Abstract

Genomics and proteomics have held out the promise of individualized medicine for the last 10 or 20 years, but clinical medicine has not yet delivered on this promise. Some cancers, such as breast cancer and some hematologic malignancies, have been at the forefront of individualized therapeutic approaches by integrating molecular biomarkers into treatment decision algorithms. Until recently, the treatment of colorectal cancer (CRC) has lagged behind these other cancers in this regard and therapeutic decisions have been solely empirical. Data from various clinical trials and translational studies have now opened the door for individualized treatment approaches by identifying patients with metastatic CRC who are most likely to benefit from antibodies against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab. Activating mutations of KRAS, a downstream mediator of EGFR signaling, has been shown to render EGFR antibodies ineffective, such that analyzing tumors for these mutations has become mandatory before the use of EGFR antibodies is considered in CRC. Beyond KRAS, several additional biomarkers are currently being investigated as potential positive or negative predictors for the efficacy of EGFR-targeted therapy. Most of these markers are alterations of molecules integrated in the EGFR pathway. This review will focus on the type and quality of evidence that has been gathered to date to predict resistance to monoclonal antibodies against EGFR in CRC.

Epidermal growth factor receptor (EGFR) is a transmembrane receptor-tyrosine kinase expressed on the surface of nonmalignant and malignant epithelial cells including colorectal cancer (CRC). EGFR is placed at the intersection of the environmental and intracellular effectors that regulate cell growth, cell migration, differentiation, dedifferentiation, and angiogenesis. Its position at the top of a complex and crucial signaling network has made it an especially attractive therapeutic target. Two types of drugs have been developed to inhibit EGFR signaling: tyrosine kinase inhibitors, to inhibit receptor signaling at the intracellular component of the protein [reviewed by Hammermann and colleagues in this issue (1)] and monoclonal antibodies (mAb) to prevent the interaction of the receptor with its ligands.

Although small molecule tyrosine kinase inhibitors have so far failed to show proof of efficacy in CRC, mAbs against EGFR have been integrated into algorithms for the treatment of advanced CRC for more than 5 years. Cetuximab, a mouse-human chimeric immunoglobulin G (IgG1) antibody, and panitumumab, a human IgG2 antibody, have both consistently yielded response rates of 10 to 15% as single agents in unselected patients with advanced CRC and higher in combination with conventional chemotherapy (2). In addition, the use of cetuximab in a salvage therapy setting without cross-over significantly improved overall survival in advanced CRC (3, 4). Until recently, the decision to use an EGFR mAb in an individual patient was solely based on clinical, empiric considerations. This situation has dramatically changed over the last 2 years. Although positive predictive molecular markers for the efficacy of EGFR antibodies remain elusive, recent evidence has implicated activating mutations in genes downstream of EGFR such as KRAS and BRAF as effectors of resistance (4–6). These findings have already allowed enrichment of a patient population more likely to benefit from cetuximab and panitumumab, and other molecular markers of components integrated in the EGFR-mediated signaling cascade are currently being investigated as positive and negative predictors of efficacy. This review will focus on the type and quality of evidence that has been gathered to date to predict responsiveness and resistance to mAb against EGFR in CRC.

Molecular Predictors of Efficacy of EGFR Antibodies

CRC represents a heterogenous group of diseases (Table 1). It has recently become possible to molecularly classify groups into subsets with similar genetic and/or epigenetic characteristics.
One step on the way toward individualized treatment decisions is to identify biomarkers that correlate with patient survival (prognostic markers) and/or treatment response (predictive markers). Subsets include cancers that are linked to microsatellite instability, chromosomal instability, and the CpG island methylator phenotype. Each of these has been correlated or inversely correlated with a signature of mutations in tumor suppressors or oncogenes (7). Molecular alterations found reproducibly and consistently in CRC include mutations of p53, PIK3CA, KRAS, APC (8), COX-2, LINE-1, MGMT, p27, p16, FASN, AURAK, and BRAF. Some of these markers, namely KRAS, PIK3CA, and BRAF, are downstream effectors of EGFR-signaling (Fig. 1). This makes them particularly promising candidate predictors of anti-EGFR-targeted therapy because they are less likely than less consistently found molecular alterations to be “passenger mutations” instead of tumor-driving mutations. Also, more data are available on the prognostic value of these downstream effectors, making it easier to distinguish this effect from potential predictive significance for response to a given therapy. Other components of the EGFR signaling network, including PTEN, amphiregulin, epiregulin, EGFR gene copy number, as well as polymorphisms of Fc-receptors are intriguing candidates as predictive markers with varying degree of evidence based on currently available data.

One of the challenges in determining the predictive and prognostic value of molecular markers is that alterations in these effectors can be interrelated. Although it is well-established that KRAS and BRAF mutations are mutually exclusive, a recent study by Lambrechts and colleagues (9) in 276 patients with metastatic CRC (mCRC) showed that about 18% of tumors carrying a KRAS mutation (KRAS-mut) and 6% of tumors with a BRAF mutation (BRAF-mut) also carried a PIK3CA mutation. In this study, KRAS, BRAF, and PI3KCA mutations were significantly associated with clinical outcome after cetuximab-based therapy in a univariate analysis. However, in a multivariate analysis, only KRAS and BRAF were found to be significant. This finding highlights the importance of multivariate analyses to definitively identify the predictive and prognostic value of investigated molecular markers.

