PIK3CA Mutations Are Not a Major Determinant of Resistance to the Epidermal Growth Factor Receptor Inhibitor Cetuximab in Metastatic Colorectal Cancer

Hans Prenen, Jef De Schutter, Bart Jacobs, Wendy De Roock, Bart Biesmans, Bart Claes, Diether Lambrechts, Eric Van Cutsem, and Sabine Tejpar

Abstract

Purpose: It has been reported that activating KRAS mutations negatively affect response to anti–epidermal growth factor receptor (EGFR) monoclonal antibodies in metastatic colorectal cancer. The mutation status of signaling molecules downstream of the EGFR target is thus crucial to predict clinical benefit to EGFR-targeted therapies. Other mechanisms of resistance to EGFR inhibitors could involve activating mutations of the other main EGFR effector pathway, i.e., the PI3K/PTEN/AKT pathway.

Experimental Design: We analyzed the PIK3CA and KRAS mutation status in a large group (\(n = 200\)) of chemorefractory metastatic colorectal cancers treated with cetuximab (Erbitux) in monotherapy or in combination with irinotecan, and correlated the mutation status with outcome.

Results: Twenty-three (12%) of the 200 samples carried 1 of the PIK3CA mutations included in our assay. We found no correlation between the presence of a PIK3CA mutation and impaired response to cetuximab.

Conclusions: Our findings do not provide any evidence for a strong role of PIK3CA mutations as a single marker in determining response to cetuximab in chemorefractory metastatic colorectal cancer.

The identification of patients with colorectal cancer that respond to the epidermal growth factor receptor (EGFR) inhibitors cetuximab and panitumumab remains a major challenge.

It is well-established that activation of the EGFR pathway triggers a complex program of intracellular signals. The two major pathways activated by the EGFR are the KRAS/RAF/MEK/ERK 1/2 pathway, which controls gene transcription, cell-cycle progression, and cell proliferation, and the PI3K/PTEN/AKT pathway, which activates a cascade of antiapoptotic and prosurvival signals. It has been shown recently that KRAS, a small G-protein downstream of EGFR, can acquire activating mutations, which isolate the KRAS/RAF/MEK/ERK1/2 pathway from its receptor and thereby render EGFR inhibitors ineffective. As a result, activating KRAS mutations negatively affect response to anti-EGFR monoclonal antibodies in metastatic colorectal cancer (1–3). The mutation status of signaling molecules downstream of the EGFR target is thus crucial to predict clinical benefit to EGFR-targeted therapies.

Although patients without a KRAS mutation exhibit a much better response to EGFR inhibitors, not all of them respond to treatment. For instance, the response rate to cetuximab in advanced colorectal cancer patients with wild-type (WT) KRAS tumors was still limited to only 40% (3). Other mechanisms of resistance to EGFR inhibitors thus exist, and could for instance involve activating mutations of the other main EGFR effector pathway, i.e., the PI3K/PTEN/AKT pathway. Indeed, a recent report by Perrone et al. (4) investigated a cohort of 54 colorectal cancer tumors treated with cetuximab, and identified 4 mutations in the PIK3CA gene, which is the p110α catalytic subunit of PI3K, in patients not responding to cetuximab, thereby suggesting that mutations in PIK3CA cause resistance to cetuximab.

In the current study, we further investigated the PIK3CA and KRAS mutation status in a much larger group (\(n = 200\)) of chemorefractory metastatic colorectal cancers treated with cetuximab (Erbitux) in monotherapy or in combination with irinotecan, and correlated the mutation status with outcome. Our aim was to investigate a possible role of PIK3CA mutations in resistance to cetuximab in KRAS WT patients.

Patients and Methods

Patients and treatment. Two hundred irinotecan-refractory patients with metastatic colorectal adenocarcinoma who received cetuximab (Erbitux; Merck Serono) in monotherapy or in combination with irinotecan in several clinical trials (salvage, Bond, Babel, Everest; \(n = 197\); refs. 5–7), or outside of a clinical trial in the same setting.
**Translational Relevance**

The identification of patients with colorectal cancer that respond to the epidermal growth factor receptor (EGFR) inhibitor cetuximab and panitumumab remains a major challenge. It has been shown recently that activating KRAS mutations negatively affect response to anti-EGFR monoclonal antibodies in metastatic colorectal cancer. Although patients without a KRAS mutation exhibit a much better response to EGFR inhibitors, not all of them respond to treatment. Other mechanisms of resistance to EGFR inhibitors could involve activating mutations of the other main EGFR effector pathway, i.e., the PI3K/PTEN/AKT pathway. In the current study, we investigated the PIK3CA and KRAS mutation status in a large group (n = 200) of chemorefractory metastatic colorectal cancer treated with cetuximab (Erbilux) in monotherapy or in combination with irinotecan, and correlated the mutation status with outcome. Our findings did not provide any evidence for a strong role of PIK3CA mutations as a single marker in determining response to cetuximab.

