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 × 10⁻⁶) 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...
and PI3K-PTEN-AKT pathways. Through these, EGFR regulates multiple cellular processes including apoptosis, growth, proliferation, differentiation, and migration (6). Cetuximab binds to the extracellular binding domain of EGFR, thereby preventing its ligands binding to the receptor and triggering receptor internalization, thus inhibiting downstream signaling. Response to cetuximab has been suggested to be limited to patients with colorectal cancers wild-type for KRAS. Given the high-frequency of colorectal cancers that are KRAS mutant, guidelines are now in place which recommend testing of KRAS mutation status before treatment with anti-EGFR agents (7). However, recent data have indicated that not all somatic mutations within KRAS are refractory to cetuximab, and patients with G13D have longer overall survival (OS) and progression-free survival (PFS) after treatment, as compared with patients with other KRAS mutations (8, 9). Other genes within the EGFR pathway may also affect response to cetuximab, with efficacy likely to be dependent upon an absence of somatic mutations in BRAF, NRAS, and exon 20 of PIK3CA (10).

During embryologic development, the right colon (cecum, ascending colon, proximal two-thirds of the transverse colon) arises from the midgut, and the left colon (distal one-third of the transverse colon, descending and sigmoid colon, and rectum) from the hindgut. Differences exist in the macroscopic pathology, histopathology, and molecular biologic patterns between right-sided colon cancers (RCC) and left-sided colon cancers (LCC; 11–17). Interestingly, RCCs have been associated with peritoneal metastases and LCC with hepatic and pulmonary metastases, and this has been attributed to their different molecular biologic patterns causing distinct biologic behaviors (18).

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 or 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 REC[04/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
Microkits (Qiagen) and eluted in 50 mL 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'-GGCCCTGCTGAAAATGACTGA-3' and 5'-AGAATGTGCTCAGGACCATGAATTA-3' together with extension primer 5'-CTTGTGGCTAGTGGAGGC-3', however, this assay was subsequently modified by using the extension primers 5'-TGTGCTAGTGGAGGCAGTGGAGC-3', 5'-TGTGCTAGTGGAGGCAGTGGAGC-3', and 5'-TGTGCTAGTGGAGGCAGTGGAGC-3', as previously described (24). For codon 61 of KRAS, we used the amplification primers 5'-CTTTGGAGCAGGAACAATGTC-3' and 5'-CTCATGCTACTGCTCCCTGATG-3' together with the extension primer 5'-ATTCTCGACACAGCAGGT-3', and for codon 600 of BRAF we used the amplification primers 5'-TGCTGGCTCTAGAAGATGGA-3' and 5'-CAGGGGCCCAAATTTAATGCTG-3' together with the extension primer 5'-ATTCTCGACACAGCAGGT-3'. Reverse primers were biotinylated. PCR was conducted in 50 mL reaction volumes containing 25 mL Megamix Gold (Microzone), 10–20 ng DNA, and 10 mol/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 1 minute, followed by a final extension of 72°C for 1 minute. 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 12.5% mutant alleles, G12C and G13D in samples with 6% mutant alleles, and G12V in samples with 4% mutant alleles. Sequenom robustly detected V600E in BRAF and Q61R in NRAS in samples with 10% mutant alleles, G12C and Q61L 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%), **NRAS** 1,882/1,898 samples (99.7%), and **PIK3CA** 2,642/2,680 samples (98.8%).