**KRAS.** KRAS is a small GTPase activated by EGFR signaling. Mutations putting KRAS in a permanently activated state occur in 20 to 30% of human cancers and in approximately 40% of CRCs regardless of tumor stage (4–6, 10–12). In most cases, the mutations found in cancer cells introduce amino acid

<p>| Table 1. Characteristics of molecular markers for the activity of EGFR antibodies in CRC |
|------------------------------------------|---------------------------------|-----------------|------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Alteration</th>
<th>Test method</th>
<th>Frequency of alteration in CRC</th>
<th>Concordance of alterations between primary and metastases</th>
<th>Predictive value for EGFR mAbs</th>
<th>Prognostic value</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>Activating mutation in exon 2 (most commonly in codon 12, 13, 61)</td>
<td>Site-specific PCR, direct sequencing</td>
<td>35–45%</td>
<td>Yes</td>
<td>Yes; negative predictor</td>
<td>No</td>
<td>Mutations in BRAF and KRAS are mutually exclusive</td>
</tr>
<tr>
<td>BRAF</td>
<td>Activating mutation (V600E)</td>
<td>Site-specific PCR, direct sequencing</td>
<td>5–10%</td>
<td>Unknown</td>
<td>Likely; negative predictor</td>
<td>Yes (mutations indicate poor prognosis)</td>
<td>Mutations in BRAF and KRAS are mutually exclusive</td>
</tr>
<tr>
<td>PTEN</td>
<td>Loss of PTEN protein expression</td>
<td>IHC</td>
<td>30–50%</td>
<td>No</td>
<td>Potentially; negative predictor</td>
<td>Unknown</td>
<td>Only PTEN expression in metastases, not in primary tumor identified as potential predictive marker, no standardized IHC scoring system</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Activating mutation</td>
<td>Site-specific PCR, direct sequencing</td>
<td>10–30%</td>
<td>Unknown</td>
<td>Questionable; negative predictor</td>
<td>Questionable (mutations could indicate poor prognosis)</td>
<td>Contradictory results from different studies</td>
</tr>
<tr>
<td>EGFR Ligands (amphiregulin, epiregulin)</td>
<td>Overexpression of protein</td>
<td>Quantitative RT-PCR</td>
<td>Continuous variable</td>
<td>Unknown</td>
<td>Likely; higher ligand levels as positive predictor</td>
<td>Unknown</td>
<td>Cut-off level of continuous variable not yet well defined</td>
</tr>
<tr>
<td>FcγRIIa-FcγRIIIa polymorphism</td>
<td>Sequencing of FcγRIIa-FcγRIIIa in normal colonic tissue</td>
<td>About 40%</td>
<td>Not applicable</td>
<td>Questionable: positive predictor</td>
<td>Questionable: positive predictor</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>

Based on the provided information, one can infer that the predictive markers for EGFR antibodies in CRC include KRAS, BRAF, and PIK3CA, which are crucial in determining the efficacy of targeted therapies. Furthermore, the table presents a detailed overview of the characteristics of these molecular markers, including their frequency of alteration, concordance between primary and metastases, and their predictive and prognostic values. This data is essential for making informed decisions regarding patient treatment strategies.
changes at positions 12, 13, and 61, impairing the intrinsic ATPase activity of the protein and causing accumulation of the mutant protein in the active, GTP-bound conformation. This generated the intriguing hypothesis that a permanently activated (mutated) KRAS protein downstream of EGFR could counteract therapeutic targeting of the EGFR.

The first evidence that KRAS mutation could be a clinically relevant cause of resistance to EGFR inhibitors came from a retrospective study in 30 patients with mCRC who were treated with cetuximab alone or in combination with irinotecan and/or 5-fluorouracil (5FU; ref. 10). KRAS mutations were significantly associated with a lack of response to cetuximab (P = 0.0003) with none out of 11 patients whose tumors harbored an activating mutation responding to therapy. A year later, these initial findings were considerably strengthened by a prospectively planned, retrospectively conducted analysis of tumor samples from patients enrolled in a randomized phase III study of panitumumab monotherapy versus best supportive care (BSC; with cross-over) in patients with chemorefractory mCRC (5). Of the 463 patients originally enrolled, 409 were KRAS assessable, overall response data were available for 389 patients. Of these, 0% of patients with mutated KRAS had responded to panitumumab, whereas 17% of patients with wild-type KRAS (KRAS-wt) tumors had a response. Likewise, all benefit observed in progression-free survival, the primary endpoint of this trial, was exclusively limited to the KRAS-wt population [hazard ratio (HR) 0.45, P < 0.0001 for panitumumab versus BSC, compared with HR 0.99 in KRAS-mutated tumors].

