

PIK3CA, BRAF, and PTEN Status and Benefit from Cetuximab in the Treatment of Advanced Colorectal Cancer—Results from NCIC CTG/AGITG CO.17

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Abstract

Purpose: Cetuximab improves survival in patients with *K-ras* wild-type advanced colorectal cancer. We examined the predictive and prognostic significance of additional biomarkers in this setting, in particular *BRAF*, *PIK3CA*, and *PTEN*.

Experimental Design: Available colorectal tumor samples were analyzed from the CO.17 study. *BRAF* mutations were identified in tumor-derived DNA by direct sequencing and *PIK3CA* mutations were identified using a high-resolution melting screen with confirmation by sequencing. *PTEN* expression by immunohistochemistry (IHC) was performed on tissue microarrays. For each biomarker, prognostic and predictive effects were examined using a Cox model with tests for treatment–biomarker interaction.

Results: A total of 572 patients with pretreated colorectal cancer were randomly assigned to receive cetuximab or best supportive care (BSC). Of 401 patients assessed for *BRAF* status, 13 (3.2%) had mutations. Of 407 patients assessed for *PIK3CA* status, 61 (15%) had mutations. Of 205 patients assessed for *PTEN*, 148 (72%) were negative for IHC expression. None of *BRAF*, *PIK3CA*, or *PTEN* was prognostic for overall or progression-free survival in the BSC arm. None was predictive of benefit from cetuximab, either in the whole study population or the *K-ras* wild-type subset. In the *K-ras* wild-type subgroup, the overall survival adjusted HR according to *BRAF* mutation status was 1.39 (interaction $P = 0.69$), *PIK3CA* mutation status HR = 0.79 (interaction $P = 0.63$), and *PTEN* expression HR = 0.75 (interaction $P = 0.61$).

Conclusions: In chemotherapy-refractory colorectal cancer, neither *PIK3CA* mutation status nor *PTEN* expression were prognostic, nor were they predictive of benefit from cetuximab. Evaluation of predictive significance of *BRAF* mutations requires a larger sample size. *Clin Cancer Res*; 20(3); 744–53. ©2013 AACR.

Introduction

Our understanding of the molecular biology of cancer is advancing and this has led to the development of molecular-targeted therapies. Colorectal cancer, the third most common cancer worldwide, can be treated with the monoclonal antibody cetuximab that inhibits the epidermal growth factor receptor (EGFR; refs. 1 and 2). Cetuximab has been demonstrated to improve overall survival (OS), progression-free survival (PFS), and better preserve quality of

life in patients with *K-ras* wild-type chemotherapy-refractory advanced colorectal cancer (3). However, a significant proportion of *K-ras* wild-type cancers exhibit primary resistance to cetuximab; in other patients, secondary resistance inevitably develops and represents a major therapeutic obstacle. Avoidance of cetuximab in those that will not benefit will improve the therapeutic index and cost effectiveness of therapy. Biomarkers beyond *K-ras* may further aid in the optimal selection of patients for cetuximab. Recognition of biological mechanisms of resistance to cetuximab remains important in efforts to develop better treatment strategies.

EGFR is a trans-membrane tyrosine kinase receptor that is activated on ligand binding. The binding of the ligand to the extracellular domain of the EGFR receptor leads to phosphorylation of the tyrosine residues located in the intracellular domain. Multiple subsequent intracellular signaling pathways are subsequently activated, including the Ras/mitogen-activated protein kinase (MAPK) pathway (RAS-RAF-MAPK pathway) and the phosphoinositide 3-kinase (PI3K/Akt) pathway (PI3K-PTEN-AKT pathway).

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Translational Relevance

PIK3CA mutation status and PTEN expression within the tumor were not predictive of clinical benefit from cetuximab in the setting of metastatic colorectal cancer previously treated with chemotherapy. With only a small number of *BRAF* mutant tumors in our study, firm conclusions about the predictive relevance of this biomarker cannot be established. Accurate and clinically relevant selection and exclusion of patients for treatment with epidermal growth factor receptor monoclonal antibodies remains a critical step in developing the optimal treatment strategy for patients with colon cancer. The search for additional predictive biomarkers beyond *K-ras* continues. Within the CO.17 study, *K-ras* mutation status remains the only robust predictor of benefit. Other molecular predictive biomarkers cannot be recommended in clinical practice at the current time.

Both pathways play pivotal roles in cancer cell proliferation and resistance to apoptosis. The MAPK pathway is mainly involved in cell proliferation, differentiation, and apoptosis, whereas the PI3K/Akt pathway is important for cell survival and cancer cell invasion (4, 5).

The activation of EGFR and the downstream signaling pathways can be mediated through several mechanisms, including overexpression of the receptor, overexpression of the ligand, activating mutation of the receptor or inactivation of tumor suppressor genes. The ligands of EGFR, the receptor itself, and the downstream signaling molecules such as *K-ras*, *NRAS*, *BRAF*, *PIK3CA*, and its suppressor PTEN have all been examined as potential effectors of resistance to EGFR-targeted therapy. The mutation status of signaling molecules downstream of the EGFR target may predict clinical benefit to EGFR-targeted therapies.