<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><strong>KRAS</strong></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>
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<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>
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<tr>
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<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>
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<td>G12C</td>
<td>23/635 (3.6%)</td>
<td>14/659 (2.1%)</td>
<td>3/655 (0.5%)</td>
<td>68/1,949 (3.5%)</td>
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<td>G12R</td>
<td>6/635 (0.9%)</td>
<td>5/659 (0.8%)</td>
<td>3/655 (0.5%)</td>
<td>14/1,949 (0.7%)</td>
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<td>G12S</td>
<td>14/635 (2.2%)</td>
<td>20/659 (3.0%)</td>
<td>9/655 (1.4%)</td>
<td>43/1,949 (2.2%)</td>
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<tr>
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<td>G13C</td>
<td>3/635 (0.5%)</td>
<td>2/657 (0.9%)</td>
<td>3/652 (0.5%)</td>
<td>8/1,944 (0.4%)</td>
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<td>0/657 (0%)</td>
<td>1/652 (0.2%)</td>
<td>1/1,944 (0.1%)</td>
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<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>
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<td>Q61H</td>
<td>5/518 (1.0%)</td>
<td>8/541 (1.5%)</td>
<td>6/539 (1.1%)</td>
<td>19/1,598 (1.2%)</td>
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<tr>
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<td>Q61L</td>
<td>2/633 (0.3%)</td>
<td>3/656 (0.5%)</td>
<td>1/652 (0.2%)</td>
<td>8/1,937 (0.4%)</td>
</tr>
<tr>
<td></td>
<td>Q61R</td>
<td>3/633 (0.5%)</td>
<td>3/656 (0.5%)</td>
<td>1/652 (0.2%)</td>
<td>8/1,937 (0.4%)</td>
</tr>
<tr>
<td><strong>KRAS total</strong></td>
<td></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><strong>BRAF</strong></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>
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<tr>
<td></td>
<td>D594G</td>
<td>7/622 (1.1%)</td>
<td>5/655 (0.8%)</td>
<td>0/650 (0%)</td>
<td>12/1,926 (0.6%)</td>
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<tr>
<td><strong>BRAF total</strong></td>
<td></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>
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<tr>
<td><strong>NRAS</strong></td>
<td>G12C</td>
<td>0/621 (0%)</td>
<td>11/653 (1.7%)</td>
<td>6/634 (1.0%)</td>
<td>17/1,878 (0.9%)</td>
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<tr>
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<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>
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<tr>
<td></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></td>
<td>Q61R</td>
<td>3/633 (0.5%)</td>
<td>3/656 (0.5%)</td>
<td>2/648 (0.3%)</td>
<td>8/1,937 (0.4%)</td>
</tr>
<tr>
<td><strong>NRAS total</strong></td>
<td></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>
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<tr>
<td><strong>PIK3CA</strong></td>
<td>E542K</td>
<td>20/614 (3.3%)</td>
<td>22/638 (3.4%)</td>
<td>19/640 (3.0%)</td>
<td>60/1,932 (3.1%)</td>
</tr>
<tr>
<td></td>
<td>E545K</td>
<td>27/614 (4.4%)</td>
<td>29/637 (4.6%)</td>
<td>31/636 (4.9%)</td>
<td>87/1,887 (4.6%)</td>
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<tr>
<td></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>
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<td></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>
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<tr>
<td></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>
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<tr>
<td><strong>PIK3CA total</strong></td>
<td></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>
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<td><strong>MSI</strong></td>
<td></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>
</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, 1,907/1,965 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, G12S, G13C, G13D, G13S, G13V, Q61H, Q61L, and Q61R, and 5 remained uncharacterized), 2 BRAF mutations (D594G and V600E), 4 NRAS mutations (G12C, Q61K, Q61L, and Q61R, and one remained uncharacterized), and 5 PIK3CA mutations (E542K, E545K, Q546K, H1047L, and H1047R). Overall, KRAS mutations were found in 824/1,946 advanced 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 (114/1,101, 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 74/1,101, 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. 40/1,101, 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^-10). All of these associations remained significant after correction for multiple testing.

**Somatic mutation status and site of the primary tumor**

KRAS mutations were more common in colorectal cancers from the right colon (52.7%) as compared with those from the left colon (38.2%, P = 5.2 × 10^-5; Table 2) and BRAF mutations were more common in colorectal cancers from the transverse (28.6%) and right (17.4%) colon as compared with those from the left colon (7.8%, P = 9.8 × 10^-4 and 7.2 × 10^-3, respectively; Table 2). PIK3CA mutations were more common in colorectal cancers from the transverse colon (27.1%) as compared with the left colon (13.3%), which although significant (P = 0.01), did not withstand rigorous correction for multiple testing (Table 2).

**Somatic mutation status and site of the metastases**

We tested whether the somatic mutation status in the primary colorectal cancers correlated with the site of the metastases and initially analyzed 815 patients from COIN that had a single organ metastatic site. Of these, 547 had liver-only, 128 had lung-only, 93 had nodal-only, and 47 had peritoneal-only metastases. We generated somatic mutation data on the primary colorectal cancers from 79.3%, 75.0%, 87.1%, and 74.5% of these patients, respectively. Significantly, 57.3% of patients with lung-only metastases had KRAS mutations as compared with 36.9% of patients with liver-only metastases (P = 2.3 × 10^-4, Table 3; Fig. 1). This association was primarily driven by the mutations G12V and G12C (Supplementary Table S3). BRAF mutations were significantly more common in patients with peritoneal-only (22.2%) and nodal-only (21.0%) metastases, as compared with those with liver-only metastases.

