodal recurrence in the pelvis. Similarly, patients with peritoneal disease had extensive peritoneal metastases, which was beyond surgical resectability. All patients gave fully informed consent for their samples to be used for bowel cancer research (approved by REC04/MRE06/06). Tumor samples were collected as formalin-fixed paraffin embedded (FFPE) tissues.

Processing FFPE colorectal cancers, DNA extraction, and microsatellite instability analyses

Five-micrometer sections were cut from FFPE colorectal cancers. One section was stained with hematoxylin and eosin and visualized with a Mirax scanner. Samples containing concentrated pockets of tumor material were macrodissected using an unstained section. For samples containing limited regions of tumor, laser capture microdissection was conducted using 10 μm sections cut onto PALM membrane slides (Carl Zeiss; see Supplementary information). DNA was extracted using QIAamp DNA
Microkit (Qiagen) and eluted in 50 μL water. Microsatellite instability (MSI) status was determined using the markers BAT-25 and BAT-26.

Identification of somatic mutation "hot spots" and mutant cell lines
We queried the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic) for known common mutations in KRAS, BRAF, NRAS and PIK3CA in colorectal cancers. Cell lines known to carry variants within these genes were identified from the Sanger Cancer Cell Line Project (http://www.sanger.ac.uk/genetics/CGP/CellLines/). We tested the sensitivity of Pyrosequencing and Sequenom to detect low levels of mutant alleles using the cell lines listed in the Supplementary information. DNA extracted from these lines was quantified using a nanodrop spectrophotometer (Thermo Fisher Scientific) and serially diluted with wild-type DNA to generate known levels of mutant alleles. All dilutions were prepared and analyzed in triplicate.

Pyrosequencing
For codons 12 and 13 of KRAS, we initially used the amplification primers 5'-GGGCTCGTAAAAATGACTGA-3' and 5'-AGAATGCTGCTCAGCACTGA-3' together with extension primer 5'-CTTCTGTCAGTGGAGCCTG-3', however, this assay was subsequently modified by using the extension primers 5'-TGTGGTAGTTGGAGCTGGT-3', 5'-TGTGGTAGTTGGAGCCTG-3', and 5'-TGTGGTAGTTGGAGCCTG-3', as previously described (24). For codon 61 of KRAS, we used the amplification primers 5'-CTTGTGGTAGTTGGAGCCTG-3' and 5'-CTCAGCCAGGAAACATGTCC-3' and for codon 600 of BRAF we used the amplification primers 5'-TGCTTGAGCTCAGCAGCACTGA-3' and 5'-CAGGGCCCAAATTTCAATGCTG-3' together with the extension primer 5'-ATTCTCGACACAGCAGGT-3', and for codon 600 of BRAF we used the amplification primers 5'-TGCTTGAGCTCAGCAGCACTGA-3' and 5'-CAGGGCCCAAATTTCAATGCTG-3' together with the extension primer 5'-ATTCTCGACACAGCAGGT-3'. Reverse primers were biotinylated. PCR was conducted in 50 μL reaction volumes containing 25 μL Megamix Gold (Microzone), 10–20 ng DNA, and 10 μmol/L of primers. Thermocycling was conducted at 95°C for 10 minutes, followed by 38 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 60 seconds, followed by a final extension of 72°C for 1 minute. Unincorporated dNTPs were deactivated using 0.3U of shrimp alkaline phosphatase at 37°C for 40 minutes, and primer extension was carried out using 7–14 μmol/L of each extension primer, 1U of iPLEX termination mix and 1U of iPLEX enzyme. Reactions were cycled at 94°C for 30 seconds, followed by 40 cycles of 94°C for 5 seconds, 52°C for 5 seconds, and 80°C for 5 seconds, followed by a final extension at 72°C for 3 minutes. Extension products were spotted onto a matrix pad of a SpectroCHIP (Sequenom). After analyzing the SpectroCHIPS using a Bruker matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer, spectra were processed by the SpectroREADER software and transferred to the MassARRAY Typer 4 Analyzer. Genotyping was conducted using the MassARRAY RTTM software (Sequenom). Automated calls were validated by manual review of the raw mass spectra.