We undertook correlative biomarker analyses of *BRAF* and *PIK3CA* mutation status and PTEN expression with the primary clinical outcomes of the phase III clinical trial CO.17, a study conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) and the Australasian Gastrointestinal Trials Group (AGITG). This trial included patients with chemotherapy refractory colorectal cancer who were randomized to receive cetuximab plus best supportive care (BSC) or BSC alone. We evaluated the predictive effects and the treatment-independent prognostic significance of *BRAF*, *PIK3CA*, and PTEN.

Materials and Methods

This correlative study was designed by a protocol committee that included members of the NCIC CTG and the AGITG. The NCIC CTG analyzed the data and maintains full unrestricted rights to publication of the study data.

Patients and CO.17 trial design

CO.17, a phase III randomized controlled trial conducted by the NCIC and the AGITG, included 572 patients with

chemotherapy-refractory colorectal cancer. The trial design and eligibility criteria have been previously reported (2). Eligible patients were entered into the trial between December 2003 and August 2005. Patients were randomized to receive cetuximab plus BSC versus BSC alone. Cetuximab was administered as an initial dose of 400 mg/m² and then 250 mg/m² each week. Patients in both arms were evaluated for tumor response or progression every 8 weeks. Cetuximab therapy was continued until disease progression or intolerable toxicity.

Laboratory method

Formalin-fixed paraffin-embedded tumor tissue samples from archival specimens collected at the time of disease diagnosis were stored at a central tumor bank located at Queen's University in Kingston, Ontario, Canada. If tumor blocks were not available unstained slides were retrieved. Assays of tissue samples for *BRAF* and *PIK3CA* mutations were performed in a blinded fashion in the laboratory at the Ottawa Hospital Research Institute. PTEN expression in the tumor samples was also measured, again blinded to clinical outcome.

DNA extraction. DNA was extracted using Quick Extract FFPE DNA Extraction Kits (Epicentre Biotechnologies). Either macrodissection or laser capture microdissection was used to ensure that all samples were derived from ≥80% cancer cells. Laser capture microdissection was performed using an Arcturus XT instrument (Applied Biosystems Canada).

Control samples. Control samples for mutation analyses were prepared from cell lines with known mutation status. Cell pellets were formalin fixed and paraffin embedded and then analyzed as for tissue sections. The following cell lines were used: human colorectal cancer cell line HT29 (*BRAF* heterozygous V600E mutation), human breast cancer cell line MCF7 (heterozygous *PIK3CA* exon 9 E545K mutation), and human breast cancer cell line T47D (heterozygous *PIK3CA* exon 20 H1047R mutation). All cell lines were from the American Type Culture Collection, were used at less than 6 months after thawing, and were routinely checked for the absence of mycoplasma. The American Type Culture Collection authenticates cell lines using Short Tandem Repeat profiling.

***BRAF* mutation analyses.** The mutation status of exon 15 in the *BRAF* gene was assessed using a nested PCR procedure and sequencing. First round PCRs were performed with outside primers (forward primer 5'-CTCTCA-TAATGCTGCTCTGATAGG-3'; reverse primer 5'-T AGT AAC TCA GCA GCA TCT CAG G-3'). Second round PCRs with M13-tagged primers were performed with inside primers (forward primer 5'-CAC GAC GTT GTA AAA CGA CTG CTT GCT CTG ATA GGA AAA TG-3'; reverse primer 5'-GGA TAA CAA TTT CAC ACA GGA TCT CAG GGC CAA AAAT-3'). Second round PCR products were sequenced directly at Stem Core Laboratories, Ottawa Hospital Research Institute, using Big Dye Terminator v 3.1 Chemistry and an Applied Biosystems 3730 DNA Analyzer. Control samples of HT29 and MCF7 DNA were included in each sequencing run.

PIK3CA high-resolution melt mutation analyses. The mutation status of exons 9 and 20 in the *PIK3CA* gene was assessed using a nested PCR procedure and a high-resolution melting screen. The first round of PCR was performed in a multiplexed fashion with outside primers for both exon 9 (forward primer 5'-CTG TGA ATC CAG AGG GGA AA-3'; reverse primer 5'-GCA CTT ACC TGT GAC TCC ATA GAA-3') and exon 20 (forward primer 5'-TGA GCA AGA GGCTTT GGA GT-3'; reverse primer 5'-CCT ATG CAA TCG GTC TTT GC-3') combined in the same reaction. The second round of the nested PCR and high-resolution melt analysis was performed separately for exon 9 (inside forward primer 5'-AAG GGA AAA TGA CAA AGA ACA G-3'; inside reverse primer 5'-CAC TTA CCT GTG ACT CCA TAG AA-3') and exon 20 (inside forward primer 5'-GCA AGA GGCTTT GGA TTT C-3'; inside reverse primer 5'-TTT TCA GTT CAA TGC ATG CTG-3') using a Corbett Rotorgene 6000 and Syto9 dye. Control samples of MCF7 and T47D DNA were included in each high-resolution melt analysis run. All samples that were positive for mutations by high-resolution melting were confirmed by sequence analysis.

