PIK3CA Mutations in Colorectal Cancer Are Associated with Clinical Resistance to EGFR-Targeted Monoclonal Antibodies

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Abstract

The monoclonal antibodies (moAb) panitumumab and cetuximab target the epidermal growth factor receptor (EGFR) and have proven valuable for the treatment of metastatic colorectal cancer (mCRC). EGFR-mediated signaling involves two main intracellular cascades: on one side KRAS activates BRAF, which in turn triggers the mitogen-activated protein kinases. On the other, membrane localization of the lipid kinase PI3KCA counteracts PTEN and promotes AKT1 phosphorylation, thereby activating a parallel intracellular axis. Constitutive activation of KRAS bypasses the corresponding signaling cascade and, accordingly, patients with mCRC bearing KRAS mutations are clinically resistant to therapy with panitumumab or cetuximab. We hypothesized that mutations activating PIK3CA could also preclude responsiveness to EGFR-targeted moAbs through a similar mechanism. Here, we present the mutational analysis of PIK3CA and KRAS and evaluation of the PTEN protein status in a cohort of 110 patients with mCRC treated with anti-EGFR moAbs. We observed 15 (13.6%) PIK3CA and 32 (29.0%) KRAS mutations. PIK3CA mutations were significantly associated with clinical resistance to panitumumab or cetuximab; none of the mutated patients achieved objective response (P = 0.038). When only KRAS wild-type tumors were analyzed, the statistical correlation was stronger (P = 0.016). Patients with PIK3CA mutations displayed a worse clinical outcome also in terms of progression-free survival (P = 0.035). Our data indicate that PIK3CA mutations can independently hamper the therapeutic response to panitumumab or cetuximab in mCRC. When the molecular status of the PIK3CA/PTEN and KRAS pathways are concomitantly ascertained, up to 70% of mCRC patients unlikely to respond to EGFR moAbs can be identified. [Cancer Res 2009;69(5):1851–7]

Introduction

Despite the introduction of new treatments, the 5-year survival rate for metastatic colorectal cancer (mCRC) remains below 10% (1). Additional active agents, as well as further insights about the mechanisms of resistance to current therapeutics, are needed to improve clinical outcome. Treatment options for mCRC nowadays include the chimeric IgG1 monoclonal antibody (moAb) cetuximab and the fully humanized IgG2 moAb panitumumab (2, 3). Both molecules bind to the extracellular domain of the epidermal growth factor receptor (EGFR), leading to inhibition of its downstream signaling, and providing a meaningful clinical benefit. However, this is limited to ≤20% of patients (3–5). Others and we have previously shown that KRAS mutations (that affect signaling downstream of the EGFR) can independently impair the efficacy of anticancer therapy with panitumumab or cetuximab (6–8). The majority of patients with mCRC resistant to anti-EGFR moAbs have tumors with activating mutations of KRAS. However, only a fraction of those with wild-type KRAS tumors, although larger than in the unselected population (8–10), respond to treatment, thus suggesting a role for additional mechanisms of resistance.

The PIK3CA gene is mutated in ~20% of CRCs (11). PIK3CA mutations occurring in the “hotspots” located in exon 9 (E542K, E545K) and exon 20 (H1047R) are oncogenic in CRC cellular models (12). The PIK3CA gene encodes for a lipid kinase that regulates, alongside with KRAS, signaling pathways downstream of the EGFR. Moreover, the p110α subunit of PI3K, encoded by PIK3CA, can be activated by interaction with RAS proteins (13). PI3K-initiated signaling is normally inhibited by phosphatase and tensin homologue deleted on chromosome ten (PTEN). In breast cancer patients, PTEN protein loss, evaluated by immunohistochemistry (14) or by the signature gene stathmin (15), predicts poor prognosis (15) and resistance to the anti-HER2 moAb trastuzumab (14). In CRC, we have previously reported that loss of PTEN expression, which occurs in 30% of sporadic cases, may be associated with lack of response to cetuximab (16). Whether and to what extent the occurrence of PIK3CA mutations affects responsiveness of mCRC patients to anti EGFR moAbs is presently unknown.