Sanger sequencing
Sanger sequencing of codons 12, 13, and 61 of KRAS and codon 600 of BRAF was carried out as described in the Supplementary Information.

Statistical analyses
Correlation between somatic mutations and the sites of metastases were carried out using the χ2 test (or Fisher exact test for n < 5). Time-to-event curves for analysis of OS and PFS were estimated using the Kaplan–Meier method. Hazard ratios (HR), confidence intervals (CI), and P values were estimated using the log-rank method.

Results
Sensitivities of the assays
For Pyrosequencing, we initially designed an assay with a single-extension primer to detect mutations at codons 12 and 13 of KRAS. However, we found that G12C was not robustly detected in samples with 25% mutant alleles, so the assay was redesigned to a three-extension primer system (24). This modified assay robustly detected G12A and G12D in samples with 4% mutant alleles, and G12V in samples with 2% mutant alleles. Sequenom robustly detected V600E in BRAF and Q61R in NRAS in samples with 10% mutant alleles, G12C and G12L in KRAS and E542K, E545K, and H1047R in PIK3CA in samples with 6% mutant alleles, and G12V in KRAS in samples with 4% mutant alleles (Supplementary Table S2).

Tumor samples
We collected FFPE tumor blocks from 2,161/2,445 (88.4%) patients. One-hundred and eighty-five (8.6%) blocks contained insufficient tumor material for processing. For the remaining samples, 1,893 (95.8%) were from the primary colorectal cancer and 66 (3.3%) and 17 (0.9%) were from the liver and lymph node metastases, respectively.
Pyrosequencing and sequenom genotyping

We screened for somatic mutations in KRAS (codons 12, 13, and 61) and BRAF (codon 600) using both Pyrosequencing and Sequenom, and in BRAF (codon 594), NRAS (codons 12 and 61), and PIK3CA (codons 542, 545, 546, and 1,047) using only Sequenom. In total, 1,612 samples were successfully analyzed for KRAS mutations using both technologies and 8,642/8,719 (99.1%) genotype calls were concordant (ranging from 97.8% to 99.7% depending on which mutation was analyzed). For BRAF V600E, 1,344 samples were successfully analyzed using both technologies and 1,319/1,344 (98.1%) genotype calls were concordant. Forty-three out of 77 samples with discordant KRAS calls and 12/25 samples with discordant BRAF calls were successfully Sanger sequenced to infer genotype. For the remaining calls where Sanger sequencing failed, the mutant genotype was selected (because there was an obvious mutant trace via one technology). Both technologies had high genotype success rates; 41,944/43,340 (96.8%) for Sequenom and 21,016/25,200 (83.4%) for Pyrosequencing.

Frequency and distribution of somatic mutations

In total, for KRAS we successfully genotyped 1,949/1,976 samples (98.6%), for BRAF 1,946/1,963 samples (99.1%),