According to standard reimbursement (n = 3), were screened (median age, 61 y; range, 26-89; 120 men). These 200 patients were randomly chosen according to the availability of formalin-fixed, paraffin-embedded tissue and availability of clinical data. Table 1 summarizes the number of patients in each trial and their type of treatment. Tumor assessment was done using the Response Evaluation Criteria in Solid Tumors criteria as planned in these trials. The patients showing a complete or partial response were classified as responders and those with stable disease or progressive disease as nonresponders. All the patients gave their informed consent to participate in these clinical trials.

**Samples and mutational analysis.** The analysis was done using formalin-fixed, paraffin-embedded tissue of the primary colorectal cancer. Seven KRAS codon 12 and 13 mutations (p.G12V, p.G12D, p.G12A, p.G12S, and p.G12R) were detected by allele specific PCR, as reported previously (3). Activating mutations in PIK3CA tend to cluster in hotspots with ~80% accounted for by oncogenic substitution in exon 20 (H1047R) and exon 9 (E542K and E545K). Known PIK3CA mutations in these exons were evaluated using the Sequenom MALDI TOF MassArray system. Briefly, genotyping assays (primers for PCR amplification and the extension probe) were designed using the Sequenom MassARRAY Assay Design 3.0 software, applying default parameters. Multiplexed PCR was done with 0.2 units of Taq polymerase and 20 ng of genomic DNA, as described previously (9).

Thermocycling was at 95°C for 15 min followed by 45 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 1 min. Unincorporated deoxynucleotide triphosphates were deactivated using shrimp alkaline phosphatase, and primer extension was carried out using extension primers, deoxynucleotide triphosphates, and Thermosequenase DNA polymerase. Reactions were cycled at 94°C for 2 min, followed by 44 cycles of 94°C for 5 s, 52°C for 5 s, and 80°C for 5 s. After the addition of a cation exchange resin to remove residual salt from the reactions, the purified primer extension reaction was loaded onto a matrix pad (3-hydroxypicoloinic acid) of a SpectroCHIP (Sequenom) and analyzed using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer. The mutations were assessed as follows: p.R88Q (c.263G>A), p.N345K (c.1035T>A), p.E542K (c.1624G>A), p.E545K, A or V (c.1633G>A, c.1634A>C, G or T), p.Q546K (c.1636C>A), p.H1047R, R or L (c.3139C>T and c.3140A>G or T). In case mutations were detected on the Sequenom, they were subsequently also confirmed by standard DNA sequencing.

**Statistical analysis.** Sample size calculation was done using the following parameters: α, 0.05; power, 85%; global PIK3CA mutation rate, 10%; response rate in KRAS WT tumors, 40%; and prevalence of KRAS mutation, 40% (3). With these assumptions, a sample of 200 patients would be able to detect a decrease in response rate from 40% to 7% or lower. This calculation was done using pwr package for R (function: pwr.2p2n.test).

A two-sided Fisher’s exact test was used to evaluate the association between PIK3CA, KRAS mutations, and response to cetuximab. The progression-free survival (PFS) and the overall survival (OS) were estimated by the Kaplan-Meier method, and the groups were compared with the log-rank test. All statistical tests were two-sided. A P value of <0.05 was considered statistically significant. Statistical analyses were done using SPSS software version 16.0 (SPSS, Inc.).

**Results**

**Mutation analysis.** KRAS mutations were detected in 1 of 38 (2.6%) responders and 76 of 161 (47%) nonresponders. Twenty-three (12%) of the 200 samples carried 1 of the PIK3CA mutations included in our assay. These missense mutations were found in exon 1 [p.R88Q (n = 1)], exon 9 [p.N345K (n = 1), p.E542K (n = 5), p.E545K (n = 11), and p.Q546K (n = 1)], and exon 20 [p.H1047R (n = 3)]. One sample carried a mutation in both exon 9 (p.E545K) and 20 (p.H1047R). All mutations were confirmed by sequencing; however, it must be noted that for at least 8 samples (4%), the mutation may have been missed by sequencing if Sequenom data had not been available. One new mutation, A1565G, was identified by sequencing and was not present on the Sequenom assay. This patient also showed an E542K mutation and was thereby classified in the PIK3CA mutant group.