Most likely because of the high rate of cross-over from BSC to panitumumab upon progression (75% of patients), no statistically significant prolongation of overall survival was found for panitumumab in KRAS-wt CRC. Retrospective analysis of KRAS mutational status from tumor tissue from several randomized phase III trials (Table 2), thereafter, has provided more evidence that the negative predictive value of mutant KRAS status is 100% and that KRAS-wt type status is necessary, but not sufficient for response to cetuximab: (1) The phase III CRYSTAL trial, comparing FOLFIRI ± cetuximab in the first-line setting in mCRC, in which 45% of patients enrolled in the trial (540/1,198) were tested for KRAS (13); (2) The EPIC trial, comparing irinotecan ± cetuximab in second-line therapy, in which 23% of tumors (300/1,298) were tested for KRAS (14); (3) The randomized phase III NCIC-017 trial, comparing BSC ± cetuximab monotherapy, in which 65% (366/572) of tumors were tested for KRAS mutations (4). The results from these randomized trials all showed that response to cetuximab was restricted to patients whose tumors harbored KRAS-wt.

Data from more recently published studies (Table 3) even suggest that adding cetuximab or panitumumab to the treatment regimen of patients whose tumor has a KRAS mutation might decrease the overall efficacy of therapy (12, 15, 16). In the Dutch CAIRO-2 phase III trial, 755 patients with chemorefractory mCRC were treated with capecitabine, oxaliplatin, and bevacizumab and randomized to receive additional cetuximab or the three-drug combination only (12). The influence of KRAS status on response was tested as a specified secondary endpoint in 69% of patients by direct sequencing of KRAS exon 2. Progression free survival (PFS) in patients with an activating KRAS mutation was shorter than in those with a KRAS-wt-type tumor if treated with cetuximab (PFS 8.1 versus 12.5 months, P = 0.004). Also, the response rate of patients with mutant KRAS receiving cetuximab was inferior to that in patients treated with the three-drug combination alone (46% versus 61%,

![Fig. 1. EGFR signaling pathway and candidate predictive molecular markers for the activity of EGFR antibodies in CRC. Candidate predictive biomarkers that are also part of the EGFR signaling cascade are shown in pink. Dimerization of EGFR monomers and activation of the EGFR pathway (represented by lighting bolts) occurs after ligand binding. Ligand binding and therefore dimerization of the EGFR monomer and activation of the EGFR pathway is inhibited by EGFR-directed antibodies.](image-url)
P = 0.03). Thus, patients with mutated KRAS are not simply resistant to cetuximab therapy, but the drug, used in the wrong population of patients, has the potential for considerable harm, although for yet unknown reasons. It has thus become evident that our linear view of pathways does not do justice to the considerable complexity of the signaling networks. Instead, when faced with interference with one or several of its components, such networks might react with compensatory mechanisms with unforeseeable results.

On the basis of the above evidence for a negative predictive role of KRAS mutations on the primary tumor, the U.S. Food and Drug Administration (FDA) has recently approved a change for the indications for the usage of cetuximab and panitumumab to limit the use of these drugs to patients with tumors without KRAS mutations.

Although the results of the CAIRO-2 trial cannot exclude the possibility that an activating KRAS mutation is a negative prognostic factor, data from large groups of patients have so far failed to show any prognostic value of a mutated KRAS. From a population of 173,200 individuals enrolled in the Nurse’s Health Study and the Health Professional Follow-up study, 647 colon cancer patients with available tissue specimens were identified. Although in the univariate and multivariate analysis BRAF mutation emerged as an independent negative prognostic factor, KRAS mutational status was not associated with patient outcome (17). In another prospectively conducted observational study of 178 patients with stage III CRC and mutated KRAS enrolled in CALGB 89803, no difference was found in the 5-year disease free survival or overall survival when compared with patients with KRAS-wt (64% versus 66% and 74% versus