Table 1. Mutation frequencies, together with MSI status, according to trial-arm (Arm A - continuous oxaliplatin-based chemotherapy, Arm B - continuous chemotherapy plus cetuximab, Arm C - intermittent chemotherapy)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Arm A No. of samples with mutations/ No. successfully analyzed</th>
<th>Arm B No. of samples with mutations/ No. successfully analyzed</th>
<th>Arm C No. of samples with mutations/ No. successfully analyzed</th>
<th>Total No. of samples with mutations/ No. successfully analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>G12A</td>
<td>23/635 (3.6%)</td>
<td>11/659 (1.7%)</td>
<td>19/655 (2.9%)</td>
<td>53/1,949 (2.7%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12D</td>
<td>74/635 (11.7%)</td>
<td>94/659 (14.3%)</td>
<td>73/655 (11.1%)</td>
<td>241/1,949 (12.4%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12V</td>
<td>59/635 (9.3%)</td>
<td>82/659 (12.4%)</td>
<td>67/655 (10.2%)</td>
<td>208/1,949 (10.7%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12C</td>
<td>23/635 (3.6%)</td>
<td>14/659 (2.1%)</td>
<td>31/655 (4.7%)</td>
<td>65/1,949 (3.3%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12R</td>
<td>6/635 (0.9%)</td>
<td>5/659 (0.8%)</td>
<td>14/659 (2.1%)</td>
<td>25/1,949 (1.3%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G12S</td>
<td>14/635 (2.2%)</td>
<td>20/659 (3.0%)</td>
<td>9/659 (1.4%)</td>
<td>43/1,949 (2.2%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G13C</td>
<td>3/635 (0.5%)</td>
<td>2/657 (0.3%)</td>
<td>3/652 (0.5%)</td>
<td>8/1,944 (0.4%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G13S</td>
<td>0/635 (0%)</td>
<td>0/657 (0%)</td>
<td>1/652 (0.2%)</td>
<td>1/1,944 (0.1%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G13D</td>
<td>56/635 (8.8%)</td>
<td>54/659 (8.2%)</td>
<td>41/650 (6.3%)</td>
<td>151/1,944 (7.8%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>G13V</td>
<td>5/635 (0.8%)</td>
<td>5/659 (0.8%)</td>
<td>6/652 (0.9%)</td>
<td>12/1,944 (0.6%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>total</td>
<td>268/635 (42.2%)</td>
<td>297/659 (45.1%)</td>
<td>259/655 (39.5%)</td>
<td>824/1,949 (42.3%)</td>
</tr>
<tr>
<td>BRAF</td>
<td>V600E</td>
<td>50/632 (7.9%)</td>
<td>40/661 (6.1%)</td>
<td>64/651 (9.8%)</td>
<td>154/1,944 (7.9%)</td>
</tr>
<tr>
<td>BRAF</td>
<td>D594G</td>
<td>7/622 (1.1%)</td>
<td>5/655 (0.8%)</td>
<td>9/649 (1.4%)</td>
<td>21/1,926 (1.1%)</td>
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<tr>
<td>BRAF</td>
<td>total</td>
<td>57/632 (9.0%)</td>
<td>45/662 (6.8%)</td>
<td>73/652 (11.2%)</td>
<td>175/1,946 (9.0%)</td>
</tr>
<tr>
<td>NRAS</td>
<td>G12C</td>
<td>0/621 (0%)</td>
<td>1/653 (0.2%)</td>
<td>6/643 (1.0%)</td>
<td>7/1,908 (0.4%)</td>
</tr>
<tr>
<td>NRAS</td>
<td>Q61K</td>
<td>10/612 (1.6%)</td>
<td>12/634 (1.9%)</td>
<td>6/624 (1.0%)</td>
<td>28/1,870 (1.5%)</td>
</tr>
<tr>
<td>NRAS</td>
<td>Q61L</td>
<td>2/633 (0.3%)</td>
<td>3/656 (0.5%)</td>
<td>3/648 (0.5%)</td>
<td>8/1,937 (0.4%)</td>
</tr>
<tr>
<td>NRAS</td>