Here, we present the mutational analysis of PIK3CA and KRAS alongside with the evaluation of PTEN expression in a cohort of 110 mCRC-treated patients, to clarify how these genes affect clinical response to anti-EGFR–targeted therapies.

Materials and Methods

Patient population and treatment regimens. We analyzed 110 patients with mCRC either at Ospedale Niguarda Ca’ Granda (Milan, Italy)
cells assessed by immunohistochemistry with the Dako EGFR PharmDx kit. Patients were treated with either panitumumab (Merck Serono) or cetuximab (Merck Serono), according to the standard criteria (17). Treatment was continued until progressive disease (PD) or toxicity occurred, according to the standard criteria (17).

Clinical evaluation and tumor response criteria. Clinical response was assessed every 6 to 8 wk with radiological examination (computerized tomodensitometry or magnetic resonance imaging). The Response Evaluation Criteria in Solid Tumors (RECIST; ref. 17) were adopted for evaluation and objective tumor response was classified into partial response (PR), stable disease (SD), and PD. Patients with SD or PD were defined as nonresponders. Response to therapy was also evaluated retrospectively by independent radiologists.

Molecular analyses. Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative block, from either the primary tumor or the liver metastasis, depending on availability, containing at least 70% of neoplastic cells, was selected for each case. Genomic DNA was extracted using the QiAamp Mini kit (Qiagen) according to the manufacturer's instructions.

PTEN expression. PTEN protein expression was evaluated by immunohistochemistry on 3-μm formalin-fixed paraffin-embedded tissue sections as previously reported (16, 18) with some modifications. Briefly, anti-PTEN Ab4 (Thermo Fisher Scientific) with 1:200 dilution and PTEN Ab2 (Neomarkers) with 1:50 dilution were used at the Niguarda Hospital and at the Institute of Pathology of Locarno, respectively. PTEN protein expression was mainly detected at cytoplasmic level and very few cases also showed nuclear positivity. Tumors were considered negative, i.e., with loss of PTEN, when absence or reduction of immunostaining was seen in >50% of cells compared with internal controls (i.e., vascular endothelial cells and nerves; Supplementary Fig. S1 shows PTEN-positive and PTEN-negative representative cases). Healthy tissue, i.e., normal colon mucosa, was used as internal positive control (NEOMARKERS). PTEN expression was evaluated on tumor sections from any available block. Pretreatment biopsies were not used to avoid the technical difficulty of obtaining high-quality sections. Three to five representative sections were selected and reviewed for quality and tumor content. A single representative block, containing at least 70% of neoplastic cells, was selected for each case. Genomic DNA was extracted using the QiAamp Mini kit (Qiagen) according to the manufacturer's instructions.

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Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Median age (y; range)</th>
<th>Gender (male/female)</th>
<th>Previous chemotherapy (%)</th>
<th>No. of previous cancer treatments for advanced disease prior anti-EGFR moAbs (%)</th>
<th>Cutaneous toxicity (%)</th>
<th>Clinical response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>110</td>
<td>64 (26–85)</td>
<td>71/39</td>
<td>95 (86.4)</td>
<td>None: 13 (11.8), One: 15 (13.6), Two: 48 (43.6), Three: 28 (25.4), More than three: 6 (5.5)</td>
<td>0–1: 74 (67.3), 2–3: 32 (29.1), Unknown: 4 (3.6)</td>
<td>Complete response: 21 (19.1), Partial response: 66 (60.5), Stable disease: 13 (11.8), Progressive disease: 10 (9.1)</td>
</tr>
</tbody>
</table>

*Other: In one case, primary site was small bowel, and in one case, primary tumor sites were multiple (colon and rectum).