Tissue microarrays. Digital scans of hematoxylin and eosin stained sections from formalin-fixed paraffin-embedded blocks were marked by a pathologist (M. Daneshmand) for areas of tumor and normal tissue. For each patient, three 0.6 mm cores of tumor tissue and one 0.6 mm core of normal tissue (when present in the sample) were included in the microarrays. Blocks from 217 patients were included in tissue microarrays; normal tissue was included for 37 of these. The main reason for exclusion of patients was that the several centers involved in this multicenter trial had sent slides only, rather than blocks. Some blocks were excluded if there was insufficient tumor tissue.

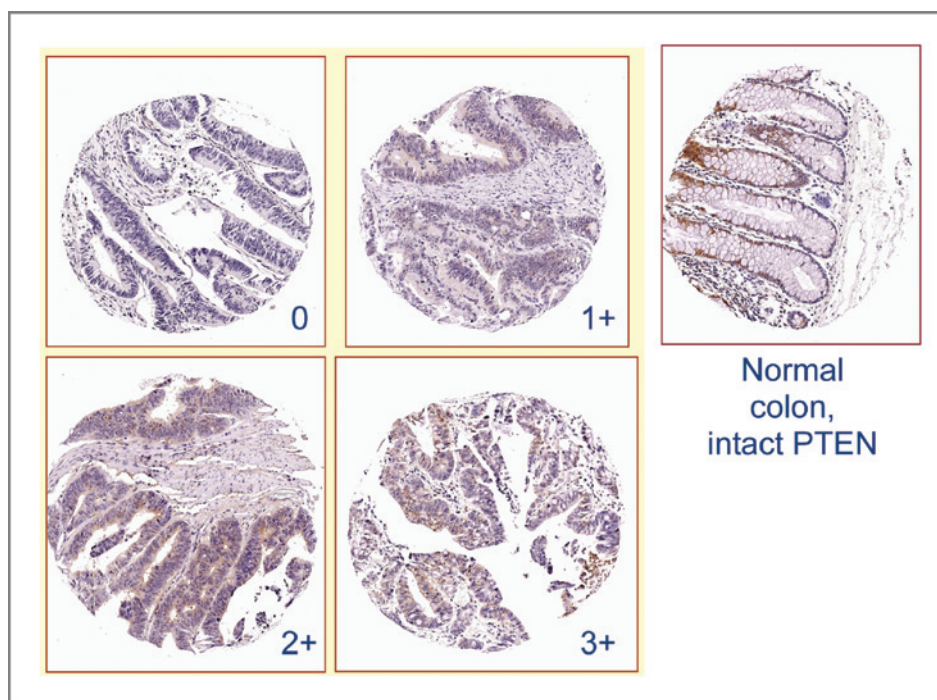
PTEN immunohistochemistry. TMAs were sectioned in Kingston, Ontario, and paraffin-dipped to preserve antigenicity. Sections (including an adjacent HE section) were shipped overnight to Ottawa, Ontario, and IHC was performed 3 days after sectioning. PTEN (138G6) rabbit monoclonal antibody from Cell Signaling Technology (cat. no. 9559) was used. The following experiments were used to validate this antibody: in Western blots, this antibody labeled a unique band of the correct size in the U87MG human glioblastoma cell line transduced with cDNA to PTEN, but did not label any bands in parental U87MG cells, which do not express PTEN because of a mutation (6); IHC on PTEN-positive cell lines MCF7 and HT29 gave a positive signal but gave no signal on PTEN-negative U87MG cells. IHC was performed as follows: after deparaffinization and rehydration, antigen retrieval was performed using Citrate-based Antigen Unmasking Solution (Vector Laboratories); sections were then blocked with 1.5% normal goat serum and incubated overnight at 4°C with a 1:50 dilution of PTEN antibody, followed by detection with EnVision+ System- HRP Labelled Polymer, Anti-Rabbit (Dako). Sections made from formalin-fixed, paraffin-embedded cell lines of known PTEN status (U87MG, HT29, and MCF7) were stained in parallel with TMA slides to serve as positive

and negative controls for IHC and to ensure consistency in staining. Digital images of TMA slides were scored by 2 pathologists (M. Daneshmand and C. Marginean) using an Aperio Scanscope with TMA Lab Microarray Analysis software (Aperio). Separate scores were given for each core for average cytoplasmic staining intensity (0, +1, +2, +3) and the percentage of cancer cells positive for cytoplasmic staining. The 2 pathologists were blinded to all clinical data and also blinded to each other's initial scores. For final analysis, scores were dichotomized into PTEN negative (no positive staining in any core) and PTEN positive (positive staining detected in any core). There was 96% concordance between the 2 pathologists' initial scores. Scoring discrepancies were resolved by side-by-side review of the relevant samples by the pathologists. Examples of PTEN IHC are shown in Fig. 1.

