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PTEN GENE EXPRESSION AND MUTATIONS IN THE PIK3CA GENE AS PREDICTORS OF CLINICAL BENEFIT TO ANTI EPIDERMAL GROWTH FACTOR RECEPTOR ANTIBODY THERAPY IN PATIENTS WITH KRAS WILD TYPE METASTATIC COLORECTAL CANCER

Arjun Sood^{1,3}, Danielle McClain^{1,4}, Radhashree Maitra^{1,3}, Atrayee Basu-Mallick^{1,3}, Raviraja Seetharam^{1,2,3}, Andreas Kaubisch^{1,2,3}, Lakshmi Rajdev^{1,2,3}, John M. Mariadason^{3,5}, Kathryn Tanaka^{1,2,4}, and Sanjay Goel^{1,2,3}

¹Montefiore Medical Center, Bronx, NY, USA

²Albert Einstein College of Medicine, Bronx, NY, USA

³Department of Medical Oncology

⁴Department of Pathology

⁵Ludwig Institute for Cancer Research, Melbourne, Australia

Abstract

PURPOSE—To identify novel genetic markers predictive of clinical benefit from epidermal growth factor receptor-directed antibody therapy in patients with metastatic colorectal cancer (mCRC).

PATIENTS AND METHODS—Seventy-six consecutive patients who received cetuximab or panitumumab, either alone or in combination with chemotherapy, with available tumor tissue were included. Tumor tissue was tested for mutations at known hotspots in the KRAS, BRAF, PIK3CA, PIK3R1, AKT1, and PTEN genes by pyrosequencing. PTEN promoter methylation status was analyzed by methylation-specific PCR, and expression determined by immunohistochemistry (IHC). Forty-four patients had 4 weeks of therapy and were considered for clinical correlates.

RESULTS—Consistent with previous studies, KRAS gene mutations were associated with a shorter progression free (PFS) and overall survival (OS). Among the KRAS wild type patients,

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Corresponding Author: Sanjay Goel, M.D., M.S., Associate Professor of Medicine, Albert Einstein College of Medicine, Department of Medical Oncology, Montefiore Medical Center, 1825 Eastchester Road, Bronx NY 10461, Ph: 718-904-2488, Fax: 718-904-2830, sgoel@montefiore.org.

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CONFLICT OF INTEREST PAGE

John Mariadason and Sanjay Goel are co-applicants on a patent filed with the USPTO on the use of PTEN and PIK3CA mutations as predictive markers of efficacy of the anti EGFR agents. This patent application is currently under review at the USPTO.

A licensing agreement has been signed with Transgenomics Inc., should this patent be granted.

None of the other authors have any conflicts to declare.

preservation of PTEN expression and PIK3CA WT status was associated with improved OS (median OS, 80.4 vs 32.5 weeks, HR: 0.33, $p=0.0008$) and a trend towards improved PFS (median PFS, 24.8 vs 15.2 weeks, HR: 0.51, $p=0.06$), compared to PTEN negative or PIK3CA mutant tumors. PTEN methylation was more common in the metastases than the primary ($p=0.02$). Simultaneous presence of methylation and mutation in the PTEN gene was associated with IHC negativity ($p=0.026$).

CONCLUSION—In addition to KRAS mutation, loss of PTEN expression (by IHC) and PIK3CA mutation is likely to be predictive of lack of benefit to anti-EGFR therapy in mCRC. PTEN promoter methylation and mutation status was predictive of PTEN expression, and may be utilized as an alternative means of predicting response to EGFR-targeted therapy.