Table 2. Univariate analysis of the association between clinical and pathologic characteristics, mutations of PIK3CA, and loss of PTEN in 110 mCRC patients treated with anti-EGFR monoclonal antibodies panitumumab or cetuximab

<table>
<thead>
<tr>
<th>PIK3CA</th>
<th>KRAS</th>
<th>PIK3CA and/or KRAS</th>
<th>PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (%)</td>
<td>Mut (%)</td>
<td>P</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>58 (81.7)</td>
<td>13 (18.3)</td>
<td>0.080</td>
</tr>
<tr>
<td>Women</td>
<td>37 (94.8)</td>
<td>2 (5.13)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤65</td>
<td>54 (87.1)</td>
<td>8 (12.9)</td>
<td>1.000</td>
</tr>
<tr>
<td>66–74</td>
<td>24 (85.7)</td>
<td>4 (14.3)</td>
<td></td>
</tr>
<tr>
<td>≥75</td>
<td>17 (89.5)</td>
<td>2 (10.5)</td>
<td></td>
</tr>
<tr>
<td>Site of T*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>48 (69.6)</td>
<td>21 (30.4)</td>
<td>0.319</td>
</tr>
<tr>
<td>Sigma-rectum</td>
<td>10 (90.9)</td>
<td>1 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>18 (66.7)</td>
<td>9 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Cutaneous rash</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1</td>
<td>49 (67.1)</td>
<td>24 (32.9)</td>
<td>0.494</td>
</tr>
<tr>
<td>2–3</td>
<td>24 (75.0)</td>
<td>8 (25.0)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: WT, wild-type; Mut, mutated.

*In one case, primary site was small bowel, and in one case, primary tumor sites were multiple (colon and rectum); P values measured by Fisher's exact test.
as internal positive control; normal endometrium was used as external positive control. The evaluations were performed without knowledge of clinical data or results of molecular analyses.

**Mutational analysis of PIK3CA and KRAS in tumor samples.** We searched for PIK3CA mutations in exons 9 and 20, and for KRAS mutations in exon 2. PIK3CA exon 9 includes codons 542 and 545, PIK3CA exon 20 codon 1047, and KRAS exon 2 codons 12 and 13, where the large majority of mutations occur in these genes (19). The list of primers used for mutational analysis is available from the authors upon request. All samples were subjected to automated sequencing by ABI PRISM 3730 (Applied Biosystems). All mutated cases were confirmed twice, starting from independent PCR reactions. In one instance (patient 55), the results from the first analysis showed a mutation in PIK3CA (E545A) that was not observed in two samples. PTEN protein assessment was performed by immunohistochemistry analysis. Among the 81 evaluated tumor specimens, 32 (39.5%) showed loss of PTEN protein expression. Results of mutational analyses and immunohistochemistry are shown in Supplementary Table S1.

**Results**

**Frequency of mutations in PIK3CA and KRAS, and loss of PTEN protein expression.** Mutational profiling of 110 colorectal tumors from patients treated with anti-EGFR moAbs led to the identification of 15 (13.6%) PIK3CA and 32 (29.0%) KRAS mutations. As expected, PIK3CA mutations were found both in exon 9 (4 cases) and in exon 20 (11 cases). Similarly, KRAS was mutated at codon 12 in 23 cases (71.9%), and at codon 13 in 18 cases (25.0%); a double point mutation involving both codons was detected in 1 case (3.1%). Concomitant PIK3CA and KRAS mutations were observed in two samples. PTEN protein assessment was performed by immunohistochemistry analysis. Among the 81 evaluated tumor specimens, 32 (39.5%) showed loss of PTEN protein. Results of mutational analyses and immunohistochemistry are shown in Supplementary Table S1.

**Clinical and pathologic characteristics according to mutations in PIK3CA or KRAS and loss of PTEN protein expression.** Analyses of the association between mutational status of PIK3CA and KRAS and PTEN expression with clinical-pathologic characteristics are shown in Table 2. No association was found between these variables and age, location of the primary tumor (i.e., colon, sigma-rectum junction, or rectum), or degree of cutaneous toxicity.