<table>
<thead>
<tr>
<th>Line of Therapy</th>
<th>First line</th>
<th>Second line</th>
<th>Third line</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>1,198</td>
<td>1,298</td>
<td>572</td>
</tr>
<tr>
<td>KRAS tested</td>
<td>540 (45%)</td>
<td>300 (23%)*</td>
<td>394 (69%)</td>
</tr>
<tr>
<td>KRAS mutated</td>
<td>36%</td>
<td>36%</td>
<td>42%</td>
</tr>
<tr>
<td>Arms</td>
<td>FOLFIRI</td>
<td>FOLFIRI + Cetux</td>
<td>FOLFOX</td>
</tr>
<tr>
<td></td>
<td>FOLFOX</td>
<td>FOLFOX + Cetux</td>
<td>Irino</td>
</tr>
<tr>
<td></td>
<td>Irino</td>
<td>Irino + Cetux</td>
<td>BSC</td>
</tr>
<tr>
<td>Response rate (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unselected population</td>
<td>39 P = 0.0038</td>
<td>36 P = 0.064</td>
<td>4 P &lt; 0.0001</td>
</tr>
<tr>
<td>KRAS-wt</td>
<td>43 P = 0.0025</td>
<td>37 P = 0.01</td>
<td>7 P = 0.61</td>
</tr>
<tr>
<td>KRAS-mut</td>
<td>40 P = 0.46</td>
<td>49 P = 0.11</td>
<td>5 P = 0.29</td>
</tr>
<tr>
<td>Response rate (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFS (mo)</td>
<td>8.0 P = 0.048, HR 0.85</td>
<td>7.2 P = 0.62, HR 0.93</td>
<td>2.6 P &lt; 0.0001, HR 0.69</td>
</tr>
<tr>
<td>Unselected population</td>
<td>8.7 P = 0.017, HR 0.68</td>
<td>7.2 P = 0.016, HR 0.57</td>
<td>2.8 P = 0.09, HR 0.77</td>
</tr>
<tr>
<td>KRAS-wt</td>
<td>8.1 P = 0.75, HR 1.07</td>
<td>8.6 P = 0.019, HR 1.83</td>
<td>2.7 P = 0.98, HR 1.0</td>
</tr>
<tr>
<td>KRAS-mut</td>
<td>18.6 P = 0.30, HR 0.93</td>
<td>N/A</td>
<td>10.0 P = 0.71, HR 0.98</td>
</tr>
<tr>
<td>OS (mo)</td>
<td>21.0 P = 0.22, HR 0.84</td>
<td>N/A</td>
<td>11.6 P = 0.18, HR 1.29</td>
</tr>
<tr>
<td>KRAS-mut</td>
<td>17.7 P = 0.85, HR 1.03</td>
<td>N/A</td>
<td>10.7 P = 0.29, HR 1.28</td>
</tr>
</tbody>
</table>

Abbreviations: PFS, median progression-free survival; OS, median overall survival; Cetux, cetuximab; P-mab, panitumumab; N/A, not available; n.s., not significant.

*Only patients treated in the United States.

Forty-seven percent cross-over from irinotecan to cetuximab-based therapy.

Seventy-six percent cross-over from BSC to panitumumab.
In the palliative setting, KRAS status was not found to influence overall survival in the BSC arm of the last-line trial comparing cetuximab versus BSC without cross-over (4). These data should lay the discussions on the potential prognostic value of KRAS mutations to rest.

**BRAF.** Another member of the RAS-RAF-MAPK pathway that might be a candidate for predicting response to therapy with EGFR-targeting mAbs is the serine/threonine protein kinase BRAF (19). BRAF mutations of codon 600 (V600E) have been found in about 5 to 10% of colon cancers, are mutually exclusive with KRAS mutations, and have been associated with unfavorable prognosis (17, 20).

Data supporting its prognostic value have emerged from analyses of large cohorts. Ogino and colleagues studied a cohort of 173,200 individuals enrolled in the Nurses’ Health Study and the Health Professional Follow-up study. Among the 649 colon cancer patients 237 (37%) had a mutation in KRAS and 105 (17%) had a mutation in BRAF, consistent with the mutation rate of these genes in other data sets. In both, the univariate and a multivariate analysis, BRAF mutations were associated with increased colon cancer-specific mortality (multivariate HR 1.97).

In the largest study indicating a predictive role for BRAF in the treatment of colon cancers with EGFR antibodies done to date, Di Nicolantonio and colleagues retrospectively analyzed tumor tissue from 113 patients who had been treated with cetuximab or panitumumab (with or without chemotherapy). Eleven patients (10%) were found to have a BRAF mutation. No patient with a BRAF mutation responded to therapy ($P = 0.029$), suggesting a negative predictive value. Although PFS and overall survival were significantly better in patients with BRAF wild-type (BRAF-wt) tumors when compared with BRAF-mut, it is not possible to judge whether this was due to the negative prognostic effect of a mutated BRAF or due to its negative predictive effect toward treatment with cetuximab, because in this series, all patients were treated with this drug. In summary, the authors considered these data hypothesis-generating, mainly in view of the small number of patients with BRAF-mut available for the study.

In a smaller series, none out of 4 patients with mutated BRAF responded to therapy (21). In another small study none out of nine patients with mutated BRAF responded to cetuximab, whereas 19 out of 57 with BRAF-wt had a response (9).