**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>
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<tbody>
<tr>
<td>KRAS</td>
<td>256/486 (52.7)</td>
<td>15/50 (30.0)</td>
<td>2.3 × 10^-2</td>
<td>128/334 (38.2)</td>
<td>5.2 × 10^-5</td>
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<td></td>
<td></td>
<td></td>
<td>4.5 × 10^-3</td>
<td>160/470 (34.0)</td>
<td>6.3 × 10^-2</td>
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<td></td>
<td></td>
<td></td>
<td>21.0 × 10^-7</td>
<td>263/599 (43.9)</td>
<td>4.0 × 10^-3</td>
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<tr>
<td>BRAF</td>
<td>85/488 (17.4)</td>
<td>4/49 (8.8)</td>
<td>5.5 × 10^-7</td>
<td>26/334 (7.8)</td>
<td>7.2 × 10^-2</td>
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<td></td>
<td></td>
<td></td>
<td>21.0 × 10^-7</td>
<td>25/595 (4.2)</td>
<td>7.8 × 10^-13</td>
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<tr>
<td>NRAS</td>
<td>14/486 (2.9)</td>
<td>1/49 (2.0)</td>
<td>1.0</td>
<td>11/335 (3.3)</td>
<td>0.74</td>
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<td>1.0</td>
<td>23/468 (4.9)</td>
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<td>18/591 (3.1)</td>
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<tr>
<td>PIK3CA</td>
<td>69/473 (14.8)</td>
<td>13/48 (27.1)</td>
<td>2.4 × 10^-2</td>
<td>44/332 (13.3)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0 × 10^-2</td>
<td>54/461 (11.7)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>62/583 (10.6)</td>
<td>5.3 × 10^-2</td>
</tr>
<tr>
<td>MSI (BRAFWT)</td>
<td>22/319 (6.9)</td>
<td>1/30 (3.3)</td>
<td>0.71</td>
<td>8/240 (3.3)</td>
<td>6.4 × 10^-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>6/357 (1.7)</td>
<td>6.8 × 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.

**Since 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 (after 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; $P = 1.3 \times 10^{-4}$), 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 ($P = 2.0 \times 10^{-4}$).

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.

### Mutation status, survival, and response to cetuximab

We have previously shown that the addition of cetuximab to standard chemotherapy did not improve OS or PFS in patients from COIN with $KRAS$, $BRAF$, and $NRAS$ wild-type tumors (25). However, exploratory analyses revealed that cetuximab had a borderline improvement in PFS among such patients when used with oxaliplatin and infusional 5-FU (OxFU; HR 0.72; 95% CI, 0.53–0.98, $P = 3.7 \times 10^{-2}$), but not oral 5-FU (HR 1.02, 95% CI, 0.82–1.26; $P = 0.88$). Here, we tested whether PIK3CA mutation status, MSI status, individual somatic $KRAS$, $BRAF$, $NRAS$, and PIK3CA mutations or somatic mutations grouped by exon, were associated with response to cetuximab in either the full cohort or those treated with OxFU.

Irrespective of the treatment arm (to search for potential prognostic effects), PIK3CA mutation status did not affect OS (HR = 0.91; 95% CI, 0.75–1.11; $P = 0.37$) or PFS (HR = 1.06; 95% CI, 0.89–1.26; $P = 0.49$). This was regardless of whether PIK3CA mutations were split between those in exon 9 (OS HR = 0.88; 95% CI, 0.70–1.2; $P = 0.31$; PFS HR = 1.07; 95% CI, 0.87–1.32; $P = 0.51$), and those in exon 20 (OS HR = 0.92; 95% CI, 0.65–1.28; $P = 0.61$; PFS HR = 1.00; 95% CI, 0.74–1.36; $P = 0.99$). MSI was associated with worse survival independent of somatic mutation status,