**Mutations in PIK3CA, KRAS, and PTEN loss are associated with lack of objective response to panitumumab or cetuximab.** The relationship between PIK3CA mutations, KRAS mutations, and PTEN expression with clinical outcome was evaluated in terms of objective tumor response, progression-free survival (PFS), and overall survival (OS).
In univariate analysis, PIK3CA mutations were significantly associated with lack of response to panitumumab or cetuximab, with none of the mutated patients achieving objective tumor response ($P = 0.038$). The same negative association was observed for KRAS mutations (9.1% of mutations among responders versus 34.5% among non responders; $P = 0.019$) and was confirmed when at least a mutation of either KRAS or PIK3CA was considered ($P = 0.001$; Table 3A). When only KRAS wild-type tumors were analyzed, the statistical association between PIK3CA mutations with lack of response to panitumumab or cetuximab was confirmed ($P = 0.016$). In bivariate analysis, PIK3CA mutations and KRAS mutations were simultaneously significant ($P = 0.0234$ and 0.0125, respectively); in multivariate logistic regression, an independent effect of PIK3CA mutations, KRAS mutations, and PTEN protein expression was also confirmed ($P = 0.0337$, 0.0029, and 0.0012, respectively; Table 3B). Our data indicate that similarly to

![Kaplan-Meier cumulative PFS on the basis of PIK3CA and KRAS mutational status and PTEN protein expression in mCRC patients treated with panitumumab and cetuximab. A, PIK3CA wild-type (wt) versus mutated; B, KRAS wild-type versus mutated; C, either PIK3CA or KRAS mutated versus both wild-type; D, PTEN loss of expression versus normal; E, either PIK3CA mutated or loss of PTEN versus both normal; F, PIK3CA wild-type versus mutated in KRAS wild-type only patients.](image-url)
KRAS, PIK3CA wild-type status represents a necessary but not sufficient condition to reach objective response. Assessment of PIK3CA mutations therefore represents an independent factor to predict clinical outcome among KRAS wild-type patients.

PIK3CA mutations and PTEN loss are negatively associated with survival in mCRCs patients treated with panitumumab or cetuximab. Analysis of survival showed that patients with tumors harboring PIK3CA mutations had a worse clinical outcome in terms of PFS, compared with wild-type tumors \( (P = 0.0035; \text{Fig. 1A}) \). Patients with KRAS mutations had a trend toward a decreased PFS \( (P = 0.0815; \text{Fig. 1B}) \). Shorter PFS was also detected in patients harboring at least a mutation of either KRAS or PIK3CA \( (P = 0.0032; \text{Fig. 1C}) \). PTEN loss was similarly associated with shorter PFS \( (P = 0.0681) \) that reached statistical significance if
this variable was combined with PIK3CA mutations (loss of PTEN and/or PIK3CA mutation; \( P = 0.0066 \); Fig. 1D–E). Accordingly, Cox multivariate survival analysis confirmed that patients with at least one alteration of either PIK3CA or PTEN had a higher risk of progression (\( P = 0.009 \)), whereas the model was not significant for KRAS mutations (\( P = 0.128 \); Table 3C). Among KRAS wild-type only patients, a decreased PFS was confirmed for patients with PIK3CA mutations in their tumors (\( P = 0.0021 \); Fig. 1F).

Neither PIK3CA mutations nor KRAS mutations were associated with OS (\( P = 0.2516 \) and 0.1127, respectively; Fig. 2A–B), although a trend toward decreased OS was evident in patients harboring at least a mutation of either KRAS or PIK3CA (\( P = 0.0645 \); Fig. 2C). PTEN loss of expression was significantly associated with worse OS (\( P = 0.0048 \)), as was the combination of PTEN loss with PIK3CA mutations (\( P = 0.0161 \); Fig. 2D–E). In KRAS wild-type tumors, PIK3CA mutations did not influence OS (\( P = 0.2921 \); Fig. 2F).