Nine of 77 (11.7%) KRAS mutants and 14 of 122 (11.5%) KRAS WT tumors harbored a PIK3CA mutation (P = 1.00), thus indicating that PIK3CA mutations occurred independently of the KRAS mutation status.

**Correlation with response.** Response was observed in 39 of 200 (19.5%) patients. As expected, almost all responders were in KRAS WT tumors (37 of 122 (30.3%)), whereas 1 response occurred in a KRAS mutant tumor [1 of 77 (1.3%)]. We found no correlation between the presence of a PIK3CA mutation and impaired response to cetuximab. PIK3CA mutations were detected in 5 of 39 (13%) of the responders and 18 of 160 (11%) of the nonresponders (P = 0.781). In KRAS WT patients, the response rate is 32 of 108 (29.6%) in PIK3CA WT versus 5 of 14 (35.7%) in PIK3CA mutants (Fisher’s Exact test, P = 0.758; Table 2). Table 3 summarizes the distribution of all mutations in the different response groups.

**Table 1. Overview of patients treated in the different clinical trials**

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Cetuximab monotherapy (no of patients)</th>
<th>Cetuximab + irinotecan (no of patients)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BABEL</td>
<td>0</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>EVEREST&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td>SALVAGE&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>BOND&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>By label</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>184</td>
<td>200</td>
</tr>
</tbody>
</table>
Cancer Therapy: Clinical

consequence, PIK3CA significant numbers of breast and other cancers (8, 11). As a PIK3CA subunit of class 1A PI3K (activating mutations of the gene that encodes the catalytic inhibition (10). Tumors EGFR independent and therefore not sensitive to EGFR negative predictive markers such as KRAS that would render a challenge. The largest success till now has been to identify a test, compared with mutants [median OS, 45.0 weeks (95% CI, 0.77)]. Similarly, the OS was significantly better in KRAS WTs versus 39 weeks (95% CI, 32.5-57.5) WT patients were 24 weeks (95% CI, 17.3-30.7) versus 18 weeks (95% CI, 16.6-19.4), and 45 weeks (95% CI, 32.5-57.5) versus 39 weeks (95% CI, 35.4-42.6; P = 0.760 and P = 0.698, respectively).

Adding the therapy (monotherapy or combination) as a covariate did not affect the results for PIK3CA. The type of therapy (combination versus monotherapy) was an independent predictive factor for OS [hazard ratio, 0.54 (0.32-0.91); P = 0.02] but not for PFS.

Discussion

Response prediction to the EGFR inhibitors remains a major challenge. The largest success till now has been to identify negative predictive markers such as KRAS that would render tumors EGFR independent and therefore not sensitive to EGFR inhibition (10).

Mutations in KRAS occur in almost 40% of the patients, and activating mutations of the gene that encodes the catalytic subunit of class 1A PI3K (PIK3CA) have also been identified in significant numbers of breast and other cancers (8, 11). As a consequence, PIK3CA and KRAS are the most commonly mutated oncogenes identified in human cancer (12, 13). However, both these oncogenic mutations play different roles in colorectal carcinogenesis: whereas KRAS mutations are involved in an earlier stage such as adenoma, PIK3CA mutations may be involved in a later stage such as invasive carcinoma.

A recent addition to the family of genes encoding resistance to EGFR inhibitors is BRAF (14). Indeed, a BRAF V600E mutation was detected in 11 patients of 80 KRAS WT patients, with none of these patients responding to EGFR inhibitors. Likewise, mutations in PIK3CA could also explain resistance to anti-EGFR treatment. Therefore, it was our aim to investigate the role of PIK3CA mutations in resistance to cetuximab in KRAS WT patients.

In our study, KRAS mutations were detected in 1 of 38 responders and 76 of 161 nonresponders (P < 0.0005), which is in line with previously published papers (1, 15, 16). Twelve percent of metastatic colorectal cancer patients carried one of the PIK3CA mutations that could be detected by our assay. The frequency and distribution of the various PIK3CA mutations detected in our colorectal cancer patients are also in agreement with previous studies (8, 17–22) and with frequencies reported in the Catalogue Of Somatic Mutations In Cancer database, with p.E542K, p.E545K, and p.H1047R being most commonly mutated. Although we agree that several groups have reported a higher mutation frequency in PIK3CA (13), others have found mutation rates comparable with our results. For instance, Nosho et al. (18) sequenced exon 9 and 20 of PIK3CA and reported a mutation frequency of 15% in 590 primary colorectal cancer. Velho et al. (17) also screened exon 9 and 20 and found a PIK3CA mutation frequency of 13.6% (n = 100). Because the Sequenom assay was focused on the most frequent mutations (80%) in exon 9 and 20, these results are in fact comparable with those reported in the literature.