Keywords

anti-EGFR; biomarker; colorectal cancer; PIK3CA mutations; PTEN; KRAS

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the US with over 52,000 deaths in 2010 [1]. The median survival of patients with metastatic CRC has improved to 24–30 months [2, 3], largely due to the availability of newer therapeutic options, including the epidermal growth factor receptor (EGFR) targeted monoclonal antibodies (mAb), cetuximab (Erbix[®], Eli Lilly, Indianapolis, IN) and panitumumab (Vectibix[®], Amgen Inc., Thousand Oaks, CA) [4, 5]. Response rates (RR) to anti-EGFR mAb range from 10–22% when used alone or in combination with irinotecan [6, 7]. Activation of the EGFR pathway occurs after ligand binding, leading to EGFR phosphorylation and oligodimerization at the cell membrane. This in turn triggers downstream signaling events including activation of the Ras/Raf/mitogen-activated protein kinase (MAPK), and the phosphoinositide-3-kinase-(PI3K)-PTEN-AKT-mTOR pathways [8, 9]. Anti-EGFR mAb inhibit these signaling pathways by inhibiting ligand binding.

The ability to predict the likelihood of clinical benefit to these agents is an area of intense research. It is well established that mutations in the KRAS gene predict for lack of response to anti-EGFR mAb [10–13]. BRAF gene mutation is considered a prognostic marker, however its predictive nature is uncertain [14–18]. Among the KRAS wild type (WT) patients, multiple studies evaluating EGFR based therapy have documented highly variable RR ranging from 17%–60% [10–12, 19–23]. This heterogeneity suggests that there may be other predictive variables, besides KRAS, that determine responsiveness to EGFR antibodies.

Our group was one of the first to demonstrate *in vitro*, that mutations in the PI3K signaling pathway also predict response to cetuximab [24]. We observed that in CRC cell lines, activating PIK3CA mutations or loss of PTEN gene expression predicted resistance to cetuximab. Since then, several clinical reports have followed, with conflicting results; with some reports suggesting a predictive role of this pathway and others refuting this finding [24–30]. Notably, a recent report suggested that mutations in exon 20 of PIK3CA specifically predicted for resistance to cetuximab [30].

Importantly, multiple components of the PI3K signaling pathway have been shown to be mutated in colon cancer, including mutations in PIK3CA, PTEN, PIK3R1 (regulatory subunit of the PIK3CA gene, p85 α [31]), and AKT1. Furthermore, loss of PTEN expression in CRC has been shown to be mediated by promoter hypermethylation [32, 33]. In this study we performed a comprehensive investigation of all components of the PI3K signaling pathway previously shown to be mutated or methylated in CRC, and linked perturbations in

the pathway with anti-EGFR mAb response. Mutation status of KRAS and BRAF were also performed as a means of validating our findings with previous reports.

PATIENTS AND METHODS

Patient selection

All patients with available archived tissue who had received cetuximab or panitumumab for the treatment of mCRC, at Montefiore Medical Center through April 2009, were identified by database searches and included in the study. Formalin fixed paraffin embedded (FFPE) tissue blocks containing at least 60% tumor tissue were identified by a clinical pathologist and used for all analyses. An IRB approved protocol was followed for the collection of patient samples and clinical information.

Mutation Analysis

DNA was isolated from FFPE blocks using the Qiaamp FFPE DNA kit (Qiagen, Valencia, CA) and analyzed for mutation at fifteen hotspots (Supplementary Table (ST)-I) by pyrosequencing using the allele quantification (AQ) mode [34]. Pyrosequencing assays were designed using PSQ Assay Design Software v1 and performed on the PSQ HS 96 platform from Qiagen. DNA was amplified using polymerase chain reaction (PCR) with biotinylated primers. The primer sequences, nucleotide dispensation order and PCR cycle settings are listed in ST-2. A threshold value for the pyrosequencing (AQ mode) was determined using a panel of 22 CRC cell lines, for which the mutation status of these loci had previously been determined by conventional Sanger sequencing. Validated pyrosequencing assays have been previously described for KRAS [35, 36] and BRAF [35, 37, 38] genes.

PTEN IHC Analysis

PTEN expression was determined by immunohistochemistry using 5- μ m thick sections from FFPE blocks. Slides were deparaffinized, rehydrated, and blocked in 3% H₂O₂ for 10 minutes followed by antigen retrieval (steaming for 30 minutes) using Dako Target Retrieval Solution (pH 9.0). Staining was performed in an automatic slide stainer (Dako LV-1 Autostainer) using the Dako universal staining system. The primary rabbit monoclonal anti-PTEN antibody (D4.3) XP (Cell Signaling) (dilution 1:50) was applied for 60 minutes at room temperature followed by secondary antibody (Dako Cytomation Envision+ system-HRP Labeled Polymer, anti rabbit) for 30 minutes and followed by DAB reagent (Cell Marque). Sections were scored semi-quantitatively by light microscopy by two pathologists, using a four-tier scoring system: 0-no staining; 1-weak staining; 2-moderate staining, and 3-strong staining (Figure II); and, additionally, the percentage of staining was given a score of 1(<10%), 2(10%–50%), or 3(>50%). The sections were subsequently classified as negative (<10%, weak or moderate staining), positive (>50%, moderate or strong staining) or indeterminate (<10%, strong; 10–50%, any staining; >50%, weak staining).

PTEN Promoter Methylation Analysis

Genomic DNA was bisulfite treated using EZ DNA Methylation kit from Zymo Research (Orange, CA). Methylation specific PCR (MSP) was performed at four independent CpG sites within the PTEN promoter previously shown to be prone to methylation in CRC (ST-3) [33, 39–41], using ZymoTaq Premix (Zymo Research) [42]. Reactions were carried out on a Peltier thermocycler in a 25 μ L volume consisting of 12.5 μ L of ZymoTaq premix solution, 2 μ L each of 10 μ M forward and reverse primers, 2 μ L of bisulfite treated DNA template with approximately 25ng of DNA and 6.5 μ L of DNase, RNase free water. Bisulfite converted genomic methylated DNA Standard (Zymo Research) and untreated genomic DNA were used as positive controls for the methylated and unmethylated PCR reactions,

respectively, and water as a negative control. Presence of a MSP product was verified by ethidium bromide staining.

Clinical Data Collection

Medical records, including cancer diagnosis, treatment history, drug toxicities, and response to treatment of all patients were reviewed independently by two medical oncologists. Objective RR was determined based on established RECIST criteria [43]. Patients who had received at least four weeks of therapy with cetuximab or panitumumab (with or without cytotoxic chemotherapy) were considered for clinical correlates. All data was tabulated using Epiinfo v 3.5 software (Centers for Disease Control, Atlanta, GA).

Statistical Analysis

Descriptive statistics was used for patient characteristics. Analysis for progression free survival (PFS) and overall survival (OS) was carried out using Graphpad Prism v 5.00 software (GraphPad Software, San Diego, CA) and reported as Kaplan-Meier curves. The log-rank test was used to determine the p value. Categorical variables were analyzed using Fisher's exact test (FET) for 2 variables and by Exact Contingency Table (ECT) for 3 variables. All tests were reported as two tailed. The primary hypothesis being tested was that loss of PTEN or presence of PIK3CA mutations will render resistance to the anti-EGFR agents. All other analysis was considered exploratory.

RESULTS

Overall response rate has a positive outcome on long term survival

A total of 76 patients (Table I) and 122 tissue samples were identified (66 primary, 56 metastases, 30 matched pairs). All tissue samples from all the patients were used for mutation analysis. Forty-four patients had ≥ 4 weeks of therapy and were considered for clinical correlates. These patients had similar clinical characteristics as the entire set of 76 patients in terms of median age, sex ratio, racial distribution, and location of primary tumor. Among the 44 patients considered for clinical correlates, 36 (82%) had irinotecan refractory disease, and 15 (34%) were treated with single agent therapy.

Four of the 44 patients evaluable for clinical response demonstrated a RECIST defined partial response (PR, 9.1%), and 18 had stable disease (SD, 40.9%). Notably, the patients with a PR appeared to have a superior PFS and OS than those without (table IV). Among the 4 patients with PR, one had a dual mutation in the KRAS (G12D) and the PIK3CA (E545K) genes. None of the other 3 patients had any detectable mutation in the KRAS, BRAF or PIK3CA genes.

Mutations in the Ras/MAPK pathway are predictive of benefit from anti-EGFR therapy in mCRC

The overall prevalence of K-Ras mutation was 42.1% and of BRAF was 7.9%, consistent with prior data [19, 22] (Table II). We identified one tumor sample that harbored mutations in both BRAF (V600E) and KRAS codon 12, and another with mutations in KRAS codons 12 and 13. Consistent with prior observations, patients who were WT for KRAS had a better PFS and OS than KRAS mutant patients (Figure I, Panels A and B; Table IV). When mutations in both KRAS and BRAF were collectively considered, PFS and OS was further improved in the WT group (Table IV). With only 4 patients exhibiting a PR, we were unable to demonstrate an association of objective response with KRAS or BRAF mutation status.

Mutations in PIK3CA are predictive of PFS when patients with mCRC are treated with anti EGFR therapy

The overall prevalence of PIK3CA mutation was 17.1% consistent with prior data [29] (Table II). We found one tumor sample that harbored mutations in both exons 9 (E545K) and 20 (H1047R) of the PIK3CA gene. As reported previously, we found a considerable overlap of KRAS mutations with PIK3CA mutations, with 25% of KRAS mutations also harboring PIK3CA mutations [24]. Patients who were WT for PIK3CA had a better PFS than those with mutant PIK3CA; however, no difference in OS was observed (Table IV).

Comprehensive Analysis of PTEN status

We next analyzed whether PTEN expression, PTEN mutation status, or PTEN promoter methylation status was predictive of response to anti-EGFR therapy.

PTEN Immunohistochemistry (IHC)—The results of the IHC studies are displayed in Table III and Figure II. Overall, PTEN expression was considered positive by IHC in 42.6% of the samples. Among the primary tumor samples, the PTEN staining revealed positive expression in 43.9%, negative in 36.4%, and indeterminate in 19.7% of the samples. In contrast, in metastatic samples, positive expression was observed in 41.1%, negative in 28.6% and indeterminate in 30.6% of the samples. Thus, there appeared to be a small difference in negativity of PTEN between primary and metastases.

Patients with preserved PTEN IHC expression (either in the primary or the metastases) appeared to have a superior OS than patients with PTEN loss, however, we were unable to demonstrate a difference in the PFS (Table IV). To determine the utility of PTEN expression as an additional biomarker, we analyzed PTEN expression specifically in KRAS WT patients. Encouragingly, we observed a more profound effect in OS and PFS (Table IV).

Truncating Mutations in the PTEN Gene—The presence of truncating PTEN mutations was assessed in the PTEN gene in 5 previously described hotspots [32, 44, 45]. Of these, we observed the presence of mutations only in exon 8 (R355X), in 27.6% of patients and 19.7% of all samples (22.7% of primary samples and 16.1% of metastases). However, neither PTEN mutations in the primary nor in metastases predicted response to anti-EGFR therapy.

Promoter Hypermethylation in the PTEN Gene—Finally, we determined whether PTEN promoter methylation predicted for clinical benefit from anti-EGFR therapy. PTEN promoter methylation was assessed using 4 pairs of primers (ST-3). Methylation was observed at only one of the 4 sites examined (Figure III). Overall, among the 76 patients, PTEN methylation at this site was observed in 28.8% of primary, and 44.6% of metastatic samples. Similar to PTEN mutations, no association between PTEN promoter methylation and benefit from anti-EGFR therapy was observed.

Collective consideration of PTEN expression and PIK3CA mutations in the KRAS WT patients

Given that PTEN loss and PIK3CA mutations are mutually exclusive [46], we analyzed the effect of perturbations of the PI3-kinase pathway in the KRAS WT patients by collectively considering the PTEN expression and PIK3CA mutation status. Patients who had preserved PTEN IHC expression (either in the primary or the metastases) and a WT PIK3CA gene, had a superior OS than patients who had PTEN loss (median OS, 80.4 vs 32.5 wk, HR: 0.33, $p=0.0008$)(Figure I, Panel C) and a very strong trend towards an improvement in PFS (median PFS, 24.8 vs 15.2 wk, HR: 0.51, $p=0.06$)(Figure I, Panel D).

In depth analysis of the 30 matched pairs of samples (from primary and metastases)

We were fortunate to have matched pairs of samples from 30 patients enabling an in depth evaluation of the PTEN gene. There were 1–3 metastases with available tissue corresponding to each primary tumor. The median time from sampling of the primary tumor to that of metastases was 2 years [range: 0 (simultaneous sampling) to 10 years]. Among the 30 pairs, PTEN expression was preserved in 15 (50%) primary and 13 (43.3%) metastases (Table III). The concordance rate of PTEN mutations, methylation and expression between primary and metastatic sites was 73.3%, 66.7% and 46.5%, respectively (ST-4). Interestingly, methylation in the primary tumor was identified in only 10 (33.3%) patients and in metastases in 18 (60%) patients (Table III, FET 0.02)].

In transitioning from primary to metastases, 9 patients demonstrated a gain in methylation and 6 patients changed from PTEN positive to negative/indeterminate. Notably, of these 6 patients, 3 demonstrated a gain in methylation. Taken together, methylation may explain loss of PTEN staining in metastases in about 50% of the patients. One of the 6 patients had a gain in PTEN mutation during the transition.

We next focused on the simultaneous presence of a truncating mutation and promoter methylation among these 30 patients. We found 5 such samples, of which 4 stained negative and 1 indeterminate for PTEN IHC. Therefore, simultaneous presence of both mutation and methylation was responsible for PTEN loss in 80% of samples. This was in contrast to samples that lacked simultaneous dual hits to the PTEN gene, where only 38% of samples were PTEN IHC negative. This difference in PTEN staining between samples with dual mutation/methylation versus none or only mutation or only methylation was statistically significant (ECT, $p=0.026$).

DISCUSSION

Biomarker driven anti-cancer therapy underwent a paradigm shift when the USFDA and ASCO recommended that the use of anti EGFR mAb in mCRC be restricted to patients with a WT KRAS gene status, because of a lack of benefit in those with a mutation [47–49]. In fact, there appears to be a negative outcome when patients with a KRAS mutation are treated with the anti-EGFR drugs [50, 51]. Furthermore, there is emerging data that not all KRAS mutations are alike, and one particular mutation, G13D, may not be a valid biomarker of exclusion after all [52]. In this retrospective study, we performed a comprehensive analysis of mutations in the PI3K/PTEN-AKT-mTOR pathway to identify further determinants of anti-EGFR treatment benefit. While we failed to identify mutations in AKT or PIK3R1 we observed that loss of expression of PTEN expression and mutations in PIK3CA, both of which hyper activate the PI3K/PTEN-AKT-mTOR pathway, are further predictors of anti-EGFR treatment response.

Consistent with our published pre-clinical observation [24], we observe that collective consideration of PIK3CA mutations and PTEN loss predict for an superior clinical outcome when treated with anti-EGFR therapy, with an improvement in OS and a strong trend in PFS, in the KRAS WT patients. A clinically meaningful approach is likely to be an evaluation for KRAS mutations, followed by PIK3CA mutation assessment and PTEN IHC staining in the KRAS WT tumors; offering anti-EGFR therapy for the KRAS WT/PIK3CA WT/PTEN positive patients. This approach could potentially wean out 60–70% of patients from therapy with these drugs, thereby markedly improving the clinical benefit rate. Thus, PTEN preservation is a potential biomarker of inclusion, while KRAS and PIK3CA are markers of exclusion, of therapy with anti-EGFR drugs. This identification of PTEN/PIK3CA status as a putative biomarker is consistent with prior reports [24, 26–29, 53–55]. Interestingly enough, one group has reported its findings on the possibility of using gene

expression profiles to characterize KRAS, BRAF or PIK3CA activated like tumors, as a further refinement of clinical therapy with anti-EGFR agents [56].

We observed an interesting overlap of mutations within the same tumor. One sample harbored mutations in both BRAF (V600E) and KRAS codon 12, and another with mutations in KRAS codons 12 and 13. Such simultaneous mutations have been reported previously, as in BRAF and KRAS, KRAS codons 12 and 61, and in KRAS codons 12 and 13 [52, 57]. It appears that this is the first report of simultaneous mutations in codons 12 and 13. Furthermore, we found one tumor sample that harbored mutations in both exons 9 (E545K) and 20 (H1047R) of the PIK3CA gene. It is likely that as more tumor samples are analyzed for multiple somatic mutations, similar overlapping simultaneous mutations will continue to be identified. The co appearance of mutations in the MAPK and PI3K pathway has been reported extensively in the literature [24, 29, 52].

Another important observation is that simultaneous consideration of PTEN methylation and truncating mutation is significantly associated with loss of PTEN expression by IHC. Moreover, careful study of the matched pairs elucidated that there was an increase in methylation in the metastases as compared to the primary. We therefore propose a hypothesize that PTEN loss requires a dual “hit”, either by homozygous mutation, or an initial allelic loss by a truncating mutation, followed by a second allelic loss by promoter methylation, that takes place between the initial diagnosis and development of metastases. This methylation event is a potential mediator of PTEN loss in the metastases and is clearly worthy of further investigations in large scale tissue studies.

The lack of an association of PTEN methylation alone with IHC expression may be explained by the fact that we interrogated four sites by the MSP and may have missed other potential important sites of methylation. Broadening the panel of primers to interrogate more CpG islands may further improve the yield of methylation detection. A second possibility is that methylation alone is insufficient to downregulate PTEN expression. For example, in a recent publication, in patients with Cowden syndrome, methylation in the PTEN promoter did not result in transcriptional repression of gene expression [58]. As regards PTEN mutations, we were unable to establish their association with overall outcome or response data. However, considering the ease of mutation testing, its role as a simple potential biomarker needs further exploration. Taken together, despite its limitations, PTEN IHC appears to remain the most robust assay to determine its expression.

The most significant finding in our study is clearly the confirmation that the loss of expression of PTEN (by IHC), and mutations in the PIK3CA gene are potential biomarkers of resistance to therapy with the anti-EGFR agents, particularly in patients with a WT KRAS in their cancer. These findings need to be further explored in large prospective randomized studies. Further identification of novel biomarkers will lead to small incremental steps towards truly individualizing therapy for patients with mCRC, thereby improving outcomes, reducing healthcare costs, and most importantly minimizing toxicity by the selective use of the most effective, and minimizing the use of drugs unlikely to benefit the patient.

CLINICAL PRACTICE POINTS

Recent recognition that patients with KRAS mutant metastatic colorectal cancer do not experience clinical benefit from the anti EGFR monoclonal antibodies cetuximab and panitumumab, and the subsequent recommendation that they be excluded from this therapy marked a paradigm shift in the way we manage patients and heralded the era of personalized therapy in cancer. However, not all KRAS WT patients derive benefit, emphasizing the urgent need for development of better predictive biomarkers. BRAF, an oncogene downstream of KRAS is purported to be a prognostic rather than a predictive

biomarker. We collected tumor rich tissue from FFPE blocks from patients who were treated with cetuximab or panitumumab at our institution, and investigated for perturbations in the alternate signaling pathway, the PI3K-mTOR-AKT-PTEN pathway that transfers signal from the cell membrane to the nucleus. Similar to our pre-clinical *in vivo* findings, we find that collective consideration of PIK3CA activating mutations and loss of PTEN expression are predictive for lack of benefit from these drugs. If adopted in clinical practice, excluding patients with KRAS and/or PIK3CA mutations or those who lack PTEN expression could narrowly define the 30–40% of patients with metastatic colorectal cancer most likely to benefit (and exclude close to 60–70% of patients) from the use of anti-EGFR therapy. These novel potent biomarkers will lead to further refinement of therapy for these patients, facilitating better outcome, a better tolerable toxicity profile, and lower cost of health care delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure I.

Effect of KRAS, PIK3CA, and PTEN gene expression status in patients

Panels A and B Kaplan Meier curves for progression free survival (PFS) and overall survival (OS) for patients based on the mutation status in the KRAS gene. There was a statistically significant difference in both PFS and OS in favor of the patients with a WT KRAS gene.

Panels C and D Kaplan Meier curves for progression free survival (PFS), and overall survival (OS) based on the PTEN expression by IHC and PIK3CA mutation status among the KRAS WT patients. There was a statistically significant difference in OS, and a strong trend towards a difference in PFS, in favor of the patients with a preserved IHC PTEN/PIK3CA WT status.

Figure II.

Panels A–D PTEN staining by IHC showing the scoring patterns; A: score of 0, B: score of 1, C: score of 2, and D: score of 3

Figure III.

Agarose gel showing examples of methylation specific PCR (MSP) using primer set 3 (Supplementary table III). Abbreviations; 100 BP: DNA ladder with position marked for the 100 base pair location; Pos Con: positive control; M: PCR product using primers to amplify methylated DNA; U: PCR product using primers to amplify unmethylated DNA; 1,2 and 4: tumor samples with methylated DNA showing both methylated and unmethylated product; 3: tumor sample with unmethylated DNA with only unmethylated product

Table I
Patient Characteristics (n=76). Summary of all patients for whom tumor tissue was available and had received therapy with an anti EGFR agent

(n=76)		
Age - median (range)		59 (34–93)
Sex	Female/Male	54/22 (71/29%)
Race	African American	32 (42%)
	Caucasian	17 (22%)
	Hispanic	12 (16%)
	Declined	8 (10%)
	Asian	2 (3%)
	Other	5 (7%)
Tumor Location	Proximal Colon	29 (39%)
	Distal Colon	36 (47%)
	Rectum	10 (13%)
	Appendix	1 (1%)
(n=44)		
# prior chemotherapy regimens	0	1 (2%)
	1	13 (23%)
	2	19 (43%)
	3	10 (23%)
	4	1 (2%)
EGFR therapy	Monotherapy	15 (34%)
	Combination	29 (66%)
EGFR therapy	Prior irinotecan	36 (82%)
	No prior irinotecan	8 (18%)

Table II
Frequency of mutations in all 76 patients, considering all 122 samples

(n=76)		
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K-ras		42.1%
Exon 2 codon 12	27.6%	
Exon 2 codon 13	13.1%	
Exon 3 codon 61	2.6%	
Braf		7.9%
PIK3CA		17.1%
E542K	3.9%	
E545K	7.9%	
R1023Q	3.9%	
H1047R	2.6%	
PTEN R355X		27.6%
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1 sample had dual mutations in BRAF and KRAS 12

1 sample had dual mutations in KRAS 12 and KRAS 13

1 sample had dual mutations in PIK3CA E545K and H1047R

Table III
PTEN IHC expression and methylation in matched pairs of samples as a function of site of tumor

		IHC Staining		Methylation Status					
		Metastases			Metastases				
		Pos.	Neg.	Ind.	Total	Primary	Present	Absent	Total
Primary	Pos.	9	3	3	15		9	1	10
	Neg.	2	4	6	12		9	11	20
	Ind.	2	0	1	3				
	Total	13	7	10	30	Total	18	12	30

Results of exploratory analysis of the effect of clinical parameters and mutation status on outcome of patients as measured by PFS and OS.

Table IV

Clinical parameter	Median PFS (weeks)	HR	p	Median OS (weeks)	HR	p
Responding patients	44.1	0.361	0.02	123.3	0.381	0.043
Non responding patients	11.4			35.2		
Kras WT	21.3	0.479	0.021	43.6	0.478	0.011
Kras mutant	8			22.1		
Kras or Braf WT	22.7	0.436	0.004	44.3	0.433	0.003
Kras and Braf mutant	8			22.1		
PIK3CA WT	15.7	0.432	0.033	37.1	0.961	0.92
PIK3CA mutant	6.3			35.3		
PTEN positive	15.3	0.9	0.734	40.7	0.496	0.012
PTEN null	13.1			32.6		
PTEN positive in Kras WT	24.4	0.65	0.258	69.2	0.339	0.0015
PTEN null in Kras WT	15.7			36.9		