Discussion

Our work, as well as that of other laboratories, has shown that almost all mCRC patients with tumors harboring KRAS mutations are resistant to treatment with the EGFR-targeted moAbs panitumumab or cetuximab (6–8). This notion has been acknowledged by European Medicines Agency (EMEA) that approved the use of panitumumab or cetuximab only in mCRC patients whose tumors display wild-type KRAS,8,9 KRAS mutations, however, only account for 30% to 40% of nonresponsive patients. The identification of additional genetic determinants of resistance to EGFR-targeted therapies in CRC is therefore clearly a priority. We noted that the mitogen-activated protein kinase kinases cascade triggered by the KRAS/BRAF pathway represents only one side of the axis on which the EGFR relies for propagation of its mitogenic stimulus. On the other side, membrane localization of the lipid kinase PIK3CA promotes AKT1 activation, ensuing to a parallel intracellular propagation of the signal. We hypothesized that, similarly to what observed for the oncogenic activation of the KRAS/MAPK pathway, the constitutive deregulation of the PIK3CA gene could bypass the EGFR-initiated signaling cascade. To test this possibility, we assessed whether tumors bearing PIK3CA mutations were resistant to EGFR-targeted therapy with moAbs. Our results indicate that PIK3CA mutations could be considered alongside with those affecting KRAS as predictors of primary resistance to EGFR moAbs therapies. PIK3CA mutations explain lack of objective response in additional 17% of KRAS wild-type patients. Furthermore, the multivariate analysis of KRAS and PIK3CA mutations showed that both alterations play an independent and significant role in predicting resistance (Table 3A). Patients with PIK3CA-mutated mCRC had worse clinical outcome in terms of PFS, and this was confirmed also for KRAS wild-type tumors (Fig. 1A and F). In addition, we show, for the first time, that loss of PTEN is associated not only with lack of objective tumor response as previously reported (16) but also with worse OS in patients with mCRC treated with panitumumab or cetuximab. Overall, our data indicate that a comprehensive analysis of both the KRAS/BRAF and PI3K pathways including KRAS and PIK3CA mutations and PTEN protein status is significantly associated with both PFS and OS, thus representing the best predictor of clinical outcome in this setting. Among the subgroup of 59 evaluable KRAS wild-type patients, this combined analysis could indeed identify an additional 44% of nonresponsive cases (Fig. 3). Thus, the combination of KRAS, PIK3CA, and PTEN analyses could lead to the identification of 70% of mCRC patients resistant to panitumumab or cetuximab.

With regard to the role of PIK3CA mutations in affecting tumor progression, a number of functional evidences suggest that PIK3CA mutations might have a relatively mild effect on the growth of the tumor (12). One possibility is that tumors carrying PIK3CA mutations may be less aggressive than those that do not and, hence, have better PFS. However, in the present study in patients with metastatic disease and particularly dismal prognosis, those carrying PIK3CA mutations have worse clinical outcome, therefore not supporting this hypothesis. In vitro studies have recently shown that PIK3CA mutation/PTEN expression status predicts response of colon cancer cell lines to cetuximab (20), thus supporting our observations on clinical samples.

The decision of health authorities (EMEA) to restrict the clinical use of panitumumab or cetuximab for patients with wild-type KRAS mCRC6,9 is expected to ameliorate the therapeutic index of these targeted agents. Nevertheless, in the KRAS wild-type
population of mCRC, the objective response rate is limited to 17% (versus 0% in KRAS mutated) for panitumumab monotherapy (8) and to 59% to 61% (versus 43–33%) for cetuximab plus either irinotecan- or oxaliplatin-based chemotherapy, respectively (9, 10). Once validated in prospective trials, the finding that deregulation of the PI3K pathway identifies mCRC patients with clinical resistance to panitumumab or cetuximab could find immediate clinical applications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

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