In colorectal cancer, deregulation of the PI3K signaling pathway is frequent (10-30%) and increases with stage (13, 17–23). Some studies reported that 8% to 24% of the tumors carried both PIK3CA and KRAS mutations, whereas other failed to report such frequencies (18–22). Our study did not show any association between the presence of a KRAS and a PIK3CA mutation.

Our data suggest that activating mutations of PI3K do not play a major role in causing resistance to the EGFR-inhibitor cetuximab because five patients with PIK3CA mutations showed an objective response to the drug and response rates

### Table 2. Summary of PIK3CA mutations in responders versus nonresponders

<table>
<thead>
<tr>
<th>Codon</th>
<th>Cases in responders</th>
<th>Cases in nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.R88Q</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>p.N345K</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>p.E542K</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>p.E545K</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>p.Q546K</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>p.H1047R</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>p.E545K/p.H1047R</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>

The PFS was significantly better in KRAS WTs compared with mutants (median PFS 24.0, weeks [95% confidence interval (95% CI), 22.0-26.0] versus 12.0 weeks [95% CI, 8.6-15.4]; Log-rank test, P < 0.0001; hazard ratio, 0.56 [95% CI, 0.41-0.77]). Similarly, the OS was significantly better in KRAS WTs compared with mutants [median OS, 45.0 weeks (95% CI, 35.9-54.1) versus 26.0 weeks (95% CI, 18.8-33.2); Log-rank test, P < 0.0001; hazard ratio, 0.50 (95% CI, 0.37-0.69)].

Median PFS (Fig. 1) and OS (Fig. 2) in PIK3CA mutant and WT patients were 24 weeks (95% CI, 17.3-30.7) versus 18 weeks (95% CI, 16.6-19.4), and 45 weeks (95% CI, 32.5-57.5) versus 39 weeks (95% CI, 35.4-42.6; P = 0.760 and P = 0.698, respectively).

Adding the therapy (monotherapy or combination) as a covariate did not affect the results for PIK3CA. The type of therapy (combination versus monotherapy) was an independent predictive factor for OS [hazard ratio, 0.54 (0.32-0.91); P = 0.02] but not for PFS.

### Table 3. Distribution of mutations throughout the response groups

<table>
<thead>
<tr>
<th>Mutation</th>
<th>n (row %)</th>
<th>CR</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS WT/PIK3K WT</td>
<td>1 (0.9)</td>
<td>31 (29)</td>
<td>55 (51)</td>
<td>21 (19)</td>
<td>108 (100)</td>
<td></td>
</tr>
<tr>
<td>KRAS WT/PIK3K mutant</td>
<td>0</td>
<td>5 (36)</td>
<td>8 (57)</td>
<td>1 (7)</td>
<td>14 (100)</td>
<td></td>
</tr>
<tr>
<td>KRAS mutant/PIK3K WT</td>
<td>0</td>
<td>1 (1)</td>
<td>42 (62)</td>
<td>25 (37)</td>
<td>68 (100)</td>
<td></td>
</tr>
<tr>
<td>KRAS mutant/PIK3K mutant</td>
<td>0</td>
<td>0</td>
<td>7 (78)</td>
<td>2 (22)</td>
<td>9 (100)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1 (0.5)</td>
<td>37 (19)</td>
<td>112 (56)</td>
<td>49 (25)</td>
<td>199* (100)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Summary of PIK3CA mutations in responders versus nonresponders.

**Table 3.** Distribution of mutations throughout the response groups.

**NOTE:** %, distribution of responses in each mutation group. Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. *KRAS data were not available for one of the patients.
were equal between KRAS WT without a PI3K mutation. There are several arguments that could explain why PIK3CA may not be major regulators of anti-EGFR resistance. First, as stated previously, PIK3CA is mutated in diverse human cancers, but the functional aspect of some of the mutations still has to be defined. In 2005, Samuels et al. (24) showed in a preclinical model that both PIK3CA E545K and H1047R mutations promote cell growth and invasion of human cancer cells. It is unclear whether other PIK3CA mutations have the same functional properties. Second, we only investigated 80% of known PIK3CA mutations that are published in literature for colorectal cancer. As shown recently in lung adenocarcinoma, genomic screening resulted in the detection of new somatic mutations (25). It is thus possible that we have missed other mutations in PIK3CA that may confer other effects. Third, it has been shown that the frequency of PIK3CA mutations increases in metastases. Because we only used primary tumors in our study, the additional incidence of PIK3CA mutations in metastatic disease could have been masked.