Statistical analysis

All statistical analyses were performed at the NCIC CTG in accordance with a prespecified statistical analysis protocol that was written before *BRAF* and *PIK3CA* mutation assessment and PTEN IHC was performed. All subjects who were randomized and had biomarker status data available were included in the analyses, representing the evaluated dataset. OS, the primary endpoint of this study, was defined as the time from randomization until death from any cause. The secondary endpoints examined were PFS, defined as the time from randomization until the first objective observation of disease progression or death from any cause, response rates, defined according to RECIST criteria, and quality of life assessed by EORTC QLQ-C30. The survival of subjects in each biomarker (*PTEN*, *BRAF*, *PIK3CA*) and/or treatment group was summarized using Kaplan-Meier curves and the difference between these groups was compared using the log-rank test with the HR and its 95% confidence interval (CI) calculated based on the Cox regression model with a single covariate. To assess whether each biomarker was an independent prognostic factor, for patients on BSC only, a multivariate Cox regression model was used, which included presence or absence of the marker and the following protocol-specified covariates: Eastern Cooperative Oncology Group (ECOG) performance status (0-1 vs. 2), gender (male vs. female), age (65 or older vs. younger than 65), baseline lactate dehydrogenase level [higher than upper normal limit (UNL) vs. UNL or less], baseline alkaline phosphatase (higher than UNL vs. UNL or less), baseline hemoglobin [less than lower limit of normal (LLN) vs. LLN or higher], number of disease sites (more than 2 vs. 2 or less), number of previous chemotherapy drug classes (more than 2 vs. 2 or less), primary tumor site (rectum only vs. colon), presence of liver metastases (yes vs. no). We used the Cox model, which included treatment, biomarker (*BRAF*, *PIK3CA*, or *PTEN*) status, and their interaction and other covariates listed above, to assess the predictive effect of the biomarker status. To assess the prognostic (treatment independent) significance of each of the 3 biomarkers, the effect of presence or absence of the marker was assessed in the BSC population only.

Figure 1. Examples of PTEN IHC scores 0 to 4, as scored by 2 pathologists. Score of 0 indicates a negative PTEN expression. PTEN positive = score 1+, 2+, 3+.



Results

A total of 572 patients were randomly assigned to receive cetuximab (287) or BSC (285). Table 1 summarizes all the baseline characteristics of the study subject according to the biomarker subgroup of interest and in the total population. We did not observe any significant differences in baseline clinical characteristics and prognostic factors

Table 1. Prognostic and predictive analyses of *BRAF*, *PIK3CA*, PTEN (prognostic significance determined in patients receiving BSC only, predictive significance determined in patients with *K-ras* wild-type tumors)

Biomarker	Prognostic analysis in BSC patients adj HR (mutant vs. wild-type), [for PTEN, negative vs. present]	
	OS	PFS
<i>BRAF</i>	1.47, $P = 0.41$	1.52, $P = 0.37$
<i>PI3KCA</i>	1.11, $P = 0.65$	1.10, $P = 0.66$
PTEN	1.13, $P = 0.70$	0.99, $P = 0.98$
	Predictive analysis in <i>KRAS</i> wild-type subset	
	adj HR (cetuximab+BSC vs. BSC), interaction P value	
	OS	PFS
<i>BRAF</i>	1.39, $P = 0.69$	1.18, $P = 0.84$
<i>PI3KCA</i>	0.79, $P = 0.63$	0.73, $P = 0.50$
PTEN	0.75, $P = 0.61$	2.47, $P = 0.088$

between the intention-to-treat population and any biomarker evaluable subsets. The biomarker groups were similar with respect to these baseline characteristics including ECOG performance status and other variables that were found to be associated with survival in the multivariate analysis. Compared with the *BRAF* wild-type group, the *BRAF* mutant group had a trend toward less prior irinotecan use (85% vs. 96%, $P = 0.066$) and fewer lines of prior therapy (1–2 lines in 19% vs. 39%, $P = 0.3$). Within the *K-ras* wild-type subset, more patients were more than 65 years (45% vs. 27%, $P = 0.031$) in the *PIK3CA* wild-type versus mutant groups. Within the *K-ras* wild-type subset, PTEN negative patients had significantly more prior radiotherapy (31% vs. 12%, $P = 0.033$), less liver involvement (82% vs. 97%, $P = 0.034$), and more nodal disease (57% vs. 30%, $P = 0.011$) than those with intact PTEN staining.

BRAF mutation analyses

In total, 418 samples were analyzed: analyses were successful in 410 of these. Of the 410, 397 were wild-type and 13 had V600E mutations (3.2% mutation frequency). A total of 359 samples were evaluable for both *BRAF* and *K-ras*; of these 12 patients had *BRAF* mutant tumors, 10 of which had wild-type *K-ras* and 2 had both *K-ras* and *BRAF* mutations. The incidence of *BRAF* mutations among *K-ras* wild-type patients was 4.8% (10/208).

Prognostic analysis (in BSC patient subset). The presence of *BRAF* mutations was associated with a nonsignificantly inferior OS (HR = 1.46, $P = 0.41$), acknowledging the low number of observed cases in our study.

Predictive analysis (in *K-ras* wild-type patient subset). There was no significant association between *BRAF*