<td>Q61R</td>
<td>3/633 (0.5%)</td>
<td>3/656 (0.5%)</td>
<td>3/648 (0.5%)</td>
<td>8/1,937 (0.4%)</td>
</tr>
<tr>
<td>NRAS</td>
<td>total</td>
<td>18/631 (2.9%)</td>
<td>32/659 (4.9%)</td>
<td>19/649 (2.9%)</td>
<td>69/1,939 (3.6%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>E542K</td>
<td>20/614 (3.3%)</td>
<td>22/638 (3.4%)</td>
<td>19/640 (3.0%)</td>
<td>60/1,984 (3.1%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>E545K</td>
<td>27/614 (4.4%)</td>
<td>29/637 (4.6%)</td>
<td>31/656 (4.9%)</td>
<td>87/1,907 (4.6%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Q546K</td>
<td>3/612 (0.5%)</td>
<td>10/630 (1.6%)</td>
<td>6/623 (1.0%)</td>
<td>19/1,885 (1.0%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>H1047L</td>
<td>10/616 (1.6%)</td>
<td>13/633 (2.1%)</td>
<td>13/639 (2.0%)</td>
<td>36/1,888 (1.9%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>H1047R</td>
<td>13/616 (2.1%)</td>
<td>14/633 (2.2%)</td>
<td>19/639 (3.0%)</td>
<td>46/1,888 (2.4%)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>total</td>
<td>71/620 (11.5%)</td>
<td>85/643 (13.2%)</td>
<td>87/644 (13.5%)</td>
<td>243/1,907 (12.7%)</td>
</tr>
<tr>
<td>MSI</td>
<td>19/502 (3.8%)</td>
<td>26/520 (5.0%)</td>
<td>21/543 (3.9%)</td>
<td>66/1,565 (4.2%)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Total numbers per locus do not exactly match individual numbers because: (i) for KRAS, 4 samples contained 2 independent mutations and 5 other samples contained uncharacterized mutations, (ii) for NRAS, one sample contained an uncharacterized mutation, and (iii) for PIK3CA, 6 samples contained 2 independent PIK3CA mutations. In those samples where genotypes were missing for rare mutations (with cumulative frequencies <1%), but where all other mutations were successfully tested as wild-type, an overall call of wild-type was made at that locus.
for NRAS 1,939/1,963 samples (98.8%), and for PIK3CA 1,907/1,963 samples (97.1%; Table 1). Fifteen samples (0.8%) consistently failed genotyping. Of the remaining 1,961 samples, 96.3% (n = 1,889) had genotypes for all of these genes and 98.8% (n = 1,938) had genotypes for at least 3 genes. In total, we detected 13 KRAS mutations (G12A, G12D, G12V, G12C, G12R, G13C, G13D, G13S, G13V, Q61H, Q61L, and Q61R, and 5 remained unrecognized), 2 BRAF mutations (D594G and V600E), 4 NRAS mutations (G12C, G61K, Q61L, and Q61R, and one remained unrecognized), and 5 PIK3CA mutations (E542K, E545K, Q546K, H1047L, and H1047R). Overall, KRAS mutations were found in 824/1,949 colorectal cancers (42.3%), BRAF mutations in 175/1,946 advanced colorectal cancers (9.0%), NRAS mutations in 69/1,939 advanced colorectal cancers (3.6%), and PIK3CA mutations in 243/1,907 advanced colorectal cancers (12.7%; Supplementary Fig. S1). Of the 243 advanced colorectal cancers with PIK3CA mutations, 162 had a mutation in exon 9 and 82 had a mutation in exon 20 (one sample carried mutations in both exons). MSI was observed in 66/1,565 (4.2%) of the successfully analyzed advanced colorectal cancers.