Given the mounting evidence for the prognostic value of BRAF-mut, which could explain the poorer PFS and overall survival in these series, withholding anti-EGFR antibodies from patients with BRAF mutations might be premature, because it is possible that the short overall survival and PFS in this patient

### Table 3. Results of randomized first-line trials investigating the addition of EGFR antibodies to chemotherapy plus bevacizumab in advanced CRC

<table>
<thead>
<tr>
<th>Trial</th>
<th>PACCE-Oxali (15)</th>
<th>PACCE-Irino (15)</th>
<th>CAIRO2 (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong></td>
<td>823</td>
<td>230</td>
<td>736</td>
</tr>
<tr>
<td><strong>KRAS tested</strong></td>
<td>664 (81%)</td>
<td>201 (87%)</td>
<td>720 (71%)</td>
</tr>
<tr>
<td><strong>KRAS mutated</strong></td>
<td>39%</td>
<td>43%</td>
<td>40%</td>
</tr>
<tr>
<td><strong>Arms</strong></td>
<td>FU/Oxali/BEV</td>
<td>FU/Oxali/BEV + P-mab</td>
<td>FU/Irino/BEV</td>
</tr>
<tr>
<td><strong>Response rate (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unselected population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS-wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS-mut</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PFS (mo)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unselected population</td>
<td>11.4 (10.0)</td>
<td>11.7 (10.1)</td>
<td>10.7 (9.4)</td>
</tr>
<tr>
<td>KRAS-wt</td>
<td>11.5 (9.8)</td>
<td>12.5 (10.0)</td>
<td>10.6 (10.5)</td>
</tr>
<tr>
<td>KRAS-mut</td>
<td>11.0 (10.4)</td>
<td>11.9 (8.3)</td>
<td>12.5 (8.1)</td>
</tr>
<tr>
<td><strong>OS (mo)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unselected population</td>
<td>24.5 (19.4)</td>
<td>20.5 (20.7)</td>
<td>20.3 (19.4)</td>
</tr>
<tr>
<td>KRAS-wt</td>
<td>24.5 (20.7)</td>
<td>19.8 (17.8)</td>
<td>22.4 (21.8)</td>
</tr>
<tr>
<td>KRAS-mut</td>
<td>19.3 (19.3)</td>
<td>20.5 (17.8)</td>
<td>24.9 (17.2)</td>
</tr>
</tbody>
</table>

NOTE: HR, 95% confidence interval.
Abbreviations: PFS, median progression-free survival; OS, median overall survival; Oxali, oxaliplatin; Irino, irinotecan; Cetux, cetuximab; P-mab, panitumumab; N/A, not available; n.s., not significant.
population could be due solely to the prognostic and not due to any presumed negative predictive effects of BRAF in the context of EGFR antibody therapy. Thus, whereas BRAF is an interesting candidate for predicting response, it has to be pointed out that the currently available studies are all limited by their retrospective nature, small numbers, and by the fact that most treatment has occurred outside of clinical trials. A prospective validation of BRAF mutations as a predictive marker for EGFR antibodies might be problematic given its prognostic value and its rare incidence. However, future analyses of ongoing (e.g., CALGB 80405, N0147, PETACC-8) and recently completed large clinical trials (e.g., COIN) could help manifest the predictive value of BRAF in the context of EGFR-targeted antibody therapy. As with KRAS mutations, most convincing will be the consistency of data, even when generated in retrospective studies.

**PIK3CA.** The PIK3CA gene encodes for the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which is a downstream effector of EGFR-mediated signaling at the head of the PI3K-Akt pathway. In its activated mutant form it can induce phosphorylation of Akt, which in turn promotes cell growth and anti-apoptosis in colorectal and other cancers. Activating mutations have been found in 10 to 30% of colon cancers. PIK3CA has recently been associated with poor prognosis in a retrospective analysis of a cohort of 450 patients with stage I to III CRC in a multivariate analysis (22). In a retrospective study of 110 patients treated with cetuximab or panitumumab-based regimens in first- to fourth-line treatment, 14% of the patients had an activating PIK3CA mutation (23). None of the 15 patients with a PIK3CA mutation had an objective response to treatment with anti-EGFR mAb compared with a response rate of 23% in the 95 patients with wild-type PIK3CA. This result translated into an association with lack of response in the univariate analysis ($P = 0.038$), as well as in a multivariate logistic regression analysis with KRAS mutations and loss of PTEN protein expression, in which PIK3CA seemed to be an independent factor ($P = 0.0337$) for lack of response to mAb against EGFR. PIK3CA mutations were negatively associated with survival and thus, seem to be negatively prognostic as well as predictive. Data conflicting with the aforementioned findings however came from a more recent and larger study by Prenen and colleagues (24). Tumors from 200 irinotecan refractory patients enrolled in different studies investigating the usefulness of anti-EGFR mAb were analyzed for activating mutations of PIK3CA. About 12% of tumors contained such a mutation, consistent with previous findings. In contrast, however, there was no correlation between PIK3CA mutations and response toward anti-EGFR mAb. In fact, five patients with PIK3CA mutations had an objective response to cetuximab. The authors conclude that PIK3CA mutations are not predictive of cetuximab response in mCRC.