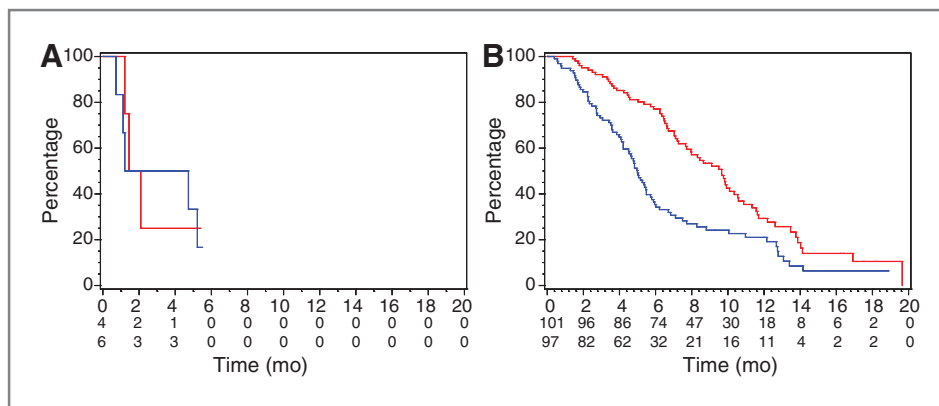


Figure 2. Kaplan–Meier curves for OS according to *BRAF* mutation status and OS by treatment within the K-ras wild-type subset. A, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and *BRAF* V600E mutations; red = cetuximab+BSC; blue = BSC alone. B, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and wild-type *BRAF*; red = cetuximab+BSC; blue = BSC alone.

mutation status and OS or PFS benefit from cetuximab (interaction P -value = 0.7; Fig. 2). Among *BRAF* wild-type patients, median OS was 9.7 versus 5.0 months for cetuximab versus BSC, respectively (HR = 0.52; $P < 0.0001$; Fig. 2). Among patients with tumor that harbored *BRAF* mutations, the median OS was 1.77 versus 2.97 months for cetuximab versus BSC (HR = 0.84; $P = 0.81$) and PFS HR was 0.76 ($P = 0.69$), but again this was limited by low frequency of *BRAF* mutations and therefore the low number of *BRAF* mutant cases. There were no tumor responses to cetuximab in the 4 patients with *BRAF* mutant versus 14% (14/101) in patients with *BRAF* wild-type status ($P = 1.0$).

PIK3CA mutation analyses

For exon 9, a total of 417 samples were analyzed: analysis was successful in all of these. Of the 417, 360 were wild type, 44 (10.6%) had E545K mutations, and 13 (3.1%) had E542K mutations. For exon 20, a total of 417 samples were analyzed: analyses were successful in 415 of these. Of the 415, 409 were wild type and 6 (1.4%) had H1047R mutations. Overall mutation frequency (either E542K, E545K, or H1047R, or a combination) was 14.7%. *PIK3CA* mutations were found in 12% versus 18% of those with *K-ras* wild-type and *K-ras* mutant status, respectively.

Prognostic analysis (in BSC patient subset). On comparison of survival outcomes according to the *PIK3CA* mutation status (any mutation versus no mutation) in the BSC

arm, there was no difference in OS (HR = 1.11, $P = 0.65$) or PFS (HR = 1.10, $P = 0.66$).

Predictive analysis (in K-ras wild-type patient subset).

The *PIK3CA* mutation status was not associated with any difference in OS or PFS benefit from cetuximab therapy in patients with K-ras wild-type tumors (interaction P -value = 0.63). Among *PIK3CA* wild type, median OS was 9.5 versus 5.1 months for cetuximab versus BSC, respectively (HR = 0.53; $P = 0.0002$; Fig. 3). Among *PIK3CA* mutant, median OS was 9.9 versus 3.6 months for cetuximab versus BSC, respectively (HR = 0.43; $P = 0.059$; Fig. 3). Tumor response rate to cetuximab was 20% versus 12% in patients with *PIK3CA* mutant versus wild-type status, respectively.

PTEN immunohistochemistry

For data analyses, results were grouped into those that were negative (0% of cells positive) and those that showed any percentage of cells positive. Of 205 evaluable samples, 148 (72%) were negative for PTEN by IHC (defined as 0% of cells positive).

Prognostic analysis (in BSC patient subset). On comparison of survival outcomes according to the PTEN status (present vs. loss) in the BSC arm, there was no difference in OS (HR = 1.13, $P = 0.70$) or PFS (HR = 0.99, $P = 0.98$).

Predictive analysis (in K-ras wild-type patient subset).

There was no significant association between PTEN status and OS or PFS benefit from cetuximab therapy (interaction