Finally, an alternate explanation for our results could be that as PIK3CA mutations are a negative prognostic factor (21), this poor prognosis could be reversed by treating those patients with cetuximab.

Sample size is important in these exploratory studies that aim to define the effect of a novel biomarker. A power analysis showed that with an expected response rate in unselected patients of 25%, our sample size (200) would have allowed to detect a significant drop in response rate to 4% or lower in PIK3CA mutants with a power of 85% at a significance level of 0.05. These shifts of response rates are in the range of those observed in the unselected versus KRAS selected population (26). However, because EGFR inhibitors are now restricted to KRAS WT patients, it is more relevant to evaluate the potential shift in response rate in the KRAS WT subgroup. Hence, with our WT cohort, we would be able to detect a drop in response rate to 7% or lower. In summary, this analysis shows that PIK3CA mutations do not have a negative predictive effect in the same order of magnitude as KRAS mutations (near 0% response rates). If they had an impact similar to KRAS, this would have been detected by our study. However, our study is not powered sufficiently to detect mild negative effects of PIK3CA mutations such as for example a reduction in response rates from 40% to 20% in KRAS WT. This would need a study with over 400 KRAS WT patients, and may be the focus of large international collaborative efforts in the future.

Theoretically KRAS and PIK3CA mutations could have the same effect, as they are one of the two (PI3K/Akt and KRAS/MAPK) effector pathways downstream of EGFR (27).

A negative predictive role for PIK3CA mutations has recently been shown in breast cancer (28) where PIK3CA mutations, present in 25% (14 of 55) of tumors, mediated resistance to the anti-HER2 monoclonal antibody trastuzumab (Herceptin) in vitro and in vivo. PIK3CA mutations contributed to an increased risk for progression (P = 0.0052); and a combined analysis of PTEN status and PIK3CA status (loss of PTEN and PI3K mutations being mutually exclusive events leading to a similar pathway deregulation) not only identified twice as many patients at increased risk for disease progression, but the combined analysis also reached statistical significance as a biomarker for prognosis after trastuzumab therapy.

The stronger single effect of PIK3CA mutations observed in breast cancer than in our series may be due to a different ERBB (HER) receptor being targeted in breast and colorectal cancer. Active receptors can stimulate the PI3K signaling pathway via binding of the p85 subunit of PI3K resulting in activation of AKT. Due to the presence of multiple binding sites for p85, ERBB3 is the most efficient activator of PI3K (29). ERBB3 has impaired kinase activity and therefore only becomes

**Fig. 1.** Kaplan Meier PFS analysis in PIK3CA mutant versus WT patients.

**Fig. 2.** Kaplan Meier OS analysis in PIK3CA mutant versus WT patients.
phosphorylated when it is dimerized with another ERBB receptor, with ERBB2 being its preferred partner (30). ERBB1 (EGFR) has no consensus sequence for the p85 subunit but can still couple to this pathway via GAB1 and Ras/raf/MEK/ERK1/2.

This could explain why the PI3K mutation status could play a more central role in breast cancer treated with HER2-targeting drugs, in contrast to colorectal cancer treated with the ERBB1 (EGFR)-targeting drugs such as cetuximab. Another likely explanation could be the fact that we assessed only a single marker and future studies should address the prognostic and predictive effect of a comprehensive analysis of the PI3K/PTEN/AKT axis in colorectal cancer. Indeed, for example methylation and, hence, silencing of the PTEN gene, resulting in activation of the PI3K/AKT pathway, is commonly seen in human cancers and can therefore also play an important role in response to EGFR inhibitors (31). To fully assess the role of the PI3K/PTEN axis in response prediction, these alterations should be taken into account.

Finally, our study was only powered to detect large differences in response rate between the two PI3K groups, similar to those observed for KRAS mutations. Large populations need to be studied to detect more subtle effects.

In conclusion, our data do not provide any evidence for a strong role of PIK3CA mutations as a single marker in determining response to cetuximab in chemorefractory metastatic colorectal cancer.

Disclosure of Potential Conflicts of Interest

The authors have received an unrestricted research grant from Merck Serono.

Acknowledgments

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References

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