KRAS and BRAF mutations were, in general, mutually exclusive (only 4 tumors carried mutations in both oncogenes, 0.2%). Colorectal cancers with mutations in both KRAS and NRAS were seen at a low level (n = 14, 0.7%; Supplementary Fig. S1); Mutations in PIK3CA were more frequently observed in those colorectal cancers with KRAS mutations (127/797, 15.9%) than in those without KRAS mutations (114/1,100, 10.4%; P = 3.1 × 10⁻⁴). This association was most striking in colorectal cancers harboring exon 9 PIK3CA mutations (88/797 cooccurred, 11.0% versus 74/1,100, 6.7% without KRAS mutation; P = 8.9 × 10⁻⁴); and was not observed in those with exon 20 PIK3CA mutations (40/797 cooccurred, 5.0%, vs. 40/1,100, 3.6% without KRAS mutation; P = 0.14). MSI was noted within all somatic mutation subgroups, although there was a significant correlation with BRAF mutations (20/139, 14.4% of BRAF-mutant tumors had MSI vs. 46/1,414, 3.3% BRAF wild-type tumors, P = 5.3 × 10⁻⁴). All of these associations remained significant after correction for multiple testing.

### Table 2. Correlation between the site of the primary and somatic mutation status

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Right colon</th>
<th>Transverse colon</th>
<th>Left colon</th>
<th>Rectosigmoid junction</th>
<th>Rectum</th>
</tr>
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<tbody>
<tr>
<td>KRAS</td>
<td>256/486 (52.7)</td>
<td>15/50 (30.0)</td>
<td>128/334 (38.2)</td>
<td>160/470 (34.0)</td>
<td>263/599 (43.9)</td>
</tr>
<tr>
<td></td>
<td>2.3 × 10⁻²</td>
<td>4.5 × 10⁻²</td>
<td>6.3 × 10⁻²</td>
<td>4.0 × 10⁻³</td>
<td>6.0 × 10⁻²</td>
</tr>
<tr>
<td>BRAF</td>
<td>85/488 (17.4)</td>
<td>14/49 (28.6)</td>
<td>26/334 (7.8)</td>
<td>24/470 (5.1)</td>
<td>25/595 (4.2)</td>
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<tr>
<td></td>
<td>5.5 × 10⁻²</td>
<td>7.2 × 10⁻²</td>
<td>2.0 × 10⁻²</td>
<td>7.8 × 10⁻¹³</td>
<td>7.8 × 10⁻¹³</td>
</tr>
<tr>
<td>NRAS</td>
<td>14/486 (2.9)</td>
<td>1/49 (2.0)</td>
<td>1/335 (0.3)</td>
<td>23/468 (4.9)</td>
<td>18/591 (3.1)</td>
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<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.10</td>
<td>0.87</td>
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<td>PIK3CA</td>
<td>69/473 (14.8)</td>
<td>13/48 (27.1)</td>
<td>44/332 (13.3)</td>
<td>54/461 (11.7)</td>
<td>62/583 (10.6)</td>
</tr>
<tr>
<td></td>
<td>2.4 × 10⁻²</td>
<td>0.47</td>
<td>0.57</td>
<td>0.19</td>
<td>5.3 × 10⁻²</td>
</tr>
<tr>
<td>MSI (BRAFWT)²</td>
<td>22/319 (6.9)</td>
<td>1/30 (3.3)</td>
<td>8/240 (3.3)</td>
<td>6/657 (1.7)</td>
<td>9/460 (2.0)</td>
</tr>
<tr>
<td></td>
<td>0.71</td>
<td>1.0</td>
<td>1.0</td>
<td>1.4 × 10⁻²</td>
<td>5.2 × 10⁻⁴</td>
</tr>
</tbody>
</table>

NOTE: Number of tumors with a mutation in the respective oncogene or with MSI against the total number of specimens tested, with percentages in parentheses.

²Since MSI and BRAF mutations significantly co-occurred, data shown for MSI BRAFWT. P values as compared with the frequency of the respective mutation in patients with a primary tumor in the right colon (²after correction for multiple testing, n = 20).
metastases \((6.7\%; P = 9.2 \times 10^{-4}\) and \(3.7 \times 10^{-5}\), respectively). MSI was also more common in patients with nodal (21.2\%) and peritoneal-only (12.9\%) metastases as compared with those with liver-only metastases (2.0\%; \(P = 4.0 \times 10^{-11}\) and \(7.5 \times 10^{-3}\), respectively), although the association with peritoneal metastases did not withstand correction for multiple testing. The association with nodal metastases was more frequent in those that also carried a \(BRAF\) mutation \((66.7\%\) as compared with 4.2\% of patients with liver-only metastases with MSI and \(BRAF\) mutations; \(P = 1.3 \times 10^{-3}\)), but was also observed in those with \(BRAF\) wild-type tumors \((11.1\%\) as compared with 1.8\% patients with liver-only tumors; \(P = 2.9 \times 10^{-4}\); Table 3).

We determined, whether these correlations were maintained in any patients with lung \((n = 784)\), nodal \((n = 906)\), or peritoneal \((n = 283)\) metastases (Supplementary Table S4). \(KRAS\) mutations were associated with lung and peritoneal metastases \((48.2\%\) and 46.5\% of patients, respectively, as compared with 36.9\% with liver-only metastases, \(P = 1.3 \times 10^{-4}\) and \(1.1 \times 10^{-2}\), respectively), \(BRAF\) mutations with peritoneal metastases \((12.7\%\) of patients as compared with 6.7\% with liver-only metastases, \(P = 6.3 \times 10^{-3}\)), \(NRAS\) mutations with lung metastases \((5.5\%\) of patients as compared with 2.5\% with liver-only metastases, \(P = 1.6 \times 10^{-3}\)), and MSI (\(BRAF\) wild-type) with peritoneal metastases \((5.1\%\) of patients as compared with 1.8\% with liver-only metastases \(P = 3.4 \times 10^{-3}\)). However, only the correlation between \(KRAS\) mutations and lung metastases was maintained after rigorous correction for multiple testing \((P = 2.0 \times 10^{-7}\).