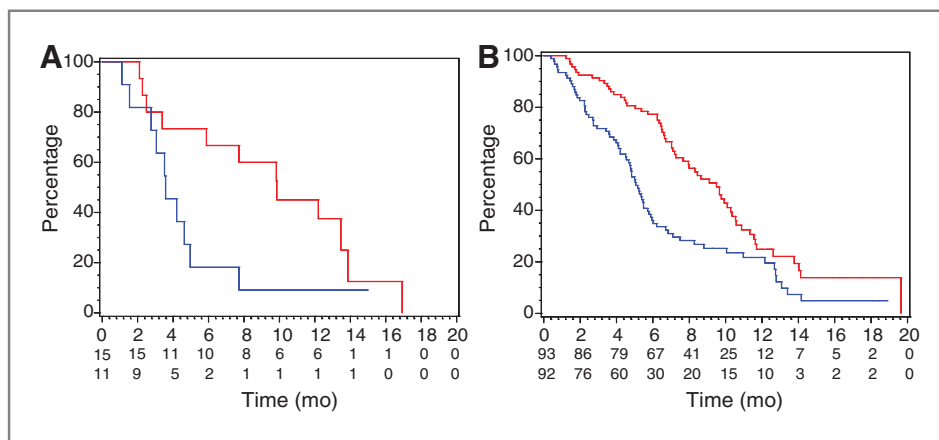
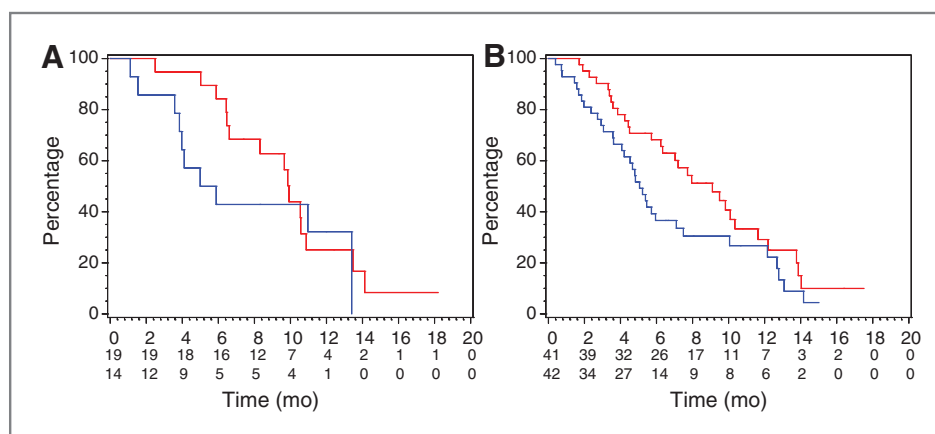


Figure 3. Kaplan–Meier curves for OS according to *PIK3CA* mutation status and OS by treatment in K-ras wild-type subset. A, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and mutated *PIK3CA*; red = cetuximab+BSC; blue = BSC alone. B, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and wild-type *PIK3CA*; red = cetuximab+BSC; blue = BSC alone.

Figure 4. Kaplan–Meier curves for OS according to PTEN expression and OS by treatment within the K-ras wild-type subset. A, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and exhibit positive PTEN expression (score 1+, 2+, 3+); red = cetuximab+BSC; blue = BSC alone. B, Kaplan–Meier curves of OS for patients with tumors that have wild-type K-ras and negative PTEN expression (score 0); red = cetuximab+BSC; blue = BSC alone.



P -value = 0.61). Among PTEN positive, the median OS was 9.9 versus 5.4 months for cetuximab versus BSC, respectively (HR = 0.66; P = 0.32; Fig. 4). Among PTEN negative, median OS was 9.1 versus 5.1 months for cetuximab versus BSC (HR = 0.63; P = 0.065; Fig. 4). Tumor response rate to cetuximab was 21% versus 15% in patients with PTEN positive versus negative staining.

These results are summarized in Tables 1 to 3.

Discussion

Neither *PIK3CA*, *PTEN*, nor *BRAF* demonstrated predictive significance in the context of advanced K-ras wild-type colorectal cancer refractory to chemotherapy and treated with cetuximab in our study. We also performed correlative analyses of the prognostic significance of these biomarkers in patients who received BSC without any chemotherapy or anti-EGFR-directed therapy. None of these biomarkers was shown to be prognostic. The number of *BRAF* cases was low, limiting the power of the findings related to this biomarker. These observations are added to an increasing "mixed bag" of published reports of these biomarkers in colorectal cancer. Our study shares several limitations common to the majority of published findings in this field. Some of the

biomarkers are observed infrequently, or biomarker subgroups are examined within another biomarker category subset, culminating in the evaluation of relatively small patient numbers. Exploration of more biomarker subgroups necessitates multiple comparisons, which increases the probability of false positive findings. The biomarkers we selected were not identified *a priori* as biological parameters of interest at the time of study commencement. The retrospective analysis is prone to bias or error. However, our study offers an ideal opportunity to elucidate the prognostic impact of these biomarkers in the absence of therapy through correlative analyses performed in the best supportive arm. The predictive analyses allow an opportunity to explore the clinical significance of these markers in this specific setting, and may inform in our appreciation of biological mechanisms of resistance. Each biomarker will be discussed in turn.

BRAF

BRAF, a signaling protein downstream of *K-ras*, with activating mutation (V600E), was found infrequently in this setting. *BRAF* mutations are known to usually be mutually exclusive of K-ras mutations in colorectal cancer (7). Previous studies have demonstrated that approximately

Table 2. Survival benefit from cetuximab for the ITT study population, by K-ras mutation status and by the mutation status of *BRAF*, *PIK3CA* and *PTEN* within the K-ras wild-type subset

Patient subset	OS adjusted HR	HR (95% CI)	Interaction P value
CO17 ITT (n = 572)		0.77 (0.64–0.92)	
K-ras MUT		0.98 (0.70–1.37)	
K-ras WT		0.55 (0.41–0.74)	P = 0.01
+BRAF WT		0.52 (0.37–0.71)	
+BRAF MUT		0.84 (0.20–3.58)	P = 0.70
+PIK3CA WT		0.53 (0.37–0.74)	
+PIK3CA MUT		0.43 (0.18–1.06)	P = 0.63
+PTEN intact		0.66 (0.29–1.52)	
+PTEN loss		0.63 (0.38–1.03)	P = 0.61

Table 3. PFS benefit from cetuximab for the ITT study population, by K-ras mutation status and by the mutation status of BRAF, PIK3CA and PTEN within the K-ras wild-type subset