**PTEN.** Several retrospective studies have analyzed different aspects of the deregulation of the tumor suppressor PTEN as a potential predictive marker for EGFR mAb responsiveness. PTEN protein expression levels were determined using immunohistochemistry (IHC) in all studies. Frattini and colleagues found that none out of 11 patients with lower PTEN expression in tumor tissue as compared with normal colonic tissue responded to a combination of cetuximab and irinotecan, whereas 10 out of 16 patients with normal expression had a partial response (25). Loupakis and colleagues did IHC on 96 primary CRCs and found no correlation with response to therapy with cetuximab plus irinotecan after irinotecan failure (26). However, when IHC was done on 59 metastases, 36% of the patients with PTEN-positive metastases had responded to therapy compared with only 5% of patients who harbored PTEN-negative metastases ($P = 0.007$). Because in the same study KRAS mutations in the primary tumor were associated with response to cetuximab and/or irinotecan, the authors conclude that a combination of KRAS mutation status and PTEN expression as determined by IHC in metastases could be a better predictive marker for patient benefit from cetuximab than KRAS mutations alone. More recently, Sartore-Bianchi and colleagues retrospectively did IHC on tissue from 81 mCRC treated with EGFR mAb-based regimens (23). Analyzing the slides in a blinded fashion they identified 32 tumors as having a reduction of PTEN expression in >50% of tumor cells, only one of which had a response to treatment. However, of three examined outcomes (response rate, PFS, overall survival), only response rate, but not PFS or overall survival, was associated with decreased PTEN expression in the univariate analysis. The two key points limiting PTEN’s value as predictive marker are: (1) In contrast to the mutation analyses of KRAS, BRAF, and PIK3CA, which generate binary “yes-no” results, evaluating PTEN protein expression by IHC and other methods will produce a continuous variable with the challenge to define cut-off and threshold levels for interpretation. In addition, IHC results are known to be affected by significant inter- and intra-observer as well as method-based variability. (2) Only PTEN expression in metastases, but not in primary tumors is associated with outcome. It is not likely that routine biopsies of metastases will be done to obtain information on PTEN expression levels, which then only serve as a weak predictive marker for EGFR-targeted therapy. Thus, it is questionable if PTEN will ever be established as a predictive marker for EGFR mAb therapy in CRC.

**Ligands.** EGFR signaling is activated through a variety of receptor-specific ligands including transforming growth factor-α (TGF-α), amphiregulin, epiregulin, and EGF. These receptor ligands are capable of autocrine and/or paracrine activation of the EGFR. Khambata-Ford and colleagues selected an unbiased approach, using transcriptional profiling with Affymetrix arrays, to screen for molecular markers that would distinguish responders from nonresponders to cetuximab (27). RNA expression profiling of metastatic lesions of 80 patients with previously treated CRC showed epiregulin and amphiregulin to positively predict response to monotherapy with cetuximab. The median PFS in patients with high levels of epiregulin or amphiregulin, as defined by signal intensity above the median, was 103 days and 115 days for epiregulin and amphiregulin, respectively, whereas the median PFS was 57 days in patients whose tumors had RNA levels below the median ($P = 0.0002$ and $P < 0.0001$). These results were confirmed by quantitative analysis of RNA transcript numbers by real-time PCR (RT-PCR).

Subsequently, the results of four different studies suggest that these ligands might indeed predict the response toward EGFR-directed antibodies: Jonker and colleagues examined whether high expression of epiregulin RNA was associated with an increased response to cetuximab using the tumor samples of
67% of the 575 patients enrolled in the randomized controlled study of cetuximab versus BSC in mCRC. Overall survival was improved in patients whose tumors had both high epiregulin expression and KRAS-wt. However, whether this effect was due to the increased epiregulin expression or was related to the KRAS status remains unclear (28). Prenen and colleagues measured gene expression for epiregulin and amphiregulin in archived tumor samples from 220 mCRC patients who were not enrolled in a prospective clinical trial. The authors describe statistically significant correlations of "ligand expression" with improved overall and PFS although it remains unclear how ligand expression was determined, what the cut-off for ligand expression was, and how it was determined. In KRAS-mut tumors, no such correlation was found (29). In another study, similar results were obtained suggesting that high amphiregulin levels were associated with better response to anti-EGFR mAb and increased survival. Tejpar and colleagues evaluated 95 tumor specimens from patients treated with cetuximab and irinotecan and analyzed RNA expression levels of epiregulin and amphiregulin by RT-PCR (30). Amphiregulin RNA levels were 2.46 fold higher in responders when compared with nonresponders (P = 0.0001). Median overall survival was higher in patients whose tumors expressed above-median levels of amphiregulin RNA when compared with those expressing below the median (43.5 weeks versus 22.9 weeks, P = 0.008). Although EGFR ligand expression creates the same principal problem as discussed for PTEN, by generating a continuous variable rather than a binary decision tool, the data available so far show remarkable consistency in relatively large series. If the determination of EGFR ligand expression levels can be standardized, it could emerge as a positive predictive marker for efficacy of EGFR mAb in CRC, pending further confirmation in future analyses.