### 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 (n%)</th>
<th>Lung-only n (n%)</th>
<th>Nodes-only n (n%)</th>
<th>Peritoneum-only n (n%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$KRAS$</td>
<td>160/434 (36.9)</td>
<td>55/96 (57.3)</td>
<td>27/81 (33.3)</td>
<td>14/35 (40.0)</td>
</tr>
<tr>
<td>$BRAF$</td>
<td>29/432 (6.7)</td>
<td>5/97 (5.2)</td>
<td>17/81 (21.0)</td>
<td>8/36 (22.2)</td>
</tr>
<tr>
<td>$NRAS$</td>
<td>11/432 (2.5)</td>
<td>2/95 (2.1)</td>
<td>3/81 (3.7)</td>
<td>0/32 (0)</td>
</tr>
<tr>
<td>$PIK3CA$</td>
<td>58/429 (13.5)</td>
<td>10/93 (10.8)</td>
<td>10/81 (12.3)</td>
<td>2/35 (5.7)</td>
</tr>
<tr>
<td>MSI ($BRAF^WT$)</td>
<td>6/328 (1.8)</td>
<td>1/77 (1.3)</td>
<td>6/54 (11.1)</td>
<td>2/24 (8.3)</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.

*since MSI and $BRAF$ mutations significantly cooccurred, data shown for MSI $BRAF^WT$, $P$ values as compared with the frequency of the respective mutation in patients with liver-only metastases (after correction for multiple testing, $n = 15$).
treatment arm, and chemotherapy regimen (adjusted OS HR = 1.60; 95% CI, 1.14–2.24; \( P = 6.6 \times 10^{-3} \), adjusted PFS HR = 1.66; 95% CI, 1.21–2.27; \( P = 1.6 \times 10^{-3} \)). Significantly, MSI was still associated with worse survival in the \( \text{BRAF} \) wild-type subgroup (adjusted OS HR = 1.89; 95% CI, 1.30–2.66; \( P = 8.5 \times 10^{-4} \), adjusted PFS HR = 1.85; 95% CI, 1.31–2.61; \( P = 5.1 \times 10^{-4} \), Fig. 2).

In terms of predicting response to cetuximab, patients with \( \text{PIK3CA} \) 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, 0.88–1.12; \( P = 0.90 \) (any chemotherapy) and HR 0.88; 95% CI, 0.71–1.09; \( P = 0.25 \) (OxFU); Supplementary Fig. S2] or somatic \( \text{KRAS} \) 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; \( P = 0.30 \) (\( \text{KRAS} \) 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, 0.68–2.43; \( P = 0.47 \), \( n = 45 \) (any chemotherapy)]. We did not observe any individual somatic \( \text{KRAS} \), \( \text{BRAF} \), \( \text{NRAS} \), or \( \text{PIK3CA} \) 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](image1.png)

**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** (\( \text{BRAF}^{\text{WT}} \)) with nodal-only metastases.

![Figure 2](image2.png)

**Figure 2.** Prognostic effect of MSI (\( \text{BRAF}^{\text{WT}} \)) in the advanced disease setting. MSI (\( \text{BRAF}^{\text{WT}} \)) was associated with worse OS (A) and PFS (B), independent of somatic mutation status, treatment arm, and chemotherapy regimen.
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
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 liver-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 then 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 practice, we 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.

We have previously reported that cetuximab had a borderline improvement in PFS amongst \( KRAS \), \( BRAF \), and \( NRAS \)-wild type patients treated with OXF1L. 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.

Disclosure of Potential Conflicts of Interest

d-Lambrechts is consultant/advisory board member and has commercial research grant from Roche and Sanofi and T.S. Maughan has honoraria from speakers’ bureau from Merck Serono. An unrestricted educational grant from Merck Serono provided additional support to this work and the trial (to J.P. Cheadle).

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Claes, T.S. Maughan, A. Meade, B. Jasani, R.A. Adams, R. Kaplan, D. Lambrechts, J.P. Cheadle

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.G. Smith, D. Fisher, T.S. Maughan, G. Peuteman, B. Harris, A. Meade, R.A. Adams, J.P. Cheadle

Writing, review, and/or revision of the manuscript: C.G. Smith, D. Fisher, T.S. Maughan, A. Meade, B. Jasani, R.A. Adams, S. Kenny, R. Kaplan, J.P. Cheadle

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.G. Smith, S. Idziaszczyk, M.D. James, A. Meade, R.A. Adams, S. Kenny, J.P. Cheadle

Study supervision: A. Meade, J.P. Cheadle

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References


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