Patient subset	PFS adjusted HR	HR (95% CI)	Interaction P value
CO17 ITT (n = 572)		0.68 (0.57–0.80)	
K-ras MUT		0.99 (0.73–1.35)	
K-ras WT		0.40 (0.30–0.54)	P < 0.001
+BRAF WT		0.41 (0.30–0.55)	
+BRAF MUT		0.76 (0.19–3.08)	P = 0.84
+PIK3CA WT		0.40 (0.29–0.56)	
+PIK3CA MUT		0.27 (0.10–0.69)	P = 0.50
+PTEN intact		0.66 (0.31–1.41)	
+PTEN loss		0.34 (0.20–0.57)	P = 0.09

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Favours Cetuximab Favours BSC

3% to 15% of advanced colorectal cancer cases carry a *BRAF* mutation, usually involving the V600E allele (8–11). In our series, the *BRAF* mutation rate was low with only 3.2% of cases harboring *BRAF* mutations. This may be related to the heavily pretreated nature of the patients in our study. *BRAF* mutations have been consistently demonstrated to predict for poorer survival regardless of therapy (i.e., negative prognostic marker) in metastatic colorectal cancer (12–15). Our findings are in keeping with this, as we observed an inferior OS in the *BRAF* mutation subgroup within the BSC arm, but given the low number of cases this was not statistically significant. We observed no responses in this *BRAF* mutation positive K-ras wild-type subgroup when treated with cetuximab. Prior studies have suggested that mutations in *BRAF* may be associated with impaired response to EGFR monoclonal antibodies and shorter PFS and OS (16–20). The role of *BRAF* mutations in predicting resistance to anti-EGFR monoclonal antibodies remains debatable. Retrospective analyses from the CRYSTAL and OPUS studies have shown that even patients with *BRAF*-mutated tumors benefit from cetuximab when used in combination with chemotherapy (13, 15, 21). In contrast, 2 studies that evaluated the role of panitumumab combined with chemotherapy, one in the first-line setting (PRIME) and the other second-line (PICCOLO) demonstrated that patients with tumors bearing *BRAF* mutations do not benefit from the addition of panitumumab (22, 23). Both of these studies confirmed the poor prognostic effect of *BRAF* mutations in both the first line and second line chemotherapy setting. A third randomized controlled trial that studied panitumumab use in the chemotherapy refractory third line advanced colorectal cancer treatment setting could not demonstrate a clear predictive influence of *BRAF* mutation status as both *BRAF* mutant and wild-type subgroups seemed to receive a possible PFS benefit from panitumumab (24). We must emphasize that our study included only 13 cases of *BRAF* mutation containing tumors. An even fewer number of these, just 4, received cetuximab. We did not observe any predictive value of *BRAF* mutation status in our study but given such low numbers firm conclusions are not possible.

PIK3CA

Besides the KRAS-BRAF-MAPK pathway, the other major downstream signaling pathway activated by EGFR is the PI3K/PTEN/AKT/mTOR signaling pathway. PIK3CA can be dysregulated by activating mutations in the PIK3CA p110 subunit or through inactivation of the tumor suppressor phosphatase and tensin homologue (PTEN) phosphatase. *PIK3CA* and PTEN mutations can coexist with KRAS and BRAF mutations. The incidence of *PIK3CA* mutations in our study was 14% is in keeping with previous reports (19, 20, 25–27). Most of these mutations were in exon 9, also in keeping with previous studies in colorectal cancer (10, 28). In our study, there was no suggestion of a prognostic impact according to *PIK3CA* mutation status in the BSC arm. Preclinical studies have suggested a possible predictive role for *PIK3CA*. Colon cancer cell lines with activating *PIK3CA* mutations or loss of PTEN expression (PTEN null) have been shown to be more resistant to cetuximab than *PIK3CA* wild-type/PTEN-expressing cell lines (29). *PIK3CA* mutant isogenic HCT116 cells have also shown increased resistance to cetuximab compared with *PIK3CA* wild-type controls (29). Despite the preclinical data, clinical findings have been inconsistent. Initial reports indicated the presence of *PIK3CA* mutations in colorectal cancer predicted for a diminished response to EGFR inhibitors (30, 31). A more recent series from Greece also reported shorter PFS associated with *PIK3CA* mutations (19). In other studies, no clear correlation between *PIK3CA* and cetuximab response or PFS has been observed. In a retrospective analysis involving 200 patients with chemotherapy refractory advanced colorectal cancer, *PIK3CA* mutations were not a major determinant of resistance to cetuximab (26). A large retrospective cohort analysis including 1,022 colorectal cancer specimens suggested that correlation with response may be present but is limited to exon 20 mutations and not exon 9 mutations (32). We observed very few exon 20 mutations in our study. A total of 57 (13.7% of those evaluable) patients in our study had tumor with exon 9 mutations and only 6 (1.4%) had exon 20 mutations. Responses were seen in patients with both *PIK3CA* mutant and wild-type tumors. We did not observe a predictive impact within the K-ras wild-type

subgroup, so again we do not have evidence to support treatment selection based on PIK3CA status. With a total of 159 deaths observed and a 12% prevalence rate of mutation within the K-ras wild-type subgroup, our study only has 80% power to detect an interaction HR of 0.26 (vs. 0.79 observed) and 2-sided 5% level.