**EGFR gene copy number.** Increased gene copy number (GCN) of the EGFR gene as determined by FISH analysis is found in 20 to 50% of colorectal tumors (31, 32). Although supported by preclinical evidence, small sample sizes and multiple hypothesis testing limit the usefulness of most clinical studies describing a correlation between GCN and response to therapy with mAb. Accordingly, the results have been somewhat contradictory. Describing an increased GCN as a positive predictive factor (33, 34), without prognostic value (32), a positive prognostic factor without predictive value (35), or of no prognostic value (27), or as a positive predictive factor but only in combination with mutated KRAS (36). The most concisely study on this subject used tumor samples from 58 out of 263 patients enrolled in a single randomized phase III trial comparing panitumumab versus BSC in chemotherapy refractory mCRC. EGFR gene amplification was never homogeneous throughout the tumor but a mean GCN of ≥2.47 copies per nucleus discriminated between responders and nonresponders and none of the patients had a response when EGFR GCN was below this value (32). The apparently arbitrary nature of this cutoff level highlights one of the challenges associated with using GCN as predictive markers for EGFR mAb. It is doubtful if these findings will be reproducible in future studies.

**FcyRIIA-FcyRIIIA polymorphisms.** Cetuximab and panitumumab are both IgG antibodies and as such could be able to initiate immune responses. Some of the antitumor activity of other clinically used mAbs, such as trastuzumab (reviewed in more detail by Pohlmann and colleagues in this issue; ref. 37) and rituximab, has been attributed to antibody-dependent cell-mediated cytotoxicity (ADCC), mediated via the Fc-receptor (FcR) that is located on immune effector cells such as natural killer cells (38). Cetuximab, an antibody of the IgG1 subtype, has the capacity to initiate ADCC, in contrast to the IgG2 antibody panitumumab. Evidence for this comes from experiments with human xenografts in nude mice (39, 40). In humans, polymorphisms of two Fc-receptors (FcyRIIa and FcyRIIIa) have been associated with better outcomes in patients treated with rituximab for follicular lymphoma and in patients treated with trastuzumab for metastatic breast cancer (41). Data from a recent study provide evidence that this might translate to patients treated with cetuximab. Bibeau and colleagues sequenced the genes for FcyRIIa and FcyRIIIa from normal colonic tissue of 69 patients with mCRC in second-line therapy with a combination of cetuximab plus irinotecan, 5FU, or oxaliplatin (42). The authors stratified the patients according to KRAS status and focused on one polymorphism in the gene for FcyRIIa (H131R) and one in the gene for FcyRIIIa (V158F). Consistent with the results for rituximab and trastuzumab, these investigators found the 158V as well as the 131H/H genotype associated with better PFS and overall survival. However, considering all combined evidence from different tumors treated with IgG1-type antibodies and taking into account a high likelihood for linkage disequilibrium between both polymorphisms, Lejeune pointed out that only the 158V genotype of the FcyRIIIa receptor is associated with better response to IgG1-antibodies (41). These data provide the preliminary evidence that ADCC could be a clinically meaningful mechanism of action of these types of antibodies and should be taken into consideration as a predictive factor for cetuximab. On the other hand, if ADCC did indeed represent a clinically meaningful mediator of activity of cetuximab by eliciting a cytotoxic immune response after binding to tumor cells, cetuximab should also show some activity in KRAS-mutated tumors, a phenomenon that so far has not been observed.

**EGFR expression and EGFR mutations.** Although overexpression of EGFR as determined by IHC is mandated in the early trials with anti-EGFR mAb and cetuximab is FDA-approved only for patients with mCRC with detectable EGF by IHC, there is a current consensus that the EGFR expression levels determined by IHC do not correlate with response (43). This statement does not imply that EGFR-directed antibodies exhibit activity in the absence of their target, it simply highlights the lack of sensitivity of IHC to identify low expression levels of EGFR that are sufficient to elicit a response to EGFR mAbs. In addition, activating EGFR mutations have not been found in patients with CRC. This is especially notable because it has been found repeatedly in different tumor types, e.g., lung cancer and head and neck cancer, as well as in model cell lines of different tumor types, that response to any therapy directed at EGFR receptors is tied to either sensitizing mutations within the receptor or its overexpression (44).

**TP53.** The prognostic and predictive significance of inactivating mutations in the tumor suppressor TP53 in CRC has been a focus of intense research but the results so far have been conflicting. Inactivating mutations are inversely associated with
microsatellite instability, which by itself is a positive prognostic factor (7). However, in a study of 3,583 CRC patients TP53 mutations did not show significant prognostic value and were a positive predictor for better survival after chemotherapy only in patients with proximal tumors (45). In a recent report by Oden-Gangloff and colleagues, the authors found 46 different p53 mutations in 41 patients (46). Compared with patients with wt-p53, these patients were more likely to have controlled disease (P = 0.037) and a longer time to progression (20 versus 12 weeks, P = 0.004). In this retrospective study, all but one patient received irinotecan in addition to cetuximab and no control group was included. Thus, these results are currently only hypothesis-generating and need to be confirmed in larger, prospectively designed studies.

Clinical Predictive Markers of Efficacy of EGFR Antibodies

EGFR-associated rash. Acne-like rash occurs in a significant portion of patients treated with cetuximab or panitumumab and has been proposed as a potential biomarker for response. Evidence for this comes from the BOND trial, in which response rate was significantly different in patients with no skin reaction as compared with any skin reaction (P = 0.005; ref. 2). In the EVEREST trial, patients with mCRC who had previously failed irinotecan-based therapy were randomized to dose escalation of cetuximab to up to 500 mg/m² or to the standard dose of 250 mg/m² weekly. The patients who tolerated and received higher doses of cetuximab experienced a higher response rate (25 to 30% versus 16%), as well as increased skin toxicity when compared with standard dose treatment. Upon further analysis, separating patients into four groups according to the grade of skin toxicity resulted in nonoverlapping curves. However, it has to be kept in mind that patients tolerating high doses of cetuximab might represent a group with an overall better performance status and thus a better PFS. In addition, it is important to note that whereas EGFR rash and KRAS status are independent, not associated markers, patients with KRAS mutated tumors will not benefit from EGFR mAb, even if they develop a severe rash.

Conclusions

Over the last 2 years several molecular markers have been investigated as potential predictors for the efficacy of anti-EGFR mAb in CRC with KRAS clearly leading the way. The significance of the KRAS story in CRC, however, goes way beyond the immediate implications of identifying patients who will not benefit from EGFR antibodies. The KRAS story opens the door toward a new understanding and definition of CRC. It has sparked the generation of biomarker-driven trial designs in this disease and has led to a growing excitement that this model may pave the way to the identification of similar biomarkers for safety and efficacy of medical therapy, with tremendous implications for the patient, clinical practice, and public health.

The results of the CAIRO2 trial clearly show the urgent need for true biomarkers that can distinguish between subgroups of patients to separate those who benefit from anti-EGFR mAb from those whose tumors are resistant and particularly those in whom anti-EGFR mAb has a detrimental effect. On the other hand, the observation of rapid and complete response to anti-EGFR mAb alone raises the possibility that some tumors might even be completely dependent on intact EGRFR signaling, similar to the dependence of chronic myeloid leukemia tumor cells on the Bcr-Abl fusion gene and c-kit mutations in gastrointestinal stromal tumor cells (reviewed by Apperley and Milojkovic and by Gramza and colleagues in this issue, respectively; refs. 47, 48), which makes it even more important to identify this subgroup. Given the enormous number of candidate markers, finding true predictors of resistance or efficacy seems a daunting task.

Ideally, once a candidate biomarker for a given drug is identified through preclinical studies and early clinical trials, the biomarker should be integrated into the definite efficacy trial of the drug. The number and nature of subgroups to be tested should be prespecified; the sensitivity, specificity, and reproducibility of the assay used to detect the biomarker should be known; randomization should be used to ensure comparability between study groups; the clinical trial should be independently reproduced, ideally with a different design. However this approach is not practical to apply to all candidates given the sheer number of potential markers that emerge from retrospective subgroup analysis. So far, none of the potential biomarkers described in this review has been tested according to these criteria and it is doubtful if the majority of these markers ever will. This raises concerns about potential bias introduced by the retrospective nature of the currently available data. Specifically, reasons for bias could include (1) not adjusting for multiple hypothesis testing, (2) poorly validated assays to assess the biomarker of interest, and (3) tumor tissue available for retrospective analysis could be nonrepresentative of the intended study population (49). The latter would be the case if, for example, informed consent was retrospectively needed for a genomic analysis of tumor specimen, which would naturally favor the inclusion of tumors of patients still alive and with a performance status good enough to give consent.

In the case of KRAS, a pragmatic approach has been proposed to further validate it as a biomarker of predictive value. An open label international phase III trial randomizing patients to FOLFIRI with or without panitumumab as first-line treatment of mCRC has recently completed accrual of 1,183 patients. Similarly, an open label randomized phase III trial investigating FOLFIRI ± panitumumab in second-line treatment of mCRC has completed accrual of 1,187 patients. Retention of tumor samples was mandatory for all patients enrolled in these trials. For both studies, the primary endpoint, PFS, has not been analyzed yet. Although not initially planned, a prospective analysis of KRAS mutational status will be added using the PCR-based DxS KRAS mutation kit. The mutational analysis will be done before analyzing clinical outcome parameters, thereby creating a situation in which the validity of KRAS as a predictive marker for panitumumab can be tested prospectively.

It is obvious that most molecular predictive and prognostic markers will likely be discovered in retrospective analyses of
tumor tissue obtained in clinical trials. Depending on the convincing consistency of the data these markers will or will not be testable in prospective clinical trials. Because of the crucial importance to identify markers that guide our therapeutic decisions, especially in the era of “targeted therapies,” future clinical trials have to be planned to allow retrospective testing of a large number of future biomarkers. This will only be possible if tissue collection becomes a mandatory component of clinical trial design.

Disclosure of Potential Conflicts of Interest
A. Grothey, consultant, BMS, Roche, Genentech, Amgen, Bayer. M. Banck has no conflicts of interest.

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Biomarkers of Resistance to Epidermal Growth Factor Receptor Monoclonal Antibodies in Patients with Metastatic Colorectal Cancer

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