We also determined, whether the correlations between somatic mutation status and site of metastases were maintained when we did not use the “liver-only metastatic group” as the reference cohort. We simply considered the frequency of the somatic mutations in any patients with, versus those without, a specific metastatic site. We found that \(KRAS\) mutations were associated with more patients with lung metastases \((P = 1.3 \times 10^{-3})\), \(BRAF\) mutations with fewer patients with liver \((P = 1.6 \times 10^{-3})\), and lung \((P = 4.0 \times 10^{-3})\) metastases, \(NRAS\) mutations with more patients with lung metastases \((P = 1.2 \times 10^{-4})\) and MSI (\(BRAF\) wild-type) with fewer patients with liver metastases \((P = 1.6 \times 10^{-3})\) (Supplementary Table S5). All of these associations remained significant after correction for multiple testing.

### Table 3. Correlation between the site of the metastases and somatic mutation status (in patients who had a single-organ metastatic site)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Liver-only n</th>
<th>Lung-only</th>
<th>Nodes-only</th>
<th>Peritoneum-only</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>160/434 (36.9)</td>
<td>27/81 (33.3)</td>
<td>14/35 (40.0)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>55/96 (57.3)</td>
<td>0.54</td>
<td>1.00 (^b)</td>
<td>(1.00)</td>
</tr>
<tr>
<td></td>
<td>2.3 (\times 10^{-4})</td>
<td>3.5 (\times 10^{-3})</td>
<td>1.3 (\times 10^{-3})</td>
<td>(P = 1.6 \times 10^{-3})</td>
</tr>
<tr>
<td>BRAF</td>
<td>29/432 (6.7)</td>
<td>17/81 (21.0)</td>
<td>8/36 (22.2)</td>
<td>9.2 (\times 10^{-4})</td>
</tr>
<tr>
<td></td>
<td>5/97 (5.2)</td>
<td>3.7 (\times 10^{-5})</td>
<td>0.92 (\times 10^{-4})</td>
<td>(P = 4.0 \times 10^{-2})</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>0.6 (\times 10^{-4})</td>
<td>0/36 (0)</td>
<td>(0.41)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>(1.00)</td>
<td>0.41</td>
<td>(1.00)</td>
</tr>
<tr>
<td>NRAS</td>
<td>11/432 (2.5)</td>
<td>3/81 (3.7)</td>
<td>0/36 (0)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>2/95 (2.1)</td>
<td>0.38</td>
<td>0.41</td>
<td>(1.00)</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
<td>1.00</td>
<td>(1.00)</td>
<td>(1.00)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>58/429 (13.5)</td>
<td>10/81 (12.3)</td>
<td>2/35 (5.7)</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>10/93 (10.8)</td>
<td>0.78</td>
<td>0.14</td>
<td>(1.00)</td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>1.00</td>
<td>(1.00)</td>
<td>(1.00)</td>
</tr>
<tr>
<td>MSI (BRAF(^{WT}))</td>
<td>6/328 (1.8)</td>
<td>6/54 (11.1)</td>
<td>2/24 (8.3)</td>
<td>9.7 (\times 10^{-2})</td>
</tr>
<tr>
<td></td>
<td>1/77 (1.3)</td>
<td>2.9 (\times 10^{-4})</td>
<td>0.14</td>
<td>(1.00)</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>(4.4 \times 10^{-3})</td>
<td>(1.00)</td>
<td>(1.00)</td>
</tr>
</tbody>
</table>

**Note:** Number of tumors with a mutation in the respective oncogene or with MSI against the total number of specimens tested, with percentages in parentheses.

\(^a\)Since MSI and \(BRAF\) mutations significantly cooccurred, data shown for MSI\(^{BRAF(\text{WT})}\), \(P\) values as compared with the frequency of the respective mutation in patients with liver-only metastases \((P = 1.6 \times 10^{-3})\) (after correction for multiple testing, \(n = 15\).
treatment arm, and chemotherapy regimen (adjusted OS HR = 1.60; 95% CI, 1.14–2.24; P = 6.6 × 10^{-3}; adjusted PFS HR = 1.66; 95% CI, 1.21–2.27; P = 1.6 × 10^{-3}). Significantly, MSI was still associated with worse survival in the BRAF wild-type subgroup (adjusted OS HR = 1.89; 95% CI, 1.30–2.6; P = 8.5 × 10^{-3}; adjusted PFS HR = 1.85; 95% CI, 1.31–2.61; P = 5.1 × 10^{-3}; Fig. 2).

In terms of predicting response to cetuximab, patients with PIK3CA wild-type tumors did not show improved OS or PFS, regardless of chemotherapy regimen [OS HR 1.01; 95% CI, 0.88–1.16; P = 0.84 (any chemotherapy) and HR 0.96; 95% CI, 0.76–1.21; P = 0.73 (OxFU); PFS HR 0.99; 95% CI, 0.88–1.2; P = 0.90 (any chemotherapy) and HR 0.88; 95% CI, 0.71–1.09; P = 0.25 (OxFU); Supplementary Fig. S2] or somatic KRAS status [OS HR 1.03; 95% CI, 0.86–1.24; P = 0.74 and PFS HR 0.92; 95% CI, 0.78–1.08; P = 0.30 (KRAS wild-type); Supplementary Fig. S3].

Patients with MSI appeared to have worse survival after treatment with cetuximab, but this was not statistically significant due to the small number of patients analysed [OS HR 1.27; 95% CI, 0.65–2.45; P = 0.49; PFS HR 1.26, 95% CI, 0.68–2.34; P = 0.47, n = 45 (any chemotherapy)]. We did not observe any individual somatic KRAS, BRAF, NRAS, or PIK3CA mutations, or mutations grouped by exons that were associated with response to cetuximab within those patients treated with any chemotherapy or OxFU after correction for multiple testing (Fig. 3).

Discussion

Here, we used Pyrosequencing and Sequenom for high-throughput somatic mutation detection in the EGFR

Figure 1. Histogram showing the distribution of somatic mutations according to the site of the metastases (in patients who had a single-organ metastatic site). KRAS mutations were associated with lung-only metastases, BRAF mutations with peritoneal and nodal-only metastases, and MSI (BRAF WT) with nodal-only metastases.

Figure 2. Prognostic effect of MSI (BRAF WT) in the advanced disease setting. MSI (BRAF WT) was associated with worse OS (A) and PFS (B), independent of somatic mutation status, treatment arm, and chemotherapy regimen.
pathway. Both assays robustly detected low levels of mutant alleles (provided that the Pyrosequencing extension primers were designed to generate de novo peaks) with all mutations being detected in samples with 12.5% mutant alleles. Previous studies have also suggested that these platforms have equal mutation detection sensitivities, with superior detection limits as compared with traditional Sanger sequencing (26). Our analyses based on approximately 10,000 somatic genotypes, showed that these platforms had 99% genotype concordance and high genotype success rates. However, the main advantage of Sequenom was the ability to multiplex the reactions and herein we describe a three-assay system to screen for 33 somatic mutations within the EGFR pathway.

Our comprehensive somatic profiling of the RAS-RAF-MAP and PI3K-PTEN-AKT subpathways in 1,976 advanced colorectal cancers has allowed us to interrogate any functional cooperations between these pathways in colorectal tumorigenesis. We found that exon 9, but not exon 20, mutations in PIK3CA were associated with KRAS mutations. The reason for this association is likely to be due to the independent biologic roles played by different mutations in PIK3CA. Exon 9 mutations lie in the helical domain of the protein, whereas exon 20 mutations lie in the kinase domain (27). Although mutations of both exons result in activated AKT signaling, they have different requirements for interaction with the PI3K regulatory subunit p85 and with GTP bound RAS. The gain-of-function coinciding with exon 9 mutations requires interaction with GTP bound RAS, but is independent of binding to p85. Conversely, mutations of exon 20 require p85 binding but are independent of GTP bound RAS (27).

Somatic profiling of other oncogenes and tumor suppressor genes within our collection of advanced colorectal cancers are likely to provide further insights into the different mechanisms of colorectal tumorigenesis.

We have previously shown that KRAS and BRAF mutations confer a poor prognosis in patients with advanced colorectal cancer irrespective of treatment (OS - KRAS mutant, 14.4 months; BRAF mutant, 8.8 months; all wild-type, 20.1 months; ref. 25). Here, we show that MSI is also associated with poor prognosis in patients with advanced colorectal cancer (OS - 9.3 months). Consistent with this, a study of 524 patients with advanced colorectal cancer showed that those with tumors with MSI had
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significantly worse survival as compared with those with tumors that were microsatellite-stable (11.1 months vs. 22.1 months, \( P < 0.001 \)) and this association was maintained in the subset of BRAF wild-type tumors (20). It is noteworthy that in the earlier stages of colorectal tumorigenesis, MSI is more frequently observed and is associated with good prognosis (19). These differences warrant further investigation and may be influenced by an interaction with the fluoropyrimidine-based treatment used in COIN.

In agreement with others (20–23), we found that KRAS mutations were highly associated with lung-only metastases and BRAF mutations with peritoneal and nodal-only metastases. In addition, we noted that MSI was associated with nodal-only metastases and all of these associations were maintained after correction for multiple testing. We also found that the associations between KRAS mutations and lung metastases, and BRAF mutations with peritoneal metastases were maintained in the larger and more heterogeneous cohort of “any” patient with those metastases, although only the KRAS association remained significant after correction for multiple testing. Even when we conducted these analyses without using a liver-only cohort as a reference group, we still found that the association between KRAS mutations and lung metastases was maintained. Our data therefore show that different somatic profiles are associated with different metastatic sites, suggesting that these profiles influence the tumor’s biologic behavior (20). It is important to note that in terms of clinical utility these profiles may be of limited use. No marker could be used to reliably predict a metastatic site, and patients are routinely monitored with computed tomography scans that include thorax, abdomen, and pelvis anyway.

Although it is clear from existing data that there is a paucity of KRAS and BRAF mutations in tumors from the left colon, it is not clear whether there is a demarcation between KRAS and BRAF mutant tumors in the ascending and transverse colons. In the COIN trial, we collected data on the location of the primary tumor using right and left colon, rectosigmoid junction, rectum, and other (in which transverse colon was often reported). Although we noted an excess of KRAS and BRAF mutations in tumors from the right colon, we also noted an excess of BRAF mutations in colorectal cancers from the transverse colon (28.6%), which was not seen for KRAS mutations. This observation should be treated with caution because the junction between midgut and hindgut (and hence the embryologic separation of the right and left colon) occurs at a variable location within the transverse colon; however, these data suggest that future studies should categorize colorectal cancers into ascending, transverse, and descending colon to help identify potential differences between colorectal cancers harboring mutations in these oncogenes. Such differences may help explain the different patterns of metastatic spread.

We have previously reported that cetuximab had a borderline improvement in PFS amongst KRAS, BRAF, and NRAS wild-type patients treated with OxelUS. In the more comprehensive analysis reported herein, this association failed to reach statistical significance (\( P = 0.06 \)). Interestingly, others have reported that patients with PIK3CA exon 20 mutations had worse response to cetuximab (10) and that different somatic mutations in KRAS have differential effects on response (8, 9). However, our data do not support either of these observations which may be a consequence of the complicating gastrointestinal toxic effects of the drugs used in the COIN trial, a mechanistic interaction between cetuximab and oxaliplatin, or the use of small cohorts of patients harboring individual somatic mutations.

References


Somatic Profiling of the Epidermal Growth Factor Receptor Pathway in Tumors from Patients with Advanced Colorectal Cancer Treated with Chemotherapy ± Cetuximab

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