PTEN

Although samples (slides or blocks) were available for 430 patients, there were blocks with sufficient material for TMA construction for 217 patients. Analysis of all patients, and patients included in the TMA, for age, gender, ECOG status, site of primary, prior chemotherapy, sites of disease, number of sites of disease, and treatment (i.e., cetuximab or BSC) did not show any significant differences between the subset of patients included in the TMA construction and the entire study trial population. PTEN is inactivated by heterozygous or homozygous gene deletion and also by promoter methylation. Loss of PTEN drives constitutive PI3-kinase pathway activation (33). Currently there is no consensus method for assessing PTEN expression in colorectal cancer. Previous studies on PTEN expression and cetuximab benefit have reported absence of expression ranging from 14% to 57% negativity for PTEN, using a variety of different antibodies and scoring systems (18, 25, 26, 34–36). The antibody used in this study was chosen as it passed a detailed validation that we performed to ensure that it recognized PTEN and did not cross-react with other antigens either by Western blotting or in IHC using identical conditions to those used for analysis of the TMAs. Using this validated antibody, 72% of patients were negative for PTEN, a higher value than reported in earlier studies. Previous studies have suggested that loss of PTEN expression may predict for resistance to EGFR-targeted monoclonal antibodies in patients with metastatic colorectal cancer (25, 31, 37). Correlative analyses have also associated loss of PTEN with poorer survival (18, 36, 38, 39). In our study, PTEN status had no clear prognostic or predictive influence. There are numerous ongoing challenges in accurately assessing PTEN status in patients. Heterogeneity of expression is one issue, and was noted in some cases in this study (both between TMA cores and within cores). A second issue is that discordance rates of 47% to 89% between primary and secondary lesions make routine interpretation of PTEN status difficult in clinical practice, and repeat biopsy of new lesions may be required if PTEN is to be used as a predictive marker (40, 41). The significance of PTEN loss may differ depending on the source of the tumor sample as one published series indicated that the predictive value of PTEN was observed only when the examined tumor material came from a metastatic lesion and not from the primary site (26).

Molecular profiling, including K-ras, BRAF, PIK3CA, PTEN, NRAS, and other relevant biomarkers has been recommended for optimal patient selection for molecular-targeted therapies, particularly EGFR-targeted monoclonal antibodies (19, 20). In preclinical studies, there seems to be a compounding effect when multiple molecular pre-

dictors of resistance are present. PIK3CA mutant/PTEN null and Ras/BRAF mutant cell lines are highly resistant to cetuximab compared with those without dual mutations/PTEN loss indicating that constitutive and simultaneous activation of the Ras and PIK3CA pathways confers maximal resistance to this agent (29). The incorporation of multiple biomarkers into a clinical management pathway needs to be carefully researched and validated with the goal of improved patient outcome to be proven. The application of multiple biomarker results, with a potential myriad of mutation permutations, may complicate the therapeutic landscape without improving patient outcomes. Results from reported series remain conflicting. Despite data indicating that PTEN and PIK3CA could have predictive applications in selecting patients for EGFR-directed monoclonal antibody therapy, our study findings did not provide any evidence to support this. With respect to BRAF, the number of observed cases was too low to allow valid correlative analyses.

Beyond patient selection for therapy, knowledge of the presence of mutations may prove valuable in developing strategies to overcome EGFR-targeted therapy resistance. PIK3CA inhibitors and phospho-AKT inhibitors are in development. Inhibition of PI3K/Akt signaling pathway restored sensitivity to gefitinib and cetuximab in HCC827-CR cells (33). PTEN may have a controlling influence on the cellular response to cetuximab. In an *in vitro* study utilizing PC3 prostate cancer cell lines, the reintroduction of PTEN to PTEN null cells significantly reduced the constitutive overexpression of phosphorylated-AKT (p-AKT) and downstream kinases (p-GSK3 β and p-P70S6 kinase) as well as phosphorylated-ERK1/2 (p-ERK1/2). This consequently restored cetuximab-induced cell growth inhibition and apoptosis induction. BRAF may have a role in secondary resistance. Restoration of sensitivity to panitumumab or cetuximab has been observed in colon cancer cell lines carrying the BRAF V600E allele that are treated with the BRAF inhibitor sorafenib (42).

For now, only K-ras remains a validated and universally accepted predictive biomarker that enables effective selection of EGFR-targeted monoclonal antibody therapy for advanced colorectal cancer. Much remains to be learned about other molecular biomarkers that may mediate treatment resistance and provide further predictive information.

Disclosure of Potential Conflicts of Interest

C.S. Karapetis is a consultant/advisory board member of Merck Serono. J.R. Zalberg has commercial research grant of BMS. N. Tebbutt is a consultant/advisory board member of Merck Serono. T.J. Price is a consultant/advisory board member of Merck. N. Pavlakis is a consultant/advisory board member of Merck. G. van Hazel is a consultant/advisory board member of Merck. No potential conflicts of interest were disclosed by the other authors.

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***PIK3CA*, *BRAF*, and *PTEN* Status and Benefit from Cetuximab in the Treatment of Advanced Colorectal Cancer —Results from NCIC CTG/AGITG CO.17**

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