Pharmacogenetics of advanced colorectal cancer treatment

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Correlation between germline polymorphisms and the efficacy of cetuximab in metastatic colorectal cancer

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Abstract

Background

Previous studies indicated that germline polymorphisms in specific genes may predict efficacy and toxicity of cetuximab in metastatic colorectal cancer (mCRC) patients.

Methods

Germline DNA was isolated from 576 mCRC patients who were treated in the phase III CAIRO2 study with chemotherapy and bevacizumab alone or with cetuximab. Associations of epidermal growth factor (*EGF*) 61A>G, EGF receptor (*EGFR*) $CA_{14\text{-}22}$, cyclin D1 (*CCND1*) 932G>A, fragment-C gamma receptor (*FCGR*) 2A 535A>G and *FCGR3A* 818A>C polymorphisms with progression-free survival (PFS) were studied with regard to *KRAS* status.

Results

In the cetuximab arm, the *FCGR3A*818C-allele was associated with decreased PFS, both overall and in the *KRAS* wild-type subgroup (HR=1.56, 95%CI=1.14-2.15 and HR=1.57, 95%CI=1.06-2.34, respectively) and decreased incidence of grade 2-3 skin toxicity (OR=0.48, 95%CI=0.24-0.94). The *EGFR*≥20 genotype was associated with decreased PFS, both overall and in the *KRAS* wild-type subgroup (HR=1.60, 95%CI=1.17-2.19 and HR=1.58, 95%CI=1.06-2.35, respectively). The *FCGR3A* and *EGFR* polymorphisms were not associated with PFS in the no-cetuximab arm. In *KRAS* mutated patients, the *EGF*61G-allele was associated with decreased PFS in the cetuximab arm, and increased PFS in the no-cetuximab arm (HR=2.22, 95%CI=1.24-3.96 and HR=0.59, 95%CI=0.36-0.98, respectively).

Conclusion

EGFR, *FCGR3A* and *EGF* polymorphisms are associated with PFS in mCRC patients treated with cetuximab, bevacizumab and chemotherapy. Confirmation is needed before these markers could be applied clinically.

Introduction

Cetuximab is an IgG_1-type chimeric monoclonal antibody that targets the epidermal growth factor receptor (EGFR). Its principal mechanism of action is the inhibition of ligand induced EGFR activation, resulting in reduced cell proliferation, cell survival and angiogenesis. Also, cetuximab may induce antibody-dependent cell-mediated cytotoxicity (ADCC) by recruitment of immune effector cells.[1]

Cetuximab is effective in patients with chemotherapy-refractory metastatic colorectal cancer (mCRC).[2,3] A modest clinical benefit was shown for cetuximab when added to first-line chemotherapy.[4-6] Recently, it has been demonstrated that the efficacy of cetuximab is limited to patients with wild-type *KRAS* tumors.[7,8] However, the *KRAS* mutation status does not completely predict the response to cetuximab and other tumor characteristics such as *BRAF* mutation status have been investigated.[9,10] The severity of acneiform skin rash is also associated with the efficacy of cetuximab[2,3], but as this adverse event occurs after therapy has started, it cannot be used to predict response before start of treatment. Therefore, additional predictive markers are needed to better identify patients who will benefit from cetuximab.

Germline polymorphisms in genes involved in the mechanism of action of cetuximab have been investigated previously.[11-14] A CA-repeat polymorphism in intron 1 of the *EGFR* gene and the single nucleotide polymorphisms (SNPs) *EGF* c.61A>G, cyclin D1 (*CCND1*) c.932G>A and fragment-C gamma receptors 2A (*FCGR2A*) c.535A>G and 3A (*FCGR3A*) c.818A>C have previously been associated with the efficacy of cetuximab in chemotherapy-refractory mCRC patients who were treated with cetuximab either as monotherapy[11,12] or in combination with irinotecan.[13,14] However, these findings have been investigated in relation to *KRAS* mutation status in only one small study.[14] Furthermore, these former studies were hypothesis generating, and lacked a control group.

To provide more robust data, we investigated the associations of these germline polymorphisms in combination with *KRAS* mutation status with the efficacy of cetuximab in a large cohort of mCRC patients who were treated in first-line with capecitabine, oxaliplatin, bevacizumab and cetuximab and included a control group treated with the same regimen but without cetuximab.

Materials and methods

Study population

Blood samples were collected from 576 of 755 previously untreated mCRC patients who participated in a multicenter prospective, randomized phase III study and were treated with capecitabine, oxaliplatin and bevacizumab or the same regimen plus

cetuximab, the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG).[15,16] Patient eligibility criteria are described in detail elsewhere.[15] Patients were stratified according to prior adjuvant chemotherapy, serum LDH, number of affected organs and per institution. Membrane expression of EGFR in the tumor was not required.

Cetuximab was administered intravenously at a dose of 400 mg/m^2 on the first day, followed by 250 mg/m^2 weekly thereafter. Dose reductions were carried out according to the study protocol. The duration of a treatment cycle was three weeks. Treatment was continued until disease progression, death or unacceptable toxicity, whichever occurred first.

The collection of a peripheral blood sample for pharmacogenetic research was pre-specified in the study protocol and required additional written informed consent. The protocol was approved by the local institutional review boards of all participating centers.

Clinical evaluation and toxicity criteria

Progression-free survival (PFS) was calculated using tumor response assessments every three cycles by CT scan according to RECIST 1.0 criteria.[15] PFS was defined as the interval from the date of randomization to the date of disease progression, death, or last follow-up, whichever occurred first. Toxicity was scored according to the National Cancer Institute Common Toxicity Criteria version 3.0. Cetuximab-related skin toxicity was defined as any skin toxicity with the exception of hand-foot syndrome.

Analysis of genetic variants

Germline DNA was isolated from peripheral white blood cells by the standard manual salting-out method. Genotyping was performed on a TaqMan 7500 (Applied Biosystems, Foster City, CA, USA) with pre-designed assays for *EGF* c.61A>G (rs4444903), *CCND1* c.932G>A (rs9344; also referred to as 870G>A), *FCGR2A* c.535A>G (rs1801274; resulting in amino-acid change of histidine to arginine at position 131) and *FCGR3A* c.818A>C (rs396991; resulting in amino-acid change of phenylalanine to valine at position 158), according to the manufacturer's protocol. Negative controls (water) were included. In addition, genotypes were confirmed on the Biomark (Fluidigm, South San Francisco, CA, USA) according to the protocol provided by the manufacturer using the same TaqMan assays. The *FCGR3A* polymorphism was also analyzed by Pyrosequencing for 15% of the samples, which confirmed the Taqman results.

The *EGFR* $(CA)_n$ polymorphism was analyzed by fragment analysis. Briefly, 10 ng of DNA was PCR amplified using primers FAM-5'-CCAAAATATTAAACCTGTCTT-3' and 5'-AACCAGGGACAGCAATCC-3'. PCR products were run on an ABI PRISM® 3730xl Analyzer and analyzed with Genemapper v3.5 software (Applied Biosystems). Plasmids with an *EGFR* insert containing 14 to 21 CA-repeats were used as a control.[17] For the purpose of this analysis, the *EGFR* CA-repeat polymorphism was dichotomized

according to the criterion applied by Zhang and colleagues.[11] Patients with two alleles containing less than 20 CA-repeats were designated 'EGFR<20', whereas patients with either one or two alleles with 20 CA-repeats or more were designated as 'EGFR≥20'.

All genotype frequencies were in Hardy-Weinberg equilibrium.

The *KRAS* mutation status was determined in patients from whom primary tumor tissue was available. Tumor DNA was extracted and *KRAS* mutation status was analyzed using a commercially available real-time PCR-based assay (DxS, Manchester, UK) and by direct sequencing.[18]

Statistical analysis

The primary objective was to assess the association of the *EGFR*, *EGF*, *CCND1*, *FCGR2A* and *FCGR3A* polymorphisms with PFS according to *KRAS* mutation status in mCRC patients treated with cetuximab added to chemotherapy and bevacizumab. The secondary objective was to assess the association between these polymorphisms and cetuximab-related skin toxicity (grade 0-1 versus 2-3).

The PFS of each polymorphism was analyzed per treatment arm. Survival curves were estimated using the Kaplan-Meier method. The hazard ratios and 95% confidence intervals (95%CI) were estimated using a multivariate Cox proportional hazards model per treatment arm, using the most appropriate of a dominant or recessive model. The effects of the genotypes were assessed with the wild-type genotype as the reference, as this is the most frequent and therefore 'normal' genotype. Since age (<65 versus ≥65 years) and gender potentially affect the influence of a genetic polymorphism[19], these factors were included in the multivariate analysis in addition to serum LDH (normal versus abnormal), which was an independent prognostic factor in the CAIRO2 study.[15] For the analysis of *KRAS* wild-type and mutant combined, *KRAS* mutation status was added to the multivariate model (wild-type versus mutant).

For patients in cetuximab arm, the association between the genotype and cetuximab-related skin toxicity (grades 0-1 versus 2-3) was analyzed and odds ratios (ORs) and 95%CIs were estimated using a univariate logistic regression model.

A Predictive Score for PFS was generated by assessing the interaction between treatment arm and previously published baseline prognostic variables for mCRC in a multivariate Cox proportional hazards model. Baseline prognostic factors for PFS were identified from a Medline search for original articles on clinical trials of mCRC patients who were treated with first-line chemotherapy.[20-25] Factors that were significantly associated ($p<0.05$) with PFS in a multivariate analysis including treatment arm were considered prognostic factor, and the cut-off values from these studies were used subsequently. Prognostic factors for OS were not included because these could also be related to subsequent lines of treatment. The resulting baseline prognostic variables were gender, age (<65 vs. ≥65 years), performance status (0 vs. 1), number of organs involved (1 vs. >1), LDH (normal vs. above normal), alkaline phosphatase

(normal vs. above normal), prior adjuvant chemotherapy (yes vs. no), white blood cell count (<8 000 vs. ≥8 000 cells per µL), hemoglobin (<11 vs. ≥ 11 g/dL) and total bilirubin (normal vs. above normal).[20-25] Additionally, the interaction terms of treatment arm and *KRAS* mutation status and the polymorphisms in *CCND1*, *EGFR*, *EGF*, *FCGR2A* and *FCGR3A* were included. Using the resulting Cox proportional hazards model, the regression coefficients of the significant interaction terms were converted into a Partial Score analogous to the method used by Chow and colleagues.[26] By using the regression coefficients of the interaction term instead of the regression coefficient of the variable itself, correction took place for cetuximab-unrelated prognostic value of the variable. A Predictive Score for a given patient was obtained by the sum of the Partial Scores.

All statistical analyses were performed using the Statistical Analysis Software version 9.1 (SAS Inc. Bethesda, Maryland, USA).

Results

Study population

Germline DNA was obtained from 576 included patients, of which 564 received the allocated treatment (282 in each arm). The baseline clinical characteristics, *KRAS* mutation status, median PFS and OS, and the incidence of cetuximab-related skin toxicity of these patients were not statistically significant different from the 172 patients of whom no blood sample was available (Table 1).

Association with outcome in the cetuximab arm

Progression free survival

KRAS wild-type patients

In the cetuximab arm, patients who were carriers of the *FCGR3A* C-allele (AC and CC genotypes combined) had a significantly decreased PFS compared with patients with the *FCGR3A* AA genotype (median PFS, 8.2 versus 12.8 months, respectively; HR 1.57; 95%CI 1.06 to 2.34; P=.025, table 2). Patients in the cetuximab arm with the *EGFR*<20 genotype had significantly decreased PFS compared with patients with the *EGFR*≥20 genotype (median PFS, 7.6 versus 12.4 months, respectively; HR 1.58; 95%CI 1.06 to 2.35; P=.024, table 2). The other polymorphisms were not significantly associated with PFS.

KRAS mutant patients

In the cetuximab arm, patients who were carriers of the *EGF* G-allele (AG and GG genotypes combined) had a significantly decreased PFS compared with patients with the *EGF* AA genotype (median PFS, 7.4 versus 13.3 months, respectively; HR 2.22; 95%CI 1.24 to 3.96; P=.007, table 3). The other polymorphisms were not significantly associated with PFS.

Table 1 Baseline clinical and demographic characteristics and clinical outcome of metastatic colorectal cancer patients treated with first-line capecitabine, oxaliplatin and bevacizumab only (arm A) or with the addition of cetuximab (arm B)

		Patients included in the pharmacogenetic analysis		Patients with no DNA available		
		Arm A (n = 282)	Arm B (n = 282)	Arm A (n = 86)	Arm B (n = 86)	P value[1]
Baseline characteristics						
Age – yr	Median	62	63	60.5	60.0	0.19
	Range	31-83	33-80	27-77	33-78	
Gender – no. (%)	Male	159 (56%)	180 (64%)	46 (53%)	53 (62%)	0.55
	Female	123 (44%)	102 (36%)	40 (47%)	33 (38%)	
KRAS mutation status – no. (%)	*KRAS* wild-type	119 (42%)	127 (45%)	36 (42%)	32 (37%)	0.23
	KRAS mutant	83 (29%)	69 (24%)	25 (29%)	29 (34%)	
	Missing	80 (28%)	86 (30%)	25 (29%)	25 (29%)	
Outcome parameters						
Progression-free survival – months	Median	10.8	10.1	9.9	7.9	0.09
	95% CI	9.7-12.5	8.6-11.0	8.4-13.4	6.7-10.5	
Overall survival – months	Median	20.4	20.5	17.9	15.6	0.08
	95% CI	17.8-24.9	18.7-22.1	15.6-31.1	11.7-20.3	
Overall worst grade cetuximab-related skin toxicity – no. (%)	grade 0 – 2	280 (99%)	207 (73%)	86 (100%)	67 (78%)	0.39
	grade 3	2 (1%)	75 (27%)	0 (0%)	19 (22%)	

[1] Comparison between patients included in the pharmacogenetic analysis versus patients not included in the pharmacogenetic analysis.

Table 2 Analysis of progression-free survival in *KRAS* wild-type patients

	Arm A[1]					Arm B[2]				
	n	median PFS[3]	HR[4]	95% CI	P	n	median PFS[3]	HR[4]	95% CI	P
FCGR3A 818A>C										
AA	55	11.7	1.00			57	12.8	1.00		
AC or CC	63	10.1	1.05	0.69-1.58	.832	65	8.2	**1.57**	**1.06-2.34**	**.025**
FCGR2A 535A>G										
AA	33	9.4	1.00			34	12.6	1.00		
AG or GG	84	12.7	0.69	0.44-1.09	.111	92	10.0	1.50	0.96-2.36	.076
EGFR CA-repeat										
EGFR<20	54	10.7	1.00			72	12.4	1.00		
EGFR≥20	63	11.7	1.10	0.73-1.67	.649	51	7.6	**1.58**	**1.06-2.35**	**.024**
EGF 61A>G										
AA	51	10.1	1.00			50	10.8	1.00		
AG or GG	66	12.5	0.67	0.44-1.03	.067	70	10.3	1.00	0.67-1.49	.999
CCND1 870G>A										
GG	39	9.6	1.00			33	12.7	1.00		
GA	54	9.7	0.93	0.57-1.50	.752	58	9.6	1.14	0.70-1.84	.603
AA	26	15.1	0.67	0.36-1.23	.193	34	9.9	1.53	0.91-2.57	.112

[1] arm A: capecitabine, oxaliplatin and bevacizumab
[2] arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
[3] median progression free survival (PFS) in months
[4] Covariates included in the multivariate model were age, gender and serum LDH. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

Table 3 Analysis of progression-free survival in *KRAS* mutant patients

	Arm A[1]					Arm B[2]				
	n	median PFS[3]	HR[4]	95% CI	P	n	median PFS[3]	HR[4]	95% CI	P
FCGR3A 818A>C										
AA	29	11.5	1.00			25	10.4	1.00		
AC or CC	52	12.5	0.77	0.46-1.28	.311	42	7.9	1.61	0.92-2.82	.097
FCGR2A 535A>G										
AA	22	12.9	1.00			23	8.1	1.00		
AG or GG	60	12.5	0.99	0.57-1.73	.985	46	9.7	1.32	0.74-2.33	.346
EGFR CA-repeat										
EGFR<20	42	10.8	1.00			29	12.1	1.00		
EGFR≥20	40	12.7	0.63	0.38-1.03	.068	38	7.4	1.71	0.98-2.98	.060
EGF 61A>G										
AA	35	10.6	1.00			29	13.3	1.00		
AG or GG	46	13.6	**0.59**	**0.36-0.98**	**.041**	39	7.4	**2.22**	**1.24-3.96**	**.007**
CCND1 870G>A										
GG	23	12.1	1.00			21	7.4	1.00		
GA	40	11.5	0.93	0.52-1.68	.816	36	9.5	1.07	0.58-1.99	.821
AA	20	13.1	0.76	0.40-1.46	.413	12	7.1	1.33	0.54-3.27	.528

[1] arm A: capecitabine, oxaliplatin and bevacizumab
[2] arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
[3] median progression free survival (PFS) in months
[4] Covariates included in the multivariate model were age, gender and serum LDH. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

KRAS wild-type and mutant combined

When the associations were assessed in the entire cohort without subdivision by *KRAS* mutation status but with *KRAS* mutation status as a covariate, patients who were carriers of the *FCGR3A* C-allele had a significantly decreased PFS compared with patients with the *FCGR3A* AA genotype (median PFS, 7.8 versus 12.1 months, respectively; HR 1.56; 95%CI 1.14 to 2.15; P=.006, table 4). Also, patients with the *EGFR*<20 genotype had a significantly decreased PFS compared with patients with the *EGFR*≥20 genotype (median PFS, 8.8 versus 10.8 months, respectively; HR 1.60; 95%CI 1.17 to 2.19; P=.003, table 4). The other polymorphisms were not significantly associated with PFS. *KRAS* mutation status was not significantly associated with PFS in the multivariate analyses. There was significant interaction between treatment arm and the *FCGR3A* and *EGFR* polymorphisms (P=.015 and P=.009, respectively).

In figure 1A and 1B, the PFS curves for the cetuximab arm are shown for *KRAS* mutation status combined with the *EGFR* and *FCGR3A* polymorphisms, respectively.

Cetuximab-related skin toxicity

In the overall cetuximab arm (i.e. not subdivided by *KRAS* mutation status), patients who were carriers of the *FCGR3A* C-allele had significantly decreased incidence of grade 2-3 cetuximab related skin toxicity compared with patients with the *FCGR3A* AA genotype (OR, 0.46; 95%CI 0.27 to 0.78; table 5). In the multivariate analysis including age, gender, *KRAS* mutation, and serum LDH, the *FCGR3A* polymorphism remained associated with the incidence of grade 2-3 skin toxicity (OR, 0.48; 95%CI 0.24 to 0.94). The other polymorphisms were not significantly associated with cetuximab related skin toxicity.

Association with outcome in the no-cetuximab arm

In the no-cetuximab arm, *KRAS* mutant patients who were carriers of the *EGF* G-allele had significantly increased PFS compared with patients with the *EGF* AA genotype (median PFS, 13.6 versus 10.6 months, respectively; HR 0.59; 95%CI 0.36 to 0.98; P=.041, table 3). The other polymorphisms were not significantly associated with PFS.

Predictive Score for PFS

The variables that showed significant interaction with treatment for the prediction of PFS were: gender (regression coefficient, 0.56), white blood cell count (WBC <8 000 vs. ≥8 000 cells per µL; regression coefficient, 0.44) and the *FCGR3A* polymorphism (AA genotype versus C-allele carriers; regression coefficient, 0.58). *KRAS* mutation status showed no significant interaction with treatment arm (regression coefficient, 0.06). A score of one point was awarded to each of the following parameters: females, *FCGR3A* C-allele carriers and patients with ≥8 000 WBCs per µL. By summarizing the Partial Scores, a Predictive Score per patient was derived, which ranged from 0 to 3. The 32

Table 4 Analysis of progression-free survival in all patients, regardless of *KRAS* mutation status

	Arm A[1]					Arm B[2]				
	n	median PFS[3]	HR[4]	95% CI	P	n	median PFS[3]	HR[4]	95% CI	P
FCGR3A 818A>C										
AA	119	10.7	1.00			112	12.1	1.00		
AC or CC	157	10.8	0.92	0.67-1.27	.630	158	7.8	**1.56**	**1.14-2.15**	**.006**
FCGR2A 535A>G										
AA	73	10.8	1.00			84	9.8	1.00		
AG or GG	204	11.3	0.81	0.57-1.14	.233	193	10.2	1.40	0.98-1.98	.062
EGFR CA-repeat										
EGFR<20	143	10.7	1.00			141	10.8	1.00		
EGFR≥20	134	11.7	0.88	0.65-1.21	.435	129	8.8	**1.60**	**1.17-2.19**	**.003**
EGF 61A>G										
AA	117	10.1	1.00			111	10.2	1.00		
AG or GG	161	12.5	**0.65**	**0.47-0.89**	**.008**	160	10.1	1.26	0.91-1.74	.158
CCND1 870G>A										
GG	83	9.7	1.00			80	10.4	1.00		
GA	137	10.6	0.91	0.63-1.31	.610	138	9.7	1.14	0.79-1.66	.485
AA	62	13.5	0.69	0.44-1.06	.092	62	9.7	1.52	0.99-2.33	.056

[1] arm A: capecitabine, oxaliplatin and bevacizumab
[2] arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
[3] median progression free survival (PFS) in months
[4] Covariates included in the multivariate model were age, gender, serum LDH and KRAS mutation status. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

Figure 1A Progression-free survival for the *EGFR* CA-repeat polymorphism and *KRAS* mutation status for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab

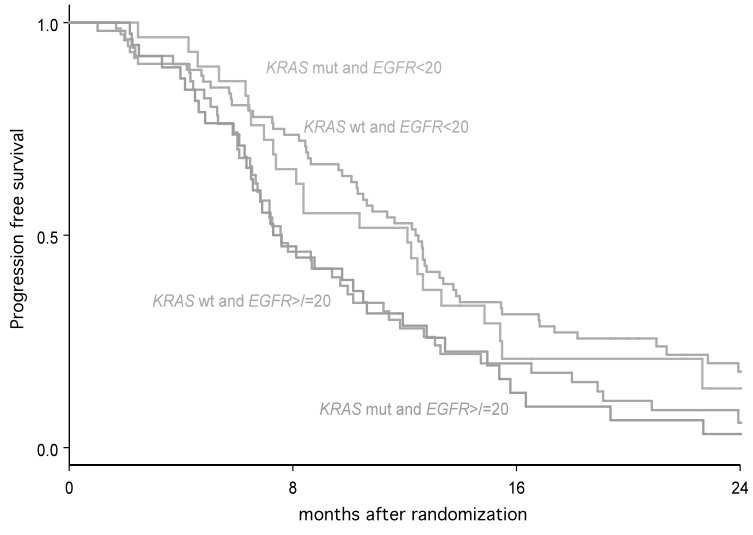
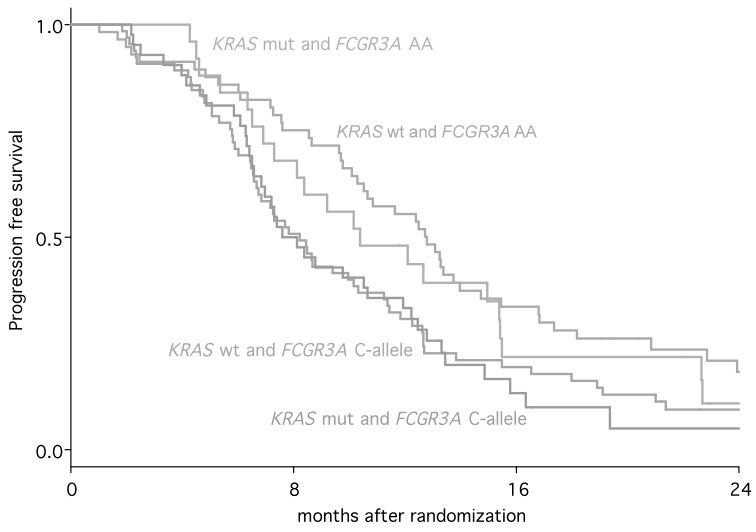


Figure 1B Progression-free survival for the *FCGR3A* 818A>C polymorphism and *KRAS* mutation status for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab



patients with a Predictive Score of 0 in the cetuximab arm had significantly improved PFS compared with all patients in the no-cetuximab arm (median PFS 15.4 versus 10.8 months, respectively; HR 0.61; 95%CI 0.39 to 0.95). Grouping of patients with a Predictive Score of 0 and 1 (a total of 142 patients) led to a non-significant improvement of PFS for the cetuximab arm compared with the no-cetuximab arm (HR 0.83; 95%CI 0.66 to 1.05).

Table 5 Analysis of the incidence of grade 2-3 cetuximab-related skin toxicity for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab

	OR[1]	95%CI	P
FCGR3A 818A>C			
AA	1.00		
AC or CC	**0.46**	**0.27 to 0.78**	**.005**
FCGR2A 535A>G			
AA	1.00		
AG or GG	1.66	0.97 to 2.84	.062
EGFR CA-repeat			
EGFR<20	1.00		
EGFR≥20	1.11	0.67 to 1.84	.693
EGF 61A>G			
AA	1.00		
AG or GG	1.28	0.76 to 2.13	.351
CCND1 870G>A			
GG	1.00		
GA	1.21	0.67 to 2.17	.535
AA	0.86	0.43 to 1.73	.679

[1] Odds ratios (OR), