Polymorphisms in Genes Involved in EGFR Turnover Are Predictive for Cetuximab Efficacy in Colorectal Cancer

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Abstract

Transmembrane receptors, such as the EGFR, are regulated by their turnover, which is dependent on the ubiquitin-proteasome system. We tested in two independent study cohorts whether SNPs in genes involved in EGFR turnover predict clinical outcome in cetuximab-treated metastatic colorectal cancer (mCRC) patients. The following SNPs involved in EGFR degradation were analyzed in a screening cohort of 108 patients treated with cetuximab in the chemorefractory setting: c-CBL (rs7105971; rs4938637; rs4938638; rs251837), EPS15 (rs17567; rs7308; rs1065754), NAE1 (rs363169; rs363170; rs363172), SH3KBP1 (rs7051590; rs5955820; rs1017874; rs11795873), SGIP1 (rs604737; rs6570808; rs7526812), UBE2M (rs895364; rs895374), and UBE2L3 (rs5754216). SNPs showing an association with response or survival were analyzed in BRAF and RAS wild-type samples from the FIRE-3 study. One hundred and

fifty-three FOLFIRI plus cetuximab-treated patients served as validation set, and 168 patients of the FOLFIRI plus bevacizumab arm served as controls. EGFR FISH was done in 138 samples to test whether significant SNPs were associated with EGFR expression. UBE2M rs895374 was significantly associated with progression-free survival (log-rank $P = 0.005$; HR, 0.60) within cetuximab-treated patients. No association with bevacizumab-treated patients ($n = 168$) could be established ($P = 0.56$; HR, 0.90). rs895374 genotype did not affect EGFR FISH measurements. EGFR recycling is an interesting mechanism of secondary resistance to cetuximab in mCRC. This is the first report suggesting that germline polymorphisms in the degradation process predict efficacy of cetuximab in patients with mCRC. Genes involved in EGFR turnover may be new targets in the treatment of mCRC. *Mol Cancer Ther; 14(10); 2374–81. ©2015 AACR.*

Introduction

Metastatic colorectal cancer (mCRC) is one of the most frequent malignancies in the Western world, with an estimated 136,830 new cases and an estimated 50,130 deaths in the United States in 2014 (1). Despite the progress that has been achieved in terms of prolonging median overall survival (OS) times during the last two decades, biomarker-driven targeted treatment is still limited to the anti-EGFR antibodies cetuximab and panitumumab. The only established biomarker in the treatment of mCRC is the negative predictive biomarker represented by the *RAS* (rat sarcoma) mutations (2–5). In first-line treatment, the combination of standard doublet chemotherapy with anti-EGFR antibodies results in response rates of 60% to 70%. In single-agent trials in further-line treatment, anti-EGFR antibodies have a response rate of 20% to 30%. But even in first-line treatment, almost one third of the tumors do not respond to treatment, and ultimately most tumors develop resistance. Therefore, other mechanisms of anti-EGFR resistance need to be identified. Receptor protein turnover regulated by the ubiquitin-proteasome system (UPS) is a potential mechanism of resistance (6).

Receptor-dependent signaling relies on the quantity of ligands binding to the respective receptors, the amount of receptors on the cell surface, and the presence of activating mutations in the

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Prior presentation: (i) ASCO Annual Meeting 2013 [J Clin Oncol 31:5s, 2013 (suppl; abstr 3557)]; (ii) ASCO Annual Meeting 2014 [J Clin Oncol 32:5s, 2014 (suppl; abstr 3571)].

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doi: 10.1158/1535-7163.MCT-15-0121

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intracellular signal pathway (7). To predict EGFR antibody resistance beyond RAS and BRAF mutations in mCRC, the expression levels of the ligands amphiregulin and epiregulin (8) and the receptor gene copy number (9) appeared to be of predictive value. But methodological problems to standardize intratumor protein measurements are unresolved. SNPs in contrast are offering a reliable and easy-to-access possibility of biomarker determination. Genomic DNA of white blood cells can be collected by standard blood draw, and tumor and genomic SNP genotypes are equivalent (10). The amount of EGFR receptors on the cell surface available for ligand binding is also regulated by processes of receptor turnover, as antibody binding to the EGFR induces receptor internalization (11). Receptor turnover can be divided into three steps: (i) receptor internalization, (ii) ubiquitination, and (iii) recycling or degradation (12). In mCRC, those processes are important for both, VEGFR (13) and EGFR (14) signaling. In short, after activation of the receptor tyrosine kinase through ligand binding and dimerization, the activated receptor is internalized by clathrin-dependent endocytosis and ubiquitinated. This terminates the tyrosine kinase activity of the activated EGFR and regulates the amount of receptors expressed on the cell surface. The final step of degradation is done by the proteasome; however, ubiquitinated receptors can be deubiquitinated by desubiquitinases and are then recycled toward the cell membrane (15). Three classes of enzymes (E1–E3) are needed for polyubiquitination. E1 enzymes are responsible for activating ubiquitin, E2 enzymes are transferring the activated ubiquitin toward the protein structure, and E3 enzymes are ligases that are able to add ubiquitin to each other. Whereas there are only some dozens of E1 and E2 enzymes, some thousands of E3

ligases are known and responsible for the substrate-specific sorting (Fig. 1). For the EGF receptor ubiquitination, the most important E3 ligase is c-CBL (casitas B-lineage lymphoma). C-CBL belongs to the class of cullin-RING ligases that are regulated by the addition of NEDD8 (neddylation; ref. 16). Neddylation is driving the turnover of EGFR toward degradation by accelerating polyubiquitination through c-CBL (17).

The following enzymes play important roles in EGFR turnover and were selected for the following study: The activated receptor is internalized by the help of c-CBL, c-CBL interacting protein of 85 kDa (CIN85), and endophilin. After being transferred into an early endosome, ubiquitination starts with the activation of ubiquitin by ubiquitin-activating enzyme (UAE1), which is then transferred to the receptor by the help of ubiquitin-conjugating enzyme (UbcH7). Polyubiquitination is done by the help of c-CBL. Epidermal growth factor receptor pathway substrate 15 (EPS15) is supporting the ligation process (18). UBC12 (NEDD8-conjugating enzyme) neddylates c-CBL by transferring activated NEDD8 to the cullin-RING motif of c-CBL (19, 20). To become functional, NEDD8 needs be activated by NAE1 (neddylation-activating enzyme 1).

This analysis aimed to (i) define subgroups of patients by SNP within genes of EGFR turnover being predictive for cetuximab efficacy and to (ii) test the predictive or prognostic value in a control cohort not treated with cetuximab.

Materials and Methods

Patients and material

SNPs with a minor allele frequency (MAF) of more than 10% in genes involved in EGFR degradation were evaluated for

Figure 1.
Growth factor receptor turnover principle. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; P, phosphorylated; c-CBL, casitas B-lineage lymphoma; CIN85, c-Cbl interacting protein of 85 kDa; UAE1, ubiquitin activating enzyme; NAE1, NEDD8 activating enzyme; EPS15, epidermal growth factor receptor substrate 15; UB, ubiquitin; N8, NEDD8; UbcH7, ubiquitin-conjugating enzyme; Ubc12, NEDD8-conjugating enzyme.
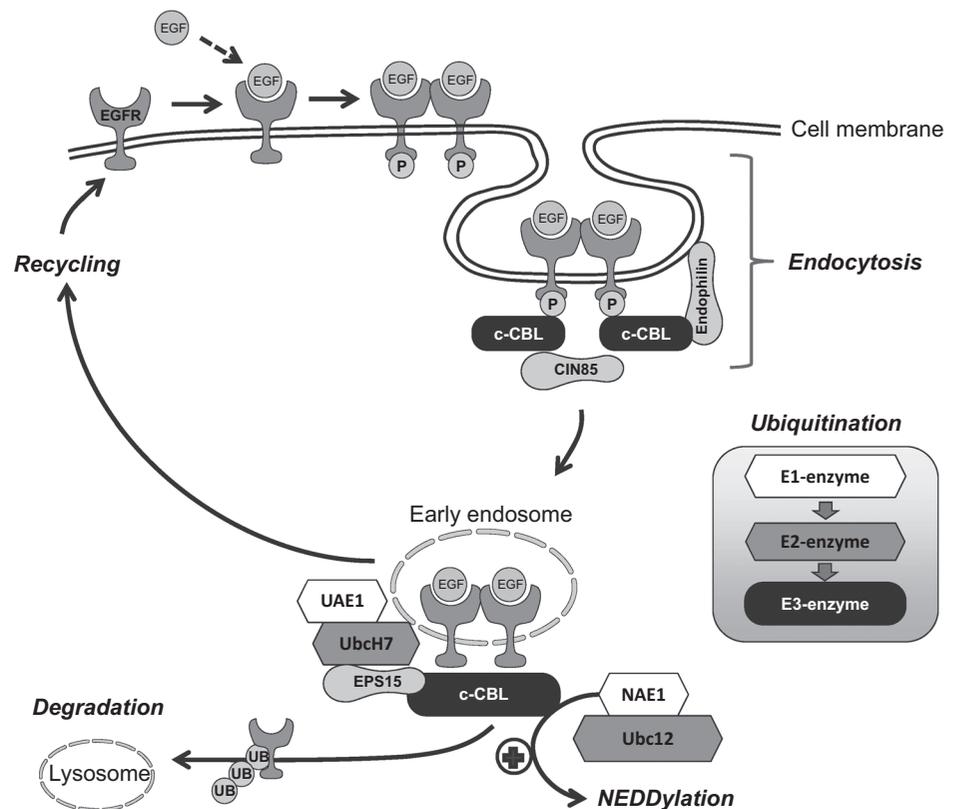
Table 1. Analyzed SNPs within degradation

Gene SNP	MAF	Allele location	Function	Forward (f) and reverse (r) primer
c-CBL				
rs2511837	49%	C/T downstream	Tag SNP	f: CAAGGCCCCCTTCCTCAT
				r: CACCCTACTTTTCAGCAGCA
rs4938637	9%	A/G upstream	Unknown	f: CAGCCTGGGTGACAGACTC
				r: GCTCTAACTCCAAGCTAAACAACC
rs4938638	44%	A/G upstream	Unknown	f: CATAAGACATGTGTACCAGGGAGT
				r: ATTCATCCTGCCACCATAGG
rs7105971	25%	G/A intron	Tag SNP	f: TGTTTGGTAAATACTTTTGTTGAATTA
				r: ACCCCACCCATCCCTAGTT
Epidermal growth factor receptor substrate 15 (EPS15)				
rs7308	21%	A/C 3′ UTR	Transcriptional regulation	f: GCACCATTTCAAGAAGAAATGAA
				r: CCAAGATAAAGAAAAATGCACCT
rs17567	21%	C/T exon 24	Protein coding, splicing regulation, transcriptional regulation, posttranslation	f: TTGCTTGGATCAGCCTCTTT
				r: TGATCCATTTCAGCCTTTCC
rs1065754	35%	G/A exon 14	Protein coding, splicing regulation, posttranslation	f: TCCCCATTTCTTCTCATTCA
				r: CACTCTCCTCCAATTCTGCTG
NEDD8-activating enzyme (NAE1)				
rs363169	44%	A/T intron	Tag SNP	f: CACACAATGGACTCAGTGACC
				r: GCCTTCTGATTCTGGGTTAATTT
rs363170	12%	A/G intron	Tag SNP	f: CACAATGCCTGATATGGCTCT
				r: TCTACTTTGGGGCGTTTTTG
rs363172	35%	A/G intron	Tag SNP	f: CTGTGAAGAGGACCTGAAAAA
				r: TGGAGATGAACAGTTTTAATGTATTT
NEDD8-conjugating enzyme (UBE2M)				
rs895374	39%	G/T downstream UBE2M Upstream CHMP2A	Transcriptional regulation	f: GAAGCCACAGGTCACTGGAT
				r: GTCACAGGTGGCATTGGAG
rs3794964	25%	A/G downstream near 3′UTR	Tag SNP	f: TGTGGAGGGTTACAGTGGTG
				r: CTCCATCCCCTTTTCCAGAT
Ubiquitin-conjugating enzyme (UBE2L3)				
rs5754216	17%	G/T intron	Tag SNP	f: TTTGGAACCAAGTCCAGGTC
				r: GGGAAGTGGGCTGAGAGAG
Endophilin (SGIP1)				
rs604737	26%	C/T exon 33	Transcriptional regulation	f: TTTGTTAAATTGTACTCTCATTCCAGT
				r: TGCAATCTACCTCTGTAAGCATT
rs657808	28%	A/G exon 33	Transcriptional regulation	f: CAAAACGCTGAGACTGAGAAT
				r: TGAATCCTAATCATCCAAAAGGA
rs7526812	14%	A/G exon 12	Protein coding, splicing regulation, transcriptional regulation, posttranslation	f: TCGGAAGAAGAAGAAGAATCACA
				r: CACACACAACACTCACCACTG
c-Cbl–interacting protein of 85 kDa (SH3KBP1)				
rs1017874	17%	C/T intron	Tag SNP	f: AAGCTACAGGAGGACAGACAGC
				r: CACTCCATCCTCGGTGACA
rs5955820	22%	C/T intron	Tag SNP	f: ACTTCCATGCTCTTAAGTTGGT
				r: TTTCAGAGAAACACTGGGAGTT
rs7051590	16%	C/G intron	Tag SNP	f: TTCCTCAAAATTTGTATGAGAAACC
				r: CAGGAGAGCAAGAGAGAGCAA
rs11795873	39%	A/G intron	Tag SNP	f: TGGGAGGAAAGCGATAACTG
				r: ATGGTCTTCCAGCCACTCAC

Abbreviations: A, adenine; C, cytosine; f, forward primer; G, guanine; NEDD8, ubiquitin-like molecule NEDD8; r, reverse primer; T, thymine; tag SNP, tagging SNP.

association with overall response rate, progression-free survival (PFS), and OS. Multivariate testing was adjusted for gender, age, primary tumor location, and treatment regimen. The training set consisted of patients from two phase II trials with histologically confirmed, *KRAS exon 2* and *BRAF* wild-type metastatic adenocarcinoma of the colon and rectum (21, 22). Patients of the screening cohort were treated with irinotecan plus cetuximab (21) or cetuximab monotherapy (22) in further-line treatment of mCRC. SNPs with significant data in multivariate analysis in the screening cohort were tested prospectively in 321 FIRE-3 *BRAF*

and *RAS* (exons 2, 3, and 4) wild-type specimens. Patients treated with FOLFIRI plus cetuximab ($n = 153$) served as validation cohort, and FOLFIRI plus bevacizumab ($n = 168$) treated patients were used as negative control.

Patients within the training set were either treated with FOLFIRI plus cetuximab [180 mg/m^2 of body-surface area (BSA) irinotecan; 400 mg/m^2 BSA leucovorin; 400 mg/m^2 BSA 5-fluorouracil as an intravenous bolus followed by a continuous infusion over 46 hours of 2,400 mg/m^2 BSA) and cetuximab (400 mg/m^2 BSA initial dose followed by a weekly dose of

250 mg/m^2 BSA; ref. 21) or cetuximab single agent (400 mg/m^2 BSA initial dose followed by a weekly dose of 250 mg/m^2 BSA; ref. 22].

Patients within the FIRE-3 trial were treated with FOLFIRI (180 mg/m^2 of BSA irinotecan; 400 mg/m^2 BSA leucovorin; 400 mg/m^2 BSA 5-fluorouracil as an intravenous bolus followed by a continuous infusion over 46 hours of 2,400 mg/m^2 BSA) and either bevacizumab (5 mg/kg) or cetuximab (400 mg/m^2 BSA initial dose followed by a weekly dose of 250 mg/m^2 BSA; ref. 5).

All patients had given their written informed consent.

Methods

Formalin-fixed, paraffin-embedded (FFPE) tissue samples or venous blood samples were used to extract genomic DNA. DNA extraction was carried out using the QIAamp DNA easy Kit (Qiagen) as recommended by the manufacturer. DNA was stored at −20°C until use.

Common and potentially functional polymorphisms within the genes being crucial for EGFR turnover regulation were selected using the Ensemble database (www.ensemble.org). If no functional SNPS were identified within targeted genes, tagging (Tag) SNPs were selected by SNPinfo (http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm). The following criteria were used to select the candidate gene polymorphisms: (a) an MAF ≥10%; (b) located in the 3'UTR, 5'UTR, or coding regions of the tested genes and/or were shown to be of biologic significance according to the location within the gene or according to literature review.

The tested SNPs for EGFR turnover and their forward and reverse primers used for PCR amplification are shown in Table 1.

PCR products were analyzed using direct sequencing. The investigator (S. Stintzing) reading the sequence was blinded to the clinical results.

EGFR FISH analysis was done in 138 FFPE samples according to a standard protocol of the Institute of Pathology, University of Munich. In short, after dewaxing, rehydration, and a pepsin digestion, a commercially available test kit (Zytomed) was used following the manufacturer's recommendations. Expression analysis was done by two pathologists (D. Neureiter and R. Kemmerling). In 100 cells in each sample, signals of EGFR and the corresponding chromosome 7 were counted and were set in relation with each other.

Statistical analysis

Treatment efficacy was measured by objective response rate (ORR), PFS, and OS in both cohorts. Survival times were measured from start of chemotherapy until progression or death. ORR was assessed using response criteria in solid tumor (RECIST) as assessed by the referring physician.

Allelic distribution of all polymorphisms was tested for deviation from the Hardy–Weinberg equilibrium using the χ^2 test with 1 degree of freedom. To evaluate the prognostic value of the polymorphisms on endpoints, the associations were examined using by Kaplan–Meier curve methods and tested by the log-rank test. The Cox proportional hazards regression model with stratification factors was fitted to re-evaluate the association between polymorphisms and outcomes considering the imbalance in the distributions of baseline patient characters in both cohorts. The baseline demographic and clinical markers that remained significantly associated with endpoints in the multivariable analyses ($P < 0.1$) were included in the final model.

With 108 patients (98 and 73 events for PFS and OS, respectively) in the screening cohort, we would have 80% power to detect minimum HRs of 1.79 to 2.17 and 1.94 to 2.32 on PFS and OS, respectively, for an SNP with a range of the variant allele frequencies (0.1–0.5) in a dominant model using a 0.05 level two-sided log-rank test. The validation set consisted of 153 patients (126 and 81 events for PFS and OS, respectively), which would give us 80% chances to find the SNPs with HRs of 1.71 to 2.03 for PFS and 1.94 to 2.25 for OS using the same test.

As a control arm, 168 patients (138 and 105 events for PFS and OS, respectively) not treated with cetuximab were used. With this number, we would have 80% power to detect minimum HRs of 1.69 to 2.00 and 1.82 to 2.12 in PFS and OS, respectively, for an SNP across a range of the variant allele frequencies (0.1–0.5) in a dominant model using the same test.

Expression levels were compared using the nonparametric Kruskal–Wallis test.

The level of significance was set to 0.05, and all statistical tests were two-sided and performed using the SAS statistical package version 9.3 (SAS Institute). Linkage disequilibrium was tested for significant SNPs.

Table 2. Baseline characteristics and primary outcome measurements of the tested cohorts

	Training set further-line cetuximab (n = 108)	FIRE-3 FOLFIRI + cetuximab (n = 153)	FIRE-3 FOLFIRI + bevacizumab (n = 168)
Median age, years (range)	64 (35–110)	64 (42–76)	65 (31–76)
Gender			
Female	39.8%	24.8%	30.4%
Male	60.2%	75.2%	69.6%
ECOG PS (%)			
0	62.1%	53.6%	55.4%
1	37.9%	45.8%	43.5%
2		0.7%	1.2%
Localization			
Midgut	N/A	16.3%	17.9%
Hindgut		80.4%	78.5%
Unknown		3.3%	3.6%
ORR	20%	77.7%	60.7%
PFS (months; 95% CI)	3.7 (2.8–4.6)	10.5 (9.1–12.0)	11.3 (10.1–12.5)
OS (months; 95% CI)	10.5 (7.7–13.3)	36.4 (27.7–45.1)	27.7 (24.4–31.0)

Table 3. Results for FOLFIRI plus cetuximab-treated patients ($n = 153$)

SNP	Genotype (N)	ORR (%)	P^a	PFS, mo	P^b HR	OS, mo	P^b HR
rs895374	CC (55)	76.6	0.94	7.9	0.018	38.3	0.84
	AC (75)	79.0		12.2	0.62	33.1	1.0
	AA (23)	76.2		12.7	0.57	49.8	0.83
rs379464	CC (10)	60.0	0.07	7.8	0.59	39.4	0.96
	CT (45)	90.0		12.2	1.4	33.8	1.1
	TT (83)	77.3		12.8	1.1	38.4	0.98
rs7051590	CC (116)	78.4	0.49	10.6	0.37	36.4	0.31
	CG (7)	71.4		13.3	1.7	41.3	2.1
	GG (14)	91.7		14.1	1.8	58.5	2.6
rs2511837	CC (23)	75.0	0.38	12.9	0.58	41.0	0.72
	CT (67)	75.9		11.3	0.80	36.4	0.71
	TT (48)	86.8		10.6	0.82	33.1	0.92
rs17567	CC (5)	100	0.73	10.4	0.75	18.6	0.61
	CT (49)	78.0		13.1	1.2	36.4	1.8
	TT (88)	80.8		10.0	0.88	38.3	1.1

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymidine.
[a] χ^2 test P.
[b] log-rank test P.

Results

Baseline characteristics and primary outcome measurements of both cohorts have been published before (5, 21, 22) and are summarized in Table 2.

All tested SNPs were within the Hardy–Weinberg equilibrium and close to the expected MAFs for the Caucasian population. For quality control purposes, a random selection of 10% of the samples was re-examined for each polymorphisms, and genotype concordance rate was 100%.

Within the screening set of 108 patients, only rs895374, an SNP in the *UBE2M* gene encoding for the NEDD8-conjugating (E2) enzyme, was able to separate significantly ($P = 0.02$) different patient groups. Another four SNPs (rs379464, rs7051590, rs2511837, and rs17567) showed a trend toward a possible selection and were selected to be tested on FIRE-3 samples. The results are shown in detail in Tables 3 and 4.

Rs895374 polymorphisms defined different patient subgroups for PFS with patients being homozygous for the major allele C reaching 7.9 months and 12.3 months for those bearing the minor allele A (log-rank test $P = 0.005$; HR, 0.60; Fig. 2A). The median

OS was not different reaching 38.3 months and 33.5 months, respectively (log-rank test $P = 0.85$; HR, 0.96). After adjusted for sex, age, ECOG performance status, number of metastatic sites, and acneiform exanthema, using Cox regression analysis, PFS was still significantly in favor of the minor allele A ($P = 0.002$; HR, 0.54). For OS even after multivariate adjustment, no difference for the different genotypes could be established ($P = 0.50$; HR, 0.78).

To distinguish whether this value is prognostic or predictive, the same SNP was tested in 168 bevacizumab-treated patients. The different genotypes of rs985374 did not lead to different outcomes in patients treated with bevacizumab, for which PFS was 12.4 months and 10.5 months (log-rank test $P = 0.56$; HR, 0.90; Fig. 2B) and median OS was 28.7 months and 25.4 months, respectively (log-rank test $P = 0.53$; HR, 0.88).

Within both arms of FIRE-3 no difference could be established regarding the overall response rate, reaching 78.3% for CA and AA and 76.6% for CC in the cetuximab (two-sided Fisher exact $P = 0.83$) and 63.4% and 71.7% in the bevacizumab arm (two-sided Fisher exact $P = 0.38$), respectively.

All associations between UB2M rs895374 and clinical outcomes in first-line FOLFIRI combination with either

Table 4. Results for FOLFIRI plus bevacizumab-treated patients ($n = 168$)

SNP	Genotype (N)	ORR (%)	P^a	PFS, mo	P^b HR	OS, mo	P^b HR
rs895374	CC (65)	71.7	0.17	10.5	0.84	25.4	0.82
	AC (79)	59.2		12.5	0.90	28.6	0.88
	AA (24)	76.7		10.8	0.91	25.0	0.88
rs379464	CC (6)	80.0	0.79	6.9	0.16	13.0	0.18
	CT (45)	65.0		14.5	1.7	26.1	1.57
	TT (86)	67.1		18.0	1.4	28.9	1.49
rs7051590	CC (96)	67.4	0.73	11.3	0.65	25.9	0.11
	CG (9)	77.8		14.9	1.1	nr	0.85
	GG (21)	73.7		9.8	0.77	30.8	0.22
rs2511837	CC (47)	65.9	0.90	11.3	0.41	29.1	0.72
	CT (48)	69.0		12.4	0.87	24.9	1.06
	TT (45)	64.3		10.2	0.47	27.5	1.23
rs17567	CC (6)	50.0	0.03	12.8	0.36	27.7	0.73
	CT (48)	55.8		9.8	0.91	25.0	0.70
	TT (86)	77.2		11.5	1.3	28.1	1.1

Abbreviations: A, adenine; C, cytosine; G, guanine; nr, not reached; T, thymidine.
[a] χ^2 test P.
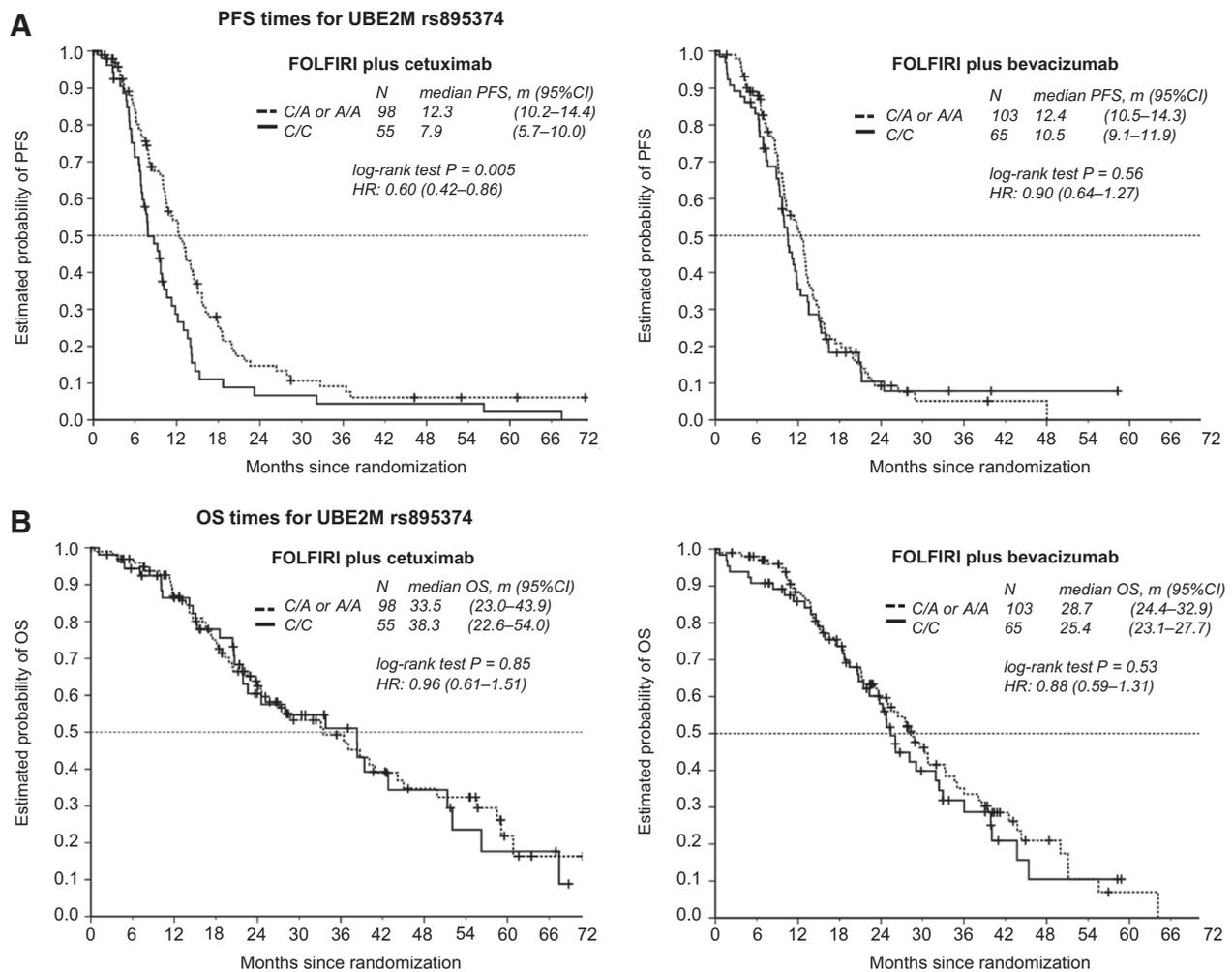[b] log-rank test P.

Figure 2.
Value of rs895374 in FOLFIRI plus cetuximab and FOLFIRI plus bevacizumab-treated patients for PFS and OS in FIRE-3. A, adenine; C, cytosine; N, number of patients; m, months; *UBE2M*, gene for NEDD8-conjugating enzyme.

cetuximab or bevacizumab in FIRE-3 are shown in Supplementary Table S1.

To test whether rs895374 genotypes are associated with EGFR gene copy number, EGFR FISH analysis was carried out. EFGR FISH readout was possible in 126 of 138 (91%) of all samples. EGFR FISH expression in relation to chromosome 7 was in median 1.022 [95% confidence interval (CI), 1.006–1.038] and was comparable for the rs895374 genotypes (Kruskal–Wallis test $P = 0.09$). Data of the association of EGFR FISH and response or survival are not shown.

There was no association between the rs895374 genotype and the occurrence of acneiform exanthema ($P = 0.48$; Supplementary Table S2).

Discussion

This is the first report of SNPs in genes involved in EGFR turnover to be analyzed for their predictive and prognostic value in the treatment of mCRC with the anti-EGFR antibody cetuximab. Rs895374 could be validated as a predictive biomarker for PFS in cetuximab-treated *RAS* and *BRAF* wild-type patients. For bevacizumab-treated patients, used as control group, neither a predictive nor a prognostic value could be demonstrated for rs895374. EGFR FISH results were not influenced by rs895374 genotypes in this analysis.

The use of anti-EGFR antibodies has been restricted to *KRAS* and *NRAS* exon 2, 3, and 4 wild-type tumors (2, 5). But even in the *RAS* wild-type population, according to RECIST, about 30% of patients do not respond to treatment. Predictive biomarkers for treatment efficacy beyond the *RAS* mutational analysis would be useful to further personalize therapy (23). Attempts using gene expression analysis for the EGFR ligands amphiregulin and epiregulin (8) or the expression of the EGFR (24, 25) have shown promising results, but methodological issues are, until now, unresolved. SNPs, on the other side, provide an ideal possibility for biomarker development as they are not influenced by the technique used for analysis, are easy to access by venous blood draw, and do not change under treatment. Rs895374 was able to predict PFS but not tumor response or OS. In other words, tumors with different genotypes of rs895374 initially respond to

cetuximab the same way, but the duration of response was different between patients bearing any "A" allele as reflected by the significantly different PFS times. This can be interpreted as a sign of developing secondary resistance to the initially successful regimen. EGFR turnover has been recognized as a possible pathway for acquired anti-EGFR resistance (6), but until now, only preclinical models have been published (26, 27). After binding to the extracellular domain of the receptor, EGFR and the respective antibody (cetuximab or panitumumab) are internalized (28). The amount of receptor to be recycled back to the cell surface is important for resistance to cetuximab/panitumumab treatment. Antibody mixtures like the novel drug Sym004 are known to enhance EGFR degradation (29) and can overcome cetuximab resistance (30).

The UPS has been increasingly recognized as a potential pathway for biomarker and drug development. Drugs inhibiting enzymes important for degradation have been developed (31), and inhibitors of neddylation are in early clinical development (32).

Rs895374 is an intergenic SNP located next to *UBE2M*, which is coding for UbcH12, the NEDD8-conjugating enzyme important for neddylation of c-CBL. Neddylation of c-CBL increases the ability of c-CBL to polyubiquitinate EGFR and therefore drives the balance of EGFR recycling and degradation toward degradation (17). In colorectal adenocarcinoma, the Colon Cancer Atlas Network (33) revealed 5.56% UBE2M mutant tumors, but the function of those mutations remains unclear. Two studies found elevated expression of UBE2M in colon adenocarcinoma when compared with normal tissue (34), but, again, relevance with regard to treatment efficacy is unclear. Furthermore, no knockout mouse model has been described so far.

In silico information provided by ENSMBL and REGULOME databases (35, 36) indicates that rs895374 alters transcriptional regulation. The polymorphism is found in a motif modulating the protein binding of the transcription factor CREBB (CREB-regulated transcription coactivator 1 binding protein; refs. 37, 38). Furthermore, rs895374 is a cis expression quantitative trait loci (eQTL) for the gene MGC2752 (CENPB DNA-binding domains containing 1 pseudogene; refs. 40, 41). A search of the GEO-Profiles database (42) revealed that MGC2725 is associated with recurrence in UICC II CRC patients linking rs895374 to CRC recurrence.

As an intergenic SNP rs895374 is also upstream *CHMP2A* (Charged multivesicular body protein 2a), which is a core component of the endosomal sorting required transport complex-III (ESCRT-III). The ESCRT machinery mediates the intracytoplasmatic sorting from endocytic bodies toward lysosomes via multivesicular bodies (MVB; ref. 43). In particular, ESCRT-III is involved in deubiquitination, clathrin-dependent endocytosis of ubiquitinated proteins, and MVB forming. All of which interfere with EGFR turnover and potentially the efficacy of cetuximab.

Although the predictive value of rs895374 could be demonstrated, several limitations have to be stated. Due to missing patient material, we cannot exclude that other mechanisms of secondary resistance, such as the outgrowth of low frequent RAS mutant tumor subclones (44), the occurrence of cetuximab-specific EGFR mutations (45), or the upregulation of other growth factor receptor pathways (26), interfere with the value of rs895374. The functional mechanism of rs895374 remains unclear, and this NEDD8 SNP needs to be tested in preclinical models to understand the biologic relevance. Therefore, the predictive value of rs895374 should be prospectively valued taking other factors of secondary resistance into account.

Receptor recycling is an important mechanism of anti-EGFR antibody resistance. EGFR receptor turnover is mediated by the UPS and modulated by the process of neddylation. This study validated for the first time the predictive value of an SNP associated with neddylation for the treatment with cetuximab in two different and independent study populations. No relation to treatment outcome could be revealed in a bevacizumab-treated control group. The antibody mixture Sym004 targeting the EGFR may overcome cetuximab resistance by urging EGFR turnover toward degradation. Inhibitors of the UPS and neddylation are already developed and in early clinical development (15). For mCRC, the UPS offers multiple targets for drug and biomarker development and should be further evaluated.

Disclosure of Potential Conflicts of Interest

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI or the NIH.

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Acknowledgments

Grant Support

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