# Polymorphisms in *Cyclooxygenase-2* and *Epidermal Growth Factor Receptor* Are Associated with Progression-Free Survival Independent of K-ras in Metastatic Colorectal Cancer Patients Treated with Single-Agent Cetuximab

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**Abstract** **Purpose:** Recently, an objective response rate of 12% was reported in a phase II study of cetuximab in patients with epidermal growth factor receptor (EGFR)-expressing metastatic colorectal cancer (mCRC) refractory to fluoropyrimidine-, oxaliplatin-, and irinotecan-based chemotherapy (IMC-0144). In this large molecular correlates study, we tested whether K-ras mutation status and polymorphisms in genes involved in the EGFR-signaling pathway were associated with clinical outcome in IMC-0144.

**Experimental Design:** We analyzed all available tissue samples from 130 of 346 mCRC patients enrolled in the IMC-0144 phase II clinical trial of cetuximab. Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor tissues, and K-ras mutation status and the genotypes were analyzed using PCR-RFLP, direct DNA-sequencing, and 5′-end [$\gamma$-$^{33}$P] ATP – labeled PCR-protocols.

**Results**: The PFS of patients with *cyclooxygenase-2 (COX-2) -765 G›C* [C/C; risk ratio (RR), 0.31; 95% confidence interval (95% CI), 0.12-0.84; $P = 0.032$], *COX-2 +8473 T›C* (C/C; RR, 0.67; 95% CI, 0.40-1.13; $P = 0.003$), *EGF +61 A›G* (G/G; RR, 0.57; 95% CI, 0.34-0.95; $P = 0.042$), and *EGFR +497 G›A* (A/G; RR, 0.82; 95% CI, 0.56-1.20; $P = 0.017$) genotypes was significantly longer compared with those with other genotypes. In addition, patients whose tumors did not have K-ras mutations showed better RR, PFS, and overall survival than patients with K-ras mutations. In multivariable analysis, *COX-2 +8473 T›C* (adjusted $P = 0.013$) and *EGFR +497 G›A* (adjusted $P = 0.010$) remained significantly associated with progression-free survival, independent of skin rash toxicity, K-ras mutation status, and Eastern Cooperative Group performance status.

**Conclusions:** Polymorphisms in *COX-2* and *EGFR* may be useful independent molecular markers to predict clinical outcome in patients with mCRC treated with single-agent cetuximab, independent of skin rash toxicity, K-ras mutation, and Eastern Cooperative Oncology Group performance status.

Colorectal cancer (CRC) is the second leading lethal malignancy in the United States. In 2008, an estimated 148,810 new cases will be diagnosed and 49,960 people will die from this disease (1). Despite recent additions to our chemotherapeutic armamentarium used to treat metastatic CRC (mCRC; ref. 2, 3), the 5-year overall survival (OS) is relatively poor, with a median survival of 18 to 21 months (4, 5). Targeted agents such as cetuximab, an IgG1 monoclonal antibody to the epidermal growth factor (EGF) receptor (EGFR), have shown relevant clinical activity as monotherapy and combined with chemotherapy in several types of human cancer (6, 7).

EGFR is overexpressed in a variety of malignancies, including up to 77% of CRC, and is associated with tumor progression and poor prognosis (8, 9). Conversely, inhibition of the EGFR pathways with anti-EGFR monoclonal antibodies blocks cell cycle progression and induces apoptosis in numerous *in vitro* and xenograft models (10 – 12). EGFR-targeted therapy with cetuximab has shown promising results in multiple phase II

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## Translational Relevance

Epidermal growth factor (EGF) receptor (EGFR) is overexpressed in up to 77% of colorectal cancer, and anti-EGFR therapy with cetuximab has shown promising results in multiple phase II clinical trials. There are several mechanisms that may lead to aberrant EGFR activation and resistance to anti-EGFR treatment; some of them include EGF overexpression and EGFR amplification, as well as activating K-ras mutations. Here, we show for the first time that germline polymorphisms of genes involved in the EGFR-signaling pathway (*cyclooxygenase-2* and *EGFR*) predict progression-free survival in metastatic colorectal patients treated with single-agent cetuximab, independently of skin rash toxicity and *K-ras* mutation status. Accordingly, the development of independent molecular markers of prognosis may not only be helpful in identifying patients who are more likely to progress, but they will also be critical in selecting more efficient treatment strategies. Larger, prospective biomarker-embedded clinical trials are needed to confirm and validate our preliminary findings.

clinical trials. Cunningham et al. (6), Saltz et al. (7), and Lenz et al. (13) reported response rates of 9.0%, 10.8%, and 11.6%, respectively, for patients with mCRC treated with single-agent cetuximab refractory to fluoropyrimidine-, oxaliplatin-, and irinotecan-based chemotherapy. All trials have thus far failed to show a significant correlation between EGFR expression, determined by immunostaining intensity and clinical outcome. In fact, antitumor activity of cetuximab was also noted in patients, whose tumors were negative for EGFR immunostaining (13).

There are several mechanisms that may lead to aberrant EGFR activation and resistance to anti-EGFR treatment; some of them include EGF overexpression and EGFR amplification, as well as activating *K-ras* and *phosphatidylinositol-3-OH kinase* mutations. These mutations in turn dysregulate mechanisms modulating tumor-angiogenesis and apoptosis that are normally controlled by multiple homeostatic mechanisms, including signals from the EGFR. As such, downstream EGFR signaling includes molecular targets, such as vascular endothelial growth factor (VEGF), a key regulator of angiogenesis, cyclin D1 (CCND1), an important mitogenic target of EGFR signaling that controls $G_1$-S cell cycle progression, and cyclooxygenase (COX)-2 (14–16), the key inducible and rate-limiting enzyme required for prostaglandin biosynthesis. Recent studies have shown that apoptosis (CCND1; ref. 17), tumor-angiogenesis, (VEGF, interleukin-8; ref. 18), and tumor-microenvironment (COX-2; ref. 18), contribute to the development of resistance to anti-EGFR therapy. Furthermore, cetuximab may exert an indirect antitumor activity by recruiting cytotoxic host effector cells such as monocytes and natural-killer cells (19). As such, antibody-dependent cell-mediated cytotoxicity has been implicated as an alternative mechanism to contribute to the antitumor activity of cetuximab, in addition to ligand/receptor blockade.

Given the recent focus on how *K-ras* mutations affect clinical outcome in mCRC and anti-EGFR therapy with cetuximab (20–22), it would be of upmost clinical relevance to identify novel molecular markers, which are independent of *K-ras* mutational status and skin rash toxicity. Based on this information, we designed a retrospective study within a cohort of a prospectively conducted phase II clinical trial (IMC-0144; ref. 13), to evaluate whether 11 functional significant polymorphisms within 8 genes involved in the EGFR pathway (Table 1), alone or in combination, were associated with clinical outcome in mCRC patients treated with single-agent cetuximab, independent of *K-ras* mutation status and skin rash toxicity.

## Patients and Methods

***Patients.*** One hundred thirty patients with histopathologically confirmed metastatic colorectal carcinoma, who either failed at least two prior chemotherapy regimens or failed adjuvant therapy plus one chemotherapy regimen for metastatic disease, were included in this study. These 130 patients were part of a phase II open-label multicenter study (IMC 0144) of cetuximab, which included a total of 346 patients (13). Due to limited tissue sampling, 130 of 346 (38%) patients were assessable to determine gene polymorphisms. All patients with available tumor tissue samples were included for correlative studies, irrespective of clinical outcome and K-ras mutation status. The present study was conducted retrospectively from prospectively obtained clinical data (IMC 0144) and was done at the University of Southern California/Norris Comprehensive Cancer Center, after approval by the Institutional Review Board of the University of Southern California for Medical Sciences. All patients provided their written informed consent for tissue and blood collection to allow study of molecular correlates.

***Clinical evaluation of response criteria.*** For patients with measurable disease, response was assessed every 6 wk during the course of the study, and criteria were based on modified WHO guidelines (13). An independent response assessment committee that was blinded to the investigator-reported measurements evaluated response to cetuximab retrospectively and assessments were reported in the study. Patients underwent weekly blood counts, and physical examinations were done at every third week. All patients received 2 wk of initial treatment with cetuximab and underwent a formal skin rash evaluation (13). A partial response required at least a 50% reduction in the sum of the bidimensional products of all measurable lesions documented at least 4 wk apart. Treatment was continued in the absence of intolerable toxicity or progressive disease, defined as at least a 25% increase in measurable disease, unequivocal growth of existing nonmeasurable disease, the appearance of one or more new lesions, or reappearance of old lesions (13).

***Candidate polymorphisms.*** The polymorphisms we tested were selected by an EGFR-pathway approach with the goal of selecting genes known to modulate EGF driven angiogenesis (Table 1). We used the following criteria to select genes for study: (*a*) that the gene be part of a pathway for which there is a credible scientific basis to support its involvement in the EGFR-signaling pathway; (*b*) that the gene has an established, well-documented genetic polymorphism; (*c*) that the frequency of the polymorphism is high enough that its effect on clinical outcome will be meaningful; and/or (4) that the polymorphism has some degree of likelihood to alter the function of the gene in a biologically relevant manner.

***Genotyping.*** Formalin-fixed and paraffin-embedded tumor samples were collected and genomic DNA was extracted using the QIAamp kit (Qiagen). The majority of the samples were tested using PCR RFLP technique. Briefly, forward and reverse primers were used for PCR amplification, PCR products were digested by restriction enzymes (New England Biolab), and alleles were separated on 4% NuSieve ethidium bromide stained agarose gel. Forward and reverse


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**Table 1.** Analyzed polymorphisms and their functional significance

| Polymorphism | Location | Minor allele frequency* | Function | Clinical significance | Reference |
|---|---|---|---|---|---|
| *FCGR2A 131 H>R (rs1801274)* | Exon 4 | 45-55% (H) | H-allele: ↑ binding affinity of FCGR2A to IgG2 and IgG1 | Mediates ADCC via FC γ receptor bearing immune effector cells<br>Associated with clinical outcome in CRC | (43) |
| *FCGR3A 158 V>F (rs396991)* | Exon 5 | 15-25% (V) | V-allele: ↑ binding affinity of FCGR3A to IgG2 and IgG1<br>Enhanced effector cell stimulation and ADCC | Mediates ADCC via FC γ receptor bearing immune effector cells<br>Associated with clinical outcome in CRC | (43) |
| *EGFR +497 G>A (rs11543848)* | Codon 497 | 25-35% (A) | A-allele: ↓ EGFR ligand binding, growth stimulation, tyrosine kinase activation | Associated with rectal cancer tumor recurrence | (44) |
| *EGFR (CA)14-23 (rs45608036)* | Intron 1 | 25-30% (≥20) | Length of CA microsatellite repeat correlates inversely with *EGFR* gene transcription | Associated with rectal cancer tumor recurrence | (44) |
| *Cyclin D1 +870 A>G (rs17852153)* | Exon 4 | 30-50% (A) | A-allele: modulates *CCND-1* mRNA splicing<br>A-allele: ↑ longer half-life of Cyclin D1 protein | Apoptosis regulatory protein<br>EGFR activates *CCND1* promoter<br>CCDN1-deregulation modulates efficacy of tyrosine kinase inhibitors<br>Resistance to cetuximab in CRC | (17) |
| *IL-8 -251 T>A (rs4073)* | 3′-UTR | 35-40% (A) | A-allele: ↑ IL-8 plasma levels | Mediator of VEGF independent angiogenesis<br>Associated with colon cancer tumor recurrence | (45) |
| *VEGF +936 C>T (rs3025039)* | 3′-UTR | 15-20% (T) | T-allele: ↓ VEGF plasma levels | Activator of angiogenesis<br>Associated with colon cancer tumor recurrence | (45) |
| *COX-2 -765 G>C (rs20417)* | 3′UTR | 15-35% (G) | C-allele: ↓ COX-2 promoter activity | Downstream effector of the EGFR pathway<br>*COX-2* overexpression is associated with poor outcome and resistance to cetuximab in CRC | (18) |
| *COX-2 +8473 T>C (rs5275)* | Exon 10 | 35-50% (G) | C-allele: ↓ mRNA stability<br>C-allele: protective effect against lung cancer | Downstream effector of the EGFR pathway<br>*COX-2* overexpression is associated with poor outcome and resistance to cetuximab in CRC | (18) |
| *EGF +61 A>G (rs4444903)* | 5′-UTR | 30-55% (A) | A-allele: ↓ EGF serum levels | EGFR ligand<br>Associated with esophageal cancer tumor recurrence | (46) |
| *NRP-1 C/T (rs3750733)* | Exon 2 | 15-20% (T) | Not known | VEGFR coreceptor<br>Associated with clinical outcome in ovarian cancer | (47) |

Abbreviations: UTR, untranslated region; FCGR, fragment c γ receptor; IL-8, interleukin-8; NRP-1, neuropilin-1; CCDN1, cyclin D1; ADCC, antibody-dependent cell-mediated cytotoxicity.
*Minor alleles are indicated in brackets.



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**Table 2.** Primer sequences, annealing temperatures, and restriction enzyme

| Gene | Forward-primer (5′-3′) | Reverse-primer (5′-3′) | Enzyme | Annealing |
|---|---|---|---|---|
| *FCGR2A*<br>*131 H>R* | GGAAAATCCCAGA<br>AATTCTCGC | CAACAGCCTGACTACCTA<br>TTACGCGGG | BstUI | 55° |
| *FCGR3A*<br>*158 V>F* | CTGAAGACACATTT<br>TTACTCCCAAA/C | TCCAAAAGCCACACTC<br>AAAGAC | n.a. | 64° |
| *EGFR*<br>*+497 G>A* | TGCTGTGACCCACT<br>CTGTCT | CCAGAAGGTTGCACT<br>TGTCC | Bst-NI | 59° |
| *EGFR*<br>*$(CA)_{14\text{-}23}$ repeat* | ACCCCAGGGCTC<br>TATGGGAA | TGAGGGCACAAGAAG<br>CCCCT | n.a. | 55° |
| *Cyclin D1*<br>*+870 A>G* | GTGAAGTTCATTTCC<br>AATCCGC | GGGACATCACCCT<br>CACTTAC | ScrFI | 61° |
| *IL-8*<br>*-251 T>A* | TTGTTCTAACACCTG<br>CCACTCT | GGCAAACCTGAGTC<br>TCACA | Mfe I | 60° |
| *VEGF*<br>*+936 C>T* | AAGGAAGAGGAGACT<br>CTGCGCAGAGC | TAAATGTATGTATGTGGG<br>TGGGTGTGTCTACAGG | Nla III | 60° |
| *COX-2*<br>*-765 G>C* | ATTCTGGCCATCGC<br>CGCTTC | CTCCTTGTTTCTTGGAAA<br>GAGACG | Aci I | 55° |
| *COX-2*<br>*+8473 T>C* | GTTTGAAATTTTAA<br>AGTACTTTTGAT | TTTCAAATTATTGTT<br>TCATTGC | BclI | 53° |
| *EGF*<br>*+61 A>G* | CATTTGCAAACAG<br>AGGCTCA | TGTGACAGAGCAA<br>GGCAAAG | Alu I | 60° |
| *Cyclin D1*<br>*+870 A>G* | GTGAAGTTCATTTCC<br>AATCCGC | GGGACATCACCCT<br>CACTTAC | ScrFI | 61° |

primer, restriction enzymes, and annealing temperatures are listed in Table 2. If no matching restriction enzyme could be found, samples were analyzed by direct sequencing. For quality assurance purposes, a total of 20% positive and negative duplicate-controls were matched for each polymorphism and were analyzed by direct DNA-sequencing where applicable. Genotype concordance was ≥99%.

The *EGFR* $(CA)_n$ repeat polymorphism was determined by a 5′-end 33p γATP–labeled PCR protocol with a few modifications. In summary, DNA template, deoxynucleotide triphosphates, 5′-end 33p γATP-labeled primer, unlabeled complementary primer, Taq Polymerase (Perkin-Elmer, Inc.), and PCR Buffer were used together in a final PCR. The reaction was carried out and the reaction products were separated on a 6% denaturing polyacrylamid DNA sequencing gel, which then was vacuum blotted for 1 h at 80°C and exposed to an XAR film (Eastman-Kodak Co.) overnight. In addition, the exact number of repeats was confirmed by direct sequencing.

***K-ras* mutation analysis.** Mutational analyses of *K-ras* were done using available genomic DNAs isolated from tumor specimens. Primers used for K-ras exons 12 to 13, coding for the tyrosine kinase domain, were published previously. The primers used to evaluate exon 12 of K-ras and exon 13 of K-ras were as follows: K-ras forward, 5′-TGA CTG AAT ATA AAC TTG TGG TAG TTG-3′, and K-ras reverse, 5′-TCG TCC ACA AAA TGA TTC TGA A-3′. PCR was done using conditions as previously described (23). PCR fragments were sequenced on an ABI 3100A Capillary Genetic Analyzer (Applied Biosystems) and analyzed in both sense and antisense directions for the presence of heterozygous mutations. DNA sequence analyzes were done by two independent investigators (G.L and W.Z.) using the ABI Sequencing Scanner v1.0 (Applied Biosystems). Appropriate positive and negative controls were included for each of the exons evaluated. Mutational analyses were done without knowledge of clinical outcome, including tumor response.

***Statistical analysis.*** The primary end points of this pharmacogenetic substudy were progression-free survival (PFS), OS, tumor response to cetuximab, and skin rash toxicity. The PFS was calculated from the time of the first date of cetuximab treatment until the first observation of disease progression or death from any cause. If a patient had not progressed or died, PFS was censored at the time of the last follow-up. The OS time was calculated as the period from the first day of cetuximab infusion or until death from any cause, at which the point data were censored.

The association between each polymorphism with OS and PFS was analyzed using Kaplan-Meier plots and the log-rank test. The distributions of polymorphisms across demographic characteristics were examined using Fisher's exact test. The associations of each polymorphism with tumor response and toxicity were summarized using contingency tables and the exact conditional test. Tumor response rate was defined as the total number of partial responses divided by the number of patients whose tumor response was evaluable.

The Benjamini and Hochberg method was used to control the false discovery rate (FDR) of multiple testing (24). In the univariate analysis, an FDR-adjusted *P* value of <0.15 was used to select polymorphisms as candidates for inclusion in the multivariable model.

With 130 patients, we would have 80% power to detect a minimum hazard ratio around 1.7 across a range of common allele frequencies (0.2-0.5) for both PFS and OS in a dominant model. For a recessive model, a minimum hazard ratio is below 3.6 when the allele frequency is 0.2 and approaches 1.8 when the allele frequency is 0.5. At the time of analysis, 23 patients (17%) were alive. Allelic distribution of all polymorphisms was tested for deviation from Hardy-Weinberg equilibrium. Multivariable analysis was conducted using Cox proportional hazards regression model. The level of significance was set to a *P* value of <0.05, and *P* values are given for 2-sided testing. All statistical test were done using the SAS statistical package version 9.1 (SAS Institute, Inc.), and Epilog Plus Version 1.0 (Epicentre Software).

## Results

Patients whose tissues samples were available for analysis of molecular correlates (*n* = 130) had a similar median PFS [1.3 months; 95% confidence interval (CI), 1.3-1.5], OS (6.3 months; 95% CI, 4.3-7.7), and response rate (9.2%; 95% CI, 4.9%-15.6%) compared with the clinical outcome of the patients without tissue samples available from the entire study population of IMC 0144 [*n* = 216; median PFS, 1.5 mo (95% CI, 1.4-2.6); OS, 6.8 months (95% CI, 5.8-8.1), and response rate of 13.0% (95% CI, 8.8%-18.2%); ref. 13]. There



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**Table 3.** Baseline patient characteristics, skin rash severity, K-ras mutation status, and clinical outcome ($n$ = 130)

| | ***n*** | **Response*** | | | **Skin-rash severity** | | |
|---|---|---|---|---|---|---|---|
| | | **PR** | **SD** | **PD** | **Grade 0** | **Grade 1** | **Grade 2-3** |
| Age, y | | | | | | | |
| ≤54 | 36 | 2 (6%) | 11 (33%) | 20 (61%) | 4 (11%) | 16 (44%) | 16 (44%) |
| 54-64 | 45 | 6 (16%) | 12 (32%) | 19 (51%) | 8 (18%) | 21 (47%) | 16 (36%) |
| ≥65 | 49 | 4 (9%) | 14 (32%) | 26 (59%) | 5 (10%) | 20 (41%) | 24 (49%) |
| *P* † | | | 0.87 | | | 0.64 | |
| Gender | | | | | | | |
| Female | 66 | 7 (12%) | 23 (38%) | 30 (50%) | 8 (12%) | 34 (52%) | 24 (36%) |
| Male | 64 | 5 (9%) | 14 (26%) | 35 (65%) | 9 (14%) | 23 (36%) | 32 (50%) |
| *P* † | | | 0.22 | | | 0.37 | |
| ECOG performance status score | | | | | | | |
| 0 | 52 | 6 (12%) | 19 (39%) | 24 (49%) | 2 (4%) | 19 (37%) | 31 (60%) |
| 1 | 76 | 6 (9%) | 18 (28%) | 40 (63%) | 14 (18%) | 37 (49%) | 25 (33%) |
| *P* † | | | 0.21 | | | <0.001 | |
| Tumor site | | | | | | | |
| Colon | 99 | 10 (11%) | 26 (30%) | 51 (59%) | 11 (11%) | 45 (45%) | 43 (43%) |
| Rectum | 31 | 2 (7%) | 11 (41%) | 14 (52%) | 6 (19%) | 12 (39%) | 13 (42%) |
| *P* † | | | 0.87 | | | 0.55 | |
| No. of prior chemotherapy regimens | | | | | | | |
| 2-3 | 58 | 4 (8%) | 16 (30%) | 33 (62%) | 4 (7%) | 31 (53%) | 23 (40%) |
| 4-5 | 60 | 6 (12%) | 18 (36%) | 26 (52%) | 11 (18%) | 24 (40%) | 25 (42%) |
| 6-8 | 12 | 2 (18%) | 3 (27%) | 6 (55%) | 2 (17%) | 2 (17%) | 8 (67%) |
| *P* † | | | 0.29 | | | 0.92 | |
| EGFR tumor immunostaining intensity | | | | | | | |
| 1+ | 79 | 8 (12%) | 19 (28%) | 41 (60%) | 12 (15%) | 36 (46%) | 31 (39%) |
| 2-3+ | 50 | 4 (9%) | 18 (40%) | 23 (51%) | 5 (10%) | 20 (40%) | 25 (50%) |
| *P* † | | | 0.67 | | | 0.24 | |
| Skin-rash severity | | | | | | | |
| Grade 0 | 17 | 0 (0%) | 0 (0%) | 7 (100%) | | | |
| Grade 1 | 57 | 6 (11%) | 16 (30%) | 31 (58%) | | | |
| Grade 2-3 | 56 | 6 (11%) | 21 (39%) | 27 (50%) | | | |
| *P* † | | | 0.087 | | | | |
| *K-ras* mutation status | | | | | | | |
| Wild-type | 88 | 12 (16%) | 26 (34%) | 39 (51%) | 12 (14%) | 38 (43%) | 38 (43%) |
| Mutant | 42 | 0 (0%) | 11 (30%) | 26 (70%) | 5 (12%) | 19 (45%) | 18 (43%) |
| *P* † | | | 0.012 | | | 1.00 | |

Abbreviations: PR, partial response; SD, stable disease; PD, progressive disease; ECOG, Eastern Cooperative Oncology Group.
*Sixteen of 130 patients (12%) were not evaluable for tumor response.
†*P* values were based on the exact conditional test for response and for skin rash severity, and the log-rank test for PFS and OS.

were 121 Caucasian (93%), 1 Hispanic (1%), 3 Asian (2%), 3 African-American (2%), and 2 other (2%) study participants. At the time of analysis, 23 (17%) patients were still alive: the follow-up for those patients ranged from 2.2 to 17.3 months (median follow-up, 12.3 months). Skin rash was observed in 87% (113 of 130) of patients. Forty-four percent ($n$ = 57) had a grade 1, and 43% ($n$ = 56) showed a grade 2 or 3 skin-reaction. Skin rash severity was significantly associated with PFS ($P < 0.001$, log-rank) and OS ($P < 0.001$, log-rank). The allelic frequencies observed for all polymorphisms analyzed were within the probability limits of Hardy-Weinberg equilibrium ($P > 0.05$, exact test for Hardy-Weinberg equilibrium). Detailed clinicopathologic and demographic characteristics are shown in Table 3.

***K-ras* mutation status and clinical outcome.** *K-ras* mutation was significantly associated with lack of response to cetuximab (Table 3). None of the 37 patients with a *K-ras* mutation whose tumor response was evaluable had a response to cetuximab, whereas 12 of the 77 wild-type *K-ras* patients were responders (0% versus 16%, respectively; $P$ = 0.012). In the 130 patients assessable for survival, PFS and OS times of patients without K-ras mutation were significantly longer compared with the PFS and OS times of mutated patients [median PFS, 1.4 month (95% CI, 1.3-2.4 month) versus 1.3 month (95% CI, 1.2-1.6 month), respectively; $P$ = 0.023; median OS, 6.6 months (95% CI, 4.3-8.9 months) versus 4.9 months (95% CI, 2.8-6.6 months), respectively; Table 3].

***EGF +61 A>G* polymorphism (rs4444903) and progression-free survival.** Genotyping for *EGF +61 A>G* was successful in 116 (89%) of 130 cases. In the other 14 (11%) patients, genotyping was not successful because of limited quantity and quality of extracted genomic DNA. *EGF +61 A>G* polymorphism showed a significant association with PFS. Patients with the *EGF +61 G/G* homozygous genotype had a median PFS of 1.4 months (95% CI, 1.3-3.9 months), compared with 1.2 months (95% CI, 1.2-1.5 months) and 1.3 months (95% CI, 1.2-2.6 months), in patients homozygous and heterozygous for the A-allele, respectively ($P$ = 0.042, log-rank test). For *EGF +61 A>G*, the FDR-adjusted



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**Table 3.** Baseline patient characteristics, skin rash severity, K-ras mutation status, and clinical outcome (*n* = 130) (Cont'd)

| PFS | | | OS | | |
|---|---|---|---|---|---|
| **Median, (95% CI)** | **Relative risk, mo (95% CI)** | | **Median, mo (95% CI)** | **Relative risk (95% CI)** | |
| 1.2 (1.2, 1.5) | 1 (Reference) | | 5.3 (3.6, 7.5) | 1 (Reference) | |
| 1.4 (1.2, 2.5) | 0.74 (0.48, 1.16) | | 7.0 (3.0, 11.5) | 0.69 (0.42, 1.13) | |
| 1.4 (1.3, 2.4) | 0.77 (0.50, 1.19) | | 6.6 (3.8, 8.8) | 0.86 (0.54, 1.38) | |
| | | 0.34 | | | 0.31 |
| 1.5 (1.3, 2.4) | 1 (Reference) | | 7.9 (5.0, 8.9) | 1 (Reference) | |
| 1.3 (1.2, 1.4) | 1.24 (0.88, 1.75) | | 4.8 (3.4, 7.0) | 1.34 (0.91, 1.96) | |
| | | 0.21 | | | 0.13 |
| 1.4 (1.2, 2.4) | 1 (Reference) | | 8.0 (5.3, 12.1) | 1 (Reference) | |
| 1.3 (1.2, 1.8) | 1.14 (0.80, 1.63) | | 4.9 (3.0, 7.0) | 1.79 (1.19, 2.68) | |
| | | 0.44 | | | 0.003 |
| 1.3 (1.2, 1.5) | 1 (Reference) | | 6.3 (3.8, 8.2) | 1 (Reference) | |
| 1.4 (1.2, 2.5) | 1.14 (0.76, 1.72) | | 5.5 (3.4, 8.7) | 0.96 (0.61, 1.52) | |
| | | 0.51 | | | 0.86 |
| 1.3 (1.2, 1.3) | 1 (Reference) | | 5.5 (3.6, 7.7) | 1 (Reference) | |
| 1.5 (1.3, 2.6) | 0.79 (0.54, 1.13) | | 5.9 (3.7, 8.2) | 1.06 (0.71, 1.58) | |
| 1.4 (1.1, 6.6) | 0.62 (0.33, 1.16) | | 12.5 (6.4, 17.7) | 0.60 (0.29, 1.22) | |
| | | 0.18 | | | 0.26 |
| 1.3 (1.2, 1.5) | 1 (Reference) | | 5.5 (3.8, 7.7) | 1 (Reference) | |
| 1.4 (1.3, 2.5) | 0.89 (0.62, 1.27) | | 7.3 (3.6, 8.7) | 0.97 (0.65, 1.43) | |
| | | 0.51 | | | 0.86 |
| 1.1 (0.9, 1.3) | 1 (Reference) | | 2.0 (1.0, 3.4) | 1 (Reference) | |
| 1.3 (1.3, 1.5) | 0.37 (0.21, 0.66) | | 6.5 (3.6, 8.7) | 0.27 (0.15, 0.48) | |
| 1.5 (1.2, 2.6) | 0.35 (0.19, 0.61) | | 7.6 (5.4, 10.0) | 0.21 (0.12, 0.39) | |
| | | <0.0001 | | | <0.0001 |
| 1.4 (1.3, 2.4) | 1 (Reference) | | 6.6 (4.3, 8.9) | 1 (Reference) | |
| 1.3 (1.2, 1.6) | 1.49 (1.01, 2.20) | | 4.9 (2.8, 6.6) | 1.59 (1.05, 2.40) | |
| | | 0.023 | | | 0.020 |

*P* value did meet the criteria for variable selection as a candidate predictor in the multivariable model (FDR-adjusted *P* = 0.11; Table 4; Fig. 1A).

***EGFR +497 G>A polymorphism (rs11543848) and progression-free survival.*** Genotyping for *EGFR +497 G>A* was successful in 122 (94%) of 130 cases. In the other 8 (6%) patients, genotyping was not successful because of limited quantity and quality of extracted genomic DNA. *EGFR +497 G>A* polymorphism showed a significant association with PFS. Patients with the *EGFR +497 A/A* homozygous genotype had a median PFS of 1.2 months (95% CI, 1.1-1.2 months), compared with 1.3 months (95% CI, 1.2-1.5 months), and 1.8 months (95% CI, 1.3-2.6 months), in patients homozygous and heterozygous for the G-allele, respectively (*P* = 0.017, log-rank test). For *EGFR +497 G>A*, the FDR-adjusted *P* value did meet the criteria for variable selection as a candidate predictor in the multivariable model (FDR-adjusted *P* = 0.094; Table 4; Fig. 1B).

***COX-2 -765 G>C polymorphism (rs20417) and progression-free survival.*** Genotyping for *COX-2 -765 G>C* was successful in 123 (95%) of 130 cases. In the other 7 (5%) patients, genotyping was not successful because of limited quantity and quality of extracted genomic DNA. *COX-2 -765 G>C* polymorphism showed a significant association with PFS. Patients with the *COX-2 -765 G/G* homozygous genotype had a median PFS of 1.3 months (95% CI, 1.2-1.5 months), compared with 1.3 month (95% CI, 1.2-2.4 months) and 5.8 month (95% CI, 3.8-9.6 months), in patients heterozygous and homozygous for the C-allele, respectively (*P* = 0.032, log-rank test). For *COX-2 -765 G>C*, the FDR-adjusted *P* value did meet the criteria for variable selection as a candidate predictor in the multivariable model (FDR-adjusted *P* = 0.11; Table 4; Fig. 1C).

***COX-2 +8473 T>C polymorphism (rs5275) and progression-free survival.*** Genotyping for *COX-2 +8473 T>C* was successful in 125 (96%) of 130 cases. In the other 5 (4%) patients, genotyping was not successful because of limited quantity and quality of extracted genomic DNA. *COX-2 +8473 T>C* polymorphism showed a significant association with PFS. Patients with the *COX-2 +8473 T/T* homozygous genotype had a median PFS of 1.4 months (95% CI, 1.3-2.6 months), compared with 1.3 months (95% CI, 1.2 to 1.4 months) and 3.8 months (95% CI, 1.2-5.8 months), in patients heterozygous and homozygous for the C-allele, respectively (*P* = 0.003, log-rank test). For *COX-2 +8473 T>C*, the FDR-adjusted *P* value did meet the criteria for variable selection as a candidate predictor in the multivariable model (FDR-adjusted *P* = 0.037; Table 4; Fig. 1D).



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**Table 4.** Genomic polymorphisms and clinical outcome in mCRC patients treated with single-agent cetuximab

| | *n* | Response* | | | | | Skin-rash severity | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | PR | SD | PD | *P* † | FDR-adjusted *P* ‡ | Grade 0 | Grade 1 | Grade 2-3 | *P* † |
| *FCGR2A 131 H>R (rs1801274)* | | | | | 0.93 | 0.92 | | | | 0.72 |
| H/H | 35 | 2 (6%) | 11 (34%) | 19 (59%) | | | 5 (14%) | 14 (40%) | 16 (46%) | |
| H/R | 29 | 4 (15%) | 8 (31%) | 14 (54%) | | | 3 (10%) | 11 (38%) | 15 (52%) | |
| R/R | 36 | 4 (12%) | 9 (26%) | 21 (62%) | | | 3 (8%) | 21 (58%) | 12 (33%) | |
| *FCGR3A 158 V>F (rs396991)* | | | | | 0.85 | 0.92 | | | | 0.13 |
| F/F | 32 | 3 (11%) | 6 (21%) | 19 (68%) | | | 4 (13%) | 10 (31%) | 18 (56%) | |
| F/V | 58 | 6 (11%) | 21 (38%) | 28 (51%) | | | 5 (9%) | 29 (50%) | 24 (41%) | |
| V/V | 37 | 3 (10%) | 9 (29%) | 19 (61%) | | | 6 (16%) | 18 (49%) | 13 (35%) | |
| *EGFR +497 G>A (rs11543848)* | | | | | 0.50 | 0.86 | | | | 0.30 |
| G/G | 66 | 5 (9%) | 20 (36%) | 31 (55%) | | | 10 (15%) | 28 (42%) | 28 (42%) | |
| A/G | 47 | 6 (14%) | 15 (34%) | 23 (52%) | | | 4 (9%) | 23 (49%) | 20 (43%) | |
| A/A | 9 | 0 (0%) | 1 (13%) | 7 (88%) | | | 1 (11%) | 2 (22%) | 6 (67%) | |
| *EGFR* $(CA)_{14\text{-}23}$ *(rs45608036)* | | | | | 0.77 | 0.92 | | | | 0.41 |
| Both repeats <20 | 54 | 6 (13%) | 12 (26%) | 29 (62%) | | | 7 (13%) | 20 (37%) | 27 (50%) | |
| Any repeats ≥20 | 63 | 5 (9%) | 22 (39%) | 30 (53%) | | | 7 (11%) | 33 (52%) | 23 (37%) | |
| *CCDN1 +870 A>G (rs17852153)* | | | | | 0.60 | 0.86 | | | | 0.36 |
| G/G | 44 | 2 (5%) | 13 (34%) | 23 (61%) | | | 4 (9%) | 19 (43%) | 21 (48%) | |
| G/A | 48 | 7 (17%) | 10 (24%) | 24 (59%) | | | 8 (17%) | 18 (38%) | 22 (46%) | |
| A/A | 34 | 2 (6%) | 13 (41%) | 17 (53%) | | | 4 (12%) | 18 (53%) | 12 (35%) | |
| *IL-8 -251 T>A (rs4073)* | | | | | 0.32 | 0.86 | | | | 0.01 |
| A/A | 35 | 3 (12%) | 5 (19%) | 18 (69%) | | | 7 (20%) | 19 (54%) | 9 (26%) | |
| A/T | 63 | 6 (10%) | 19 (33%) | 33 (57%) | | | 8 (13%) | 26 (41%) | 29 (46%) | |
| T/T | 30 | 3 (10%) | 12 (41%) | 14 (48%) | | | 1 (3%) | 12 (40%) | 17 (57%) | |
| *VEGF C+936T (rs3025039)* | | | | | 0.45 | 0.86 | | | | 0.81 |
| C/C | 89 | 7 (9%) | 25 (32%) | 45 (58%) | | | 12 (13%) | 36 (40%) | 41 (46%) | |
| C/T | 26 | 4 (17%) | 5 (21%) | 15 (63%) | | | 3 (12%) | 14 (54%) | 9 (35%) | |
| T/T | 5 | 0 (0%) | 4 (80%) | 1 (20%) | | | 0 (0%) | 3 (60%) | 2 (40%) | |
| *COX-2 -765 G>C (rs20417)* | | | | | 0.02 | 0.22 | | | | 0.72 |
| G/G | 85 | 7 (9%) | 22 (29%) | 46 (61%) | | | 9 (11%) | 38 (45%) | 38 (45%) | |
| G/C | 34 | 2 (7%) | 11 (39%) | 15 (54%) | | | 7 (21%) | 15 (44%) | 12 (35%) | |
| C/C | 4 | 3 (75%) | 1 (25%) | 0 (0%) | | | 0 (0%) | 1 (25%) | 3 (75%) | |
| *COX-2 +8473 T>C (rs5275)* | | | | | 0.62 | 0.86 | | | | 0.86 |
| T/T | 58 | 6 (11%) | 18 (34%) | 29 (55%) | | | 5 (9%) | 26 (45%) | 27 (47%) | |
| T/C | 48 | 2 (5%) | 12 (29%) | 28 (67%) | | | 9 (19%) | 24 (50%) | 15 (31%) | |
| C/C | 19 | 3 (19%) | 7 (44%) | 6 (38%) | | | 2 (11%) | 4 (21%) | 13 (68%) | |
| *EGF +61 A>G (rs4444903)* | | | | | 0.17 | 0.86 | | | | 0.93 |
| A/A | 42 | 2 (6%) | 12 (33%) | 22 (61%) | | | 6 (14%) | 18 (43%) | 18 (43%) | |
| A/G | 48 | 4 (9%) | 14 (32%) | 26 (59%) | | | 4 (8%) | 24 (50%) | 20 (42%) | |
| G/G | 26 | 5 (23%) | 6 (27%) | 11 (50%) | | | 4 (15%) | 10 (38%) | 12 (46%) | |
| *NRP-1 C/T (rs3750733)* | | | | | 0.48 | 0.86 | | | | 0.21 |
| C/C | 44 | 4 (10%) | 15 (38%) | 20 (51%) | | | 6 (14%) | 21 (48%) | 17 (39%) | |
| C/T | 51 | 5 (12%) | 13 (30%) | 25 (58%) | | | 6 (12%) | 26 (51%) | 19 (37%) | |
| T/T | 32 | 3 (10%) | 8 (27%) | 19 (63%) | | | 4 (13%) | 9 (28%) | 19 (59%) | |

*Sixteen of 130 patients (12%) were not evaluable for tumor response.
† *P* values were based on the exact conditional test for response and for skin rash severity and the log-rank test for PFS and OS.
‡ The Benjamini-Hochberg method was used to control the FDR of multiple testing. The FDR adjusted *P* values were set at <15%.

***COX-2 -765 G>C polymorphism (rs20417) and response to Cetuximab.*** *COX-2 -765 G>C* polymorphism showed a significant association with response to cetuximab. Patients homozygous for the *COX-2 -765* G-allele (*n* = 85) were more likely to experience progressive disease (61%), compared with patients carrying the G/C (progressive disease, 54%) or C/C (progressive disease, 0%) genotype ($P = 0.02$, exact-conditional test). *COX-2 -765 G>C* was not significantly associated with response,



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**Table 4.** Genomic polymorphisms and clinical outcome in mCRC patients treated with single-agent cetuximab (Cont'd)

| | PFS | | | | OS | | | |
|---|---|---|---|---|---|---|---|---|
| **FDR-adjusted $P^{\ddagger}$** | **Median, mo (95% CI)** | **Relative risk (95% CI)** | **$P^{\dagger}$** | **FDR-adjusted $P^{\ddagger}$** | **Median, mo (95% CI)** | **Relative risk (95% CI)** | **$P^{\dagger}$** | **FDR-adjusted $P^{\ddagger}$** |
| 0.93 | | | 0.50 | 0.85 | | | 0.49 | 0.87 |
| | 1.3 (1.2, 1.6) | 1 (Reference) | | | 7.5 (3.6, 8.7) | 1 (Reference) | | |
| | 1.2 (1.1, 3.9) | 0.76 (0.46, 1.27) | | | 5.3 (2.8, 8.7) | 0.73 (0.41, 1.30) | | |
| | 1.3 (1.2, 2.5) | 0.91 (0.57, 1.44) | | | 5.9 (3.7, 8.6) | 0.92 (0.55, 1.53) | | |
| 0.78 | | | 0.42 | 0.85 | | | 0.34 | 0.87 |
| | 1.3 (1.2, 1.6) | 1 (Reference) | | | 6.4 (3.4, 7.9) | 1 (Reference) | | |
| | 1.3 (1.2, 2.5) | 0.84 (0.55, 1.29) | | | 6.3 (4.4, 8.7) | 0.71 (0.45, 1.14) | | |
| | 1.3 (1.2, 1.5) | 1.08 (0.68, 1.73) | | | 4.1 (3.0, 9.3) | 0.87 (0.53, 1.44) | | |
| 0.82 | | | 0.017 | 0.094 | | | 0.65 | 0.87 |
| | 1.3 (1.2, 1.5) | 1 (Reference) | | | 5.5 (3.6, 7.6) | 1 (Reference) | | |
| | 1.8 (1.3, 2.6) | 0.82 (0.56, 1.20) | | | 7.3 (4.8, 8.7) | 0.90 (0.59, 1.37) | | |
| | 1.2 (1.1, 1.2) | 2.16 (1.06, 4.43) | | | 2.7 (1.8, 12.1) | 1.30 (0.59, 2.88) | | |
| 0.82 | | | 0.73 | 0.89 | | | 0.52 | 0.87 |
| | 1.3 (1.2, 1.5) | 1 (Reference) | | | 7.0 (4.1, 8.7) | 1 (Reference) | | |
| | 1.3 (1.3, 2.5) | 1.06 (0.73, 1.54) | | | 5.5 (3.7, 8.0) | 1.14 (0.76, 1.71) | | |
| 0.82 | | | 0.62 | 0.85 | | | 0.87 | 0.87 |
| | 1.3 (1.2, 1.6) | 1 (Reference) | | | 6.5 (3.6, 8.2) | 1 (Reference) | | |
| | 1.3 (1.2, 2.3) | 0.85 (0.56, 1.30) | | | 5.4 (3.6, 8.7) | 0.92 (0.59, 1.45) | | |
| | 1.4 (1.3, 2.8) | 0.82 (0.52, 1.29) | | | 5.5 (2.8, 8.6) | 1.05 (0.64, 1.74) | | |
| 0.054 | | | 0.14 | 0.32 | | | 0.30 | 0.87 |
| | 1.3 (1.2, 1.8) | 1 (Reference) | | | 3.4 (2.5, 6.1) | 1 (Reference) | | |
| | 1.3 (1.2, 1.5) | 0.81 (0.54, 1.24) | | | 6.6 (4.8, 8.2) | 0.85 (0.53, 1.35) | | |
| | 1.4 (1.2, 3.9) | 0.63 (0.38, 1.05) | | | 8.7 (5.3, 12.0) | 0.66 (0.38, 1.14) | | |
| 0.93 | | | 0.87 | 0.93 | | | 0.19 | 0.87 |
| | 1.3 (1.2, 1.6) | 1 (Reference) | | | 6.5 (4.9, 8.0) | 1 (Reference) | | |
| | 1.3 (1.2, 2.8) | 0.89 (0.58, 1.39) | | | 3.4 (2.7, 8.6) | 1.25 (0.77, 2.02) | | |
| | 1.3 (1.2, 5.4) | 0.99 (0.40, 2.44) | | | 14.5 (1.5, 15.0) | 0.36 (0.09, 1.48) | | |
| 0.92 | | | 0.032 | 0.11 | | | 0.48 | 0.87 |
| | 1.3 (1.2, 1.5) | 1 (Reference) | | | 5.3 (3.7, 7.9) | 1 (Reference) | | |
| | 1.3 (1.2, 2.4) | 1.03 (0.69, 1.54) | | | 5.5 (3.4, 10.0) | 0.92 (0.59, 1.43) | | |
| | 5.8 (3.8, 9.6) | 0.31 (0.12, 0.84) | | | 10.5 (10.1, 13.3) | 0.51 (0.16, 1.61) | | |
| 0.93 | | | 0.003 | 0.037 | | | 0.47 | 0.87 |
| | 1.4 (1.3, 2.6) | 1 (Reference) | | | 7.6 (5.0, 8.8) | 1 (Reference) | | |
| | 1.3 (1.2, 1.4) | 1.49 (1.01, 2.22) | | | 3.8 (2.6, 6.4) | 1.27 (0.83, 1.96) | | |
| | 3.8 (1.2, 5.8) | 0.67 (0.40, 1.13) | | | 8.7 (3.3, 12.1) | 0.98 (0.55, 1.74) | | |
| 0.93 | | | 0.042 | 0.11 | | | 0.84 | 0.87 |
| | 1.2 (1.2, 1.5) | 1 (Reference) | | | 6.4 (3.6, 8.4) | 1 (Reference) | | |
| | 1.3 (1.2, 2.6) | 0.72 (0.47, 1.10) | | | 5.0 (3.6, 8.7) | 1.13 (0.71, 1.79) | | |
| | 1.4 (1.3, 3.9) | 0.57 (0.34, 0.95) | | | 5.9 (3.0, 10.5) | 0.99 (0.57, 1.73) | | |
| 0.82 | | | 0.93 | 0.93 | | | 0.87 | 0.87 |
| | 1.3 (1.2, 2.4) | 1 (Reference) | | | 7.3 (5.5, 8.7) | 1 (Reference) | | |
| | 1.4 (1.2, 2.4) | 0.98 (0.65, 1.47) | | | 4.4 (3.6, 8.6) | 0.91 (0.58, 1.42) | | |
| | 1.3 (1.2, 2.4) | 0.92 (0.58, 1.47) | | | 5.3 (3.4, 7.5) | 1.02 (0.62, 1.68) | | |

after an FDR-adjusted $P$ value of <0.15 was used (FDR-adjusted $P = 0.22$; Table 4).

***Other tested gene polymorphisms and clinical outcome to Cetuximab.*** Other tested gene polymorphisms did not show statistically significant associations with OS, response to cetuximab, toxicity, and PFS (Table 4).

***Multiple testing using Benjamini-Hochberg method.*** After adjusting for the FDR at <15% ($P < 0.15$), *EGF +61 A>G* (FDR-adjusted $P = 0.11$), *EGFR +497 G>A* (FDR-adjusted $P = 0.094$), *COX-2 -765 G>C* (FDR-adjusted $P = 0.11$), and *COX-2 T+8473* (FDR-adjusted $P = 0.037$) were used as candidates for inclusion in the multivariable model (Table 4).

***Multivariable analysis of COX-2 +8473 T>C (rs5275), EGF +61 A>G (rs4444903), and EGFR +497 G>A (rs11543848).*** When we analyzed *COX-2 +8473 T>C* (adjusted $P = 0.013$), *EGF +61 A>G* (adjusted $P = 0.088$), and *EGFR +497 G>A*



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(adjusted $P$ = 0.010) jointly, adjusted by skin rash severity, K-ras mutation, and ECOG performance status, stratified by race, *EGFR +497 G>A* (*rs11543848*), and *COX-2 +8473 T>C* (rs5275) remained significantly associated with PFS (Table 5). Because both *COX-2* single nucleotide polymorphisms are in strong linkage disequilibrium (data not shown), *COX-2 -765 G>C* was not included into the multivariable model due to multicolinearity issues. Multivariable analysis was not conducted for OS because no polymorphism was found to be significant for OS.

## Discussion

We were able to show that germline polymorphisms of genes involved in the EGFR pathway independently predict clinical outcome in mCRC patients treated with single-agent cetuximab. To the best of our knowledge, this is the first study to show that EGFR pathway–related germline polymorphisms might be important prognostic markers in mCRC patients treated with single-agent cetuximab, independent of skin rash toxicity, and K-ras mutation status.

COX is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. The isoform COX-1 is thought to be constitutively expressed in a variety of tissues, whereas COX-2 is induced by cytokines, growth factors, mitogens, and oncoproteins (25). COX-2 is involved in the regulation of a broad range of cellular processes including tumor onset and progression, metastases, angiogenesis, and resistance to chemotherapy (26–30). The relationship between COX-2 and the EGF/EGFR signaling pathway is still

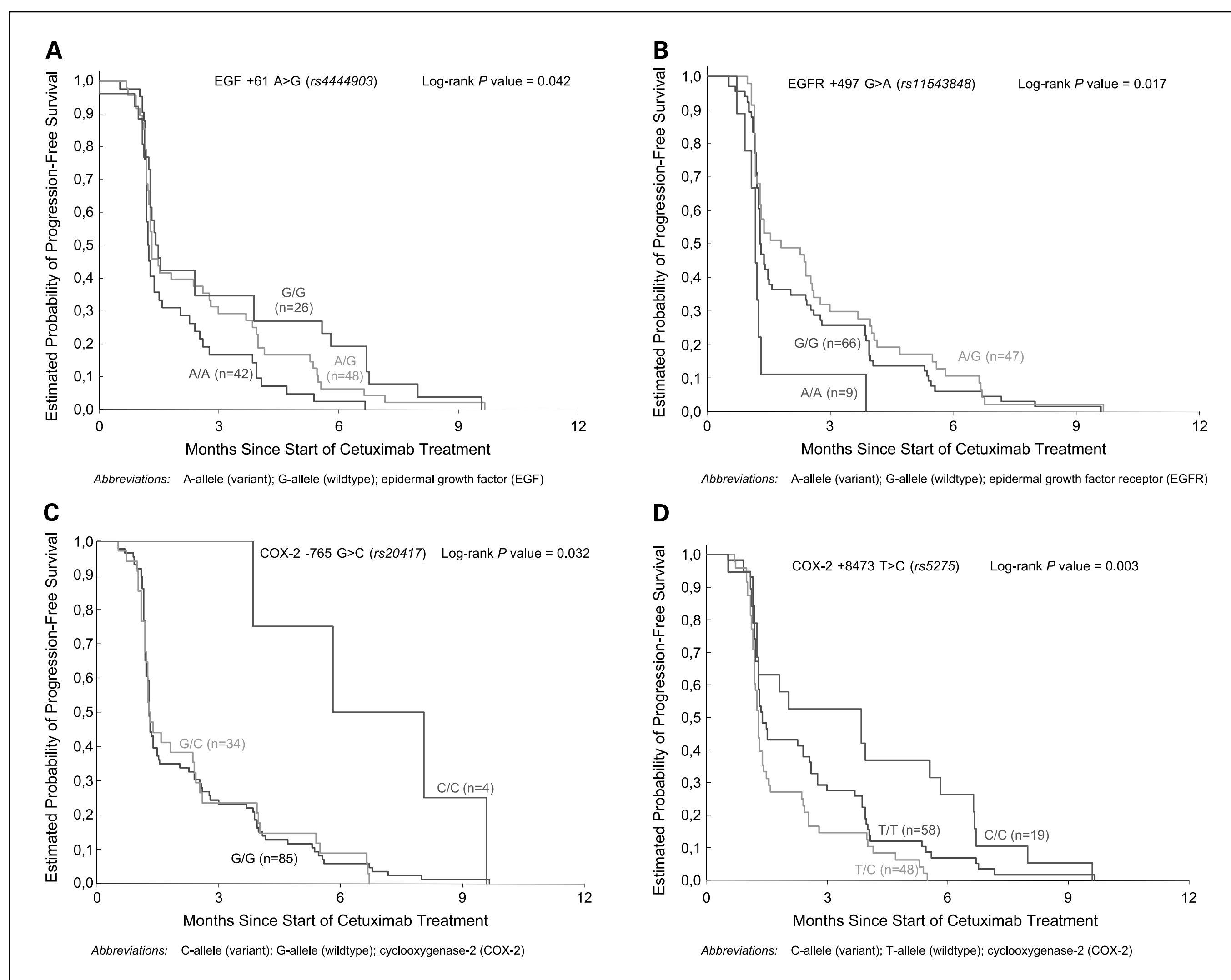


**Fig. 1.** *A,* PFS of patients with mCRC (IMC-0144) by EGF +61 A>G. Because all patients showed progressive disease, there were no censored observations. Therefore, no vertical hash marks were added to indicate the time of last follow-up for those patients who have not progressed or died at the time of the analysis of data. *B,* PFS of patients with mCRC (IMC-0144) by EGFR +497 G>A. Because all patients showed progressive disease, there were no censored observations. Therefore, no vertical hash marks were added to indicate the time of last follow-up for those patients who have not progressed or died at the time of the analysis of data. *C,* PFS of patients with mCRC (IMC-0144) by COX-2 -765 G>C. Because all patients showed progressive disease, there were no censored observations. Therefore, no vertical hash marks were added to indicate the time of last follow-up for those patients who have not progressed or died at the time of the analysis of data. *D,* PFS of patients with mCRC (IMC-0144) by COX-2 +8473 T>C. Because all patients showed progressive disease, there were no censored observations. Therefore, no vertical hash marks were added to indicate the time of last follow-up for those patients who have not progressed or died at the time of the analysis of data.



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**Table 5.** Multivariable analysis of *COX-2, EGF* and *EGFR* polymorphisms, and PFS

| | *n** | Adjusted RR (95%CI)[†] | Adjusted *P*[†] |
|---|---|---|---|
| *EGFR +497 G>A (rs11543848)* | | | 0.010[‡] |
| G/G | 60 | 1 (Reference) | |
| A/G | 43 | 0.71 (0.46, 1.08) | |
| A/A | 8 | 2.82 (1.24, 6.38) | |
| G/G + A/G vs A/A | | 3.04 (1.38-6.72) | |
| *COX-2 +8473 T>C (rs5275)* | | | 0.013[‡] |
| T/T | 50 | 1 (Reference) | |
| T/C | 43 | 1.59 (0.98, 2.58) | |
| C/C | 18 | 0.63 (0.34, 1.14) | |
| T/T + T/C vs C/C | | 0.53 (0.30-0.93) | |
| *EGF +61 A>G (rs4444903)* | | | 0.088[‡] |
| A/A | 39 | 1 (Reference) | |
| A/G | 48 | 0.70 (0.43, 1.12) | |
| G/G | 24 | 0.51 (0.28, 0.95) | |
| A/A vs A/G+G/G | | 0.64 (0.41, 1.00) | |
| Skin-rash severity | | | |
| Grade 0 | 13 | 1 (Reference) | 0.006 |
| Grade 1 | 49 | 0.27 (0.13, 0.56) | |
| Grade 2-3 | 49 | 0.28 (0.12, 0.61) | |
| *K-ras* mutation status | | | 0.45 |
| Wild-type | 72 | 1 (Reference) | |
| Mutant | 39 | 1.20 (0.75, 1.92) | |
| ECOG performance status score | | | 0.69 |
| 0 | 46 | 1 (Reference) | |
| 1 | 63 | 0.91 (0.59, 1.42) | |

*Patients with missing *EGFR +497 G>A, COX-2 +8473 T>C,* or *EGF +61 A>G* were excluded.
[†]Likelihood ratio test based on Cox proportional hazards model, adjusted by skin rash severity, *K-ras* mutation, and ECOG performance status, stratified by race, with all three polymorphisms included.
[‡]Adjusted *P* values, reflect unpooled three-group genotype comparisons.

controversial (31). COX-2 is thought to be a downstream effector of EGFR and was found to be induced by EGF-mediated stimulation of EGFR tyrosine kinase in human glioma cell lines (32). *In vivo* models by Xu et al. (32) showed that *COX-2* expression is strongly induced by p38 mitogen–activated protein kinase–mediated EGF stimulation. Other studies showed that COX-2 may be an upstream effector of EGFR in human colon cancer cells lines, suggesting that COX-2 induces colon cancer carcinogenesis by the activation of EGFR (33, 34). Furthermore, COX-2 has been reported to be a predictive and prognostic factor in a variety of malignancies (18, 26, 27). In fact, high expression levels of *COX-2* are associated with shorter OS in ovarian, head and neck, esophageal, and CRC (18, 35–37). *COX-2 -765 G>C* is a frequent single nucleotide polymorphism and is located 765 bp upstream of the COX-2 transcription start site. The *-765* C-allele was shown to be associated with significantly lower *COX-2* promoter activity and associate lower C-reactive protein plasma levels compared with the *-765* G-variant (38). Other common variants within the *COX-2* gene include the *COX-2 +8473 T>C* single nucleotide polymorphism. The *COX-2 +8473 T>C* polymorphism locates within the functional region of 3-untranslated region of the gene and, therefore, may have a potential functional relevance in carcinogenesis, perhaps through control of mRNA-stability and degradation (39, 40). The *+8473* C-allele was significantly less common in patients with lung cancer compared with healthy control patients, suggesting a protective effect against lung cancer (40). The present study found "low-expression" variants of *COX-2* (*COX-2 -765C* and *COX-2 +8473C*) to be significantly associated with higher PFS in both univariate and multivariable analysis (Tables 4 and 5). These findings are therefore consistent with previous reports by our group, demonstrating that *COX-2* mRNA overexpression is an adverse prognostic marker in mCRC (18). In addition, patients displaying the *COX-2 -765 C/C* genotype were more likely to experience partial response to cetuximab, compared with patients harboring the *-765* G-allele (log-rank test; $P = 0.02$; Table 4). Interestingly, all three patients with the *COX-2 -765 C/C* genotype and with partial response to cetuximab also showed grade 2 to 3 skin toxicity and superior PFS (median PFS, 8 months; 5.8-9.6) compared with other genotype combinations. It should be noted, however, that our study population consisted of only four patients carrying the *COX-2 -765* C-allele, and after adjustment for FDR, *COX-2 -765 G>C* did not remain significantly associated with response at the FDR of <15% level. Therefore, our data for *COX-2 -765 G>C* is tenuous and needs to be validated. Although not conclusive, our data indicate that genetic variants of *COX-2* may be prognostic and/or predictive markers for mCRC patients treated with single-agent cetuximab.

A recent study by Lu and coworkers (41) showed novel mechanisms of acquired resistance escaping treatment by cetuximab. *In vitro*, cetuximab-resistant DiFi5 CRC cells were shown to have an enhanced ubiquitination and functional degradation of EGFR (41). The authors report that CRC cells may develop acquired resistance to cetuximab via altering EGFR levels through promotion of EGFR degradation and using Src kinase–mediated cell signaling to bypass their dependency on EGFR for tumor growth and survival (41).



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*EGFR +497 G>A* is a single nucleotide polymorphism in codon 497, which has been associated with an arginine → lysine substitution in the extracellular domain within subdomain IV. Moriai et al. (42) were able to show that the lysine/lysine (A/A) genotype confers an attenuated function in EGFR ligand binding, growth stimulation, tyrosine kinase activation, and induction of proto-oncogenes. In the present study, *EGFR +497 A/A* genotype was associated with poor clinical outcome and shorter PFS, compared with other genotypes. Our findings are therefore consistent with Lu et al.'s (41) observations, as cetuximab resistance may be associated, at least in part, with intratumoral EGFR degradation. To date, *EGFR* polymorphisms have not been reported to be independently associated with PFS in mCRC patients treated with single-agent cetuximab. In our study, *EGFR +497 G>A* was found to be significantly associated with PFS in both FDR-adjusted univariate and multivariable analysis (Tables 4 and 5).

As with all clinical outcome studies, this analysis has potential limitations; First, all patients included in this study were treated with single-agent cetuximab. Therefore, it was not possible to assess genotype combinations associated with clinical outcome in an untreated control group. Second, our findings are based on a relatively small number of patients; and third, we examined eight genes within the EGFR pathway. Although it is recognized that the observed associations and patterns require confirmation with an independent data set, and no amount of reanalysis with the current data set will eliminate that need, we have taken care to (*a*) select the candidate genes with a documented role in the EGFR-signaling pathway, which have been found to be associated with prognosis in previous studies at our institution and/or in published articles (Table 1); (*b*) perform an internal validation analysis to reduce the likelihood of overanalyzing this data set; and (*c*) adjust the FDR for multiple comparisons. Nevertheless, the results of this molecular correlates study should be interpreted carefully within the context of other publications and analyses.

Notwithstanding the aforementioned limitations, we have identified polymorphisms in *COX-2*, *EGF*, and *EGFR* as potential molecular markers for clinical outcome in mCRC patients treated with single-agent cetuximab. In addition, genetic variants of *COX-2* and *EGFR* remained significantly associated with PFS in multivariable analysis, independent of skin rash toxicity and *K-ras* mutation status. Interestingly, genetic markers predicting clinical outcome seem to be different among patients with and without skin rash toxicity. In fact, only *interleukin-8 T-251A* was associated with skin rash toxicity, suggesting a specific and distinct genomic phenotype, which may be different in patients with high- and low-degree skin toxicity. In summary, this study supports the role of functional polymorphisms in *COX-2*, *EGF*, and *EGFR* in relation to PFS, which may be explained by both a predictive and/or a prognostic role of the aforementioned variants. Larger, prospective and biomarker-embedded clinical trials are needed to confirm and validate our findings.

## Disclosure of Potential Conflicts of Interest

H.-J. Lenz has received honoraria from Merck KG and Bristol-Myers Squibb. E.K. Rowinsky is employed by Imclone Systems, Inc. A. El-Khoueiry has received commercial research support from Bristol Myers Squibb. D.J. Mauro is employed by Bristol Myers Squibb. C. Langer is employed by and has an ownership interest in Bristol Myers Squibb.

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## Polymorphisms in *Cyclooxygenase-2* and *Epidermal Growth Factor Receptor* Are Associated with Progression-Free Survival Independent of K-ras in Metastatic Colorectal Cancer Patients Treated with Single-Agent Cetuximab

Georg Lurje, Fumio Nagashima, Wu Zhang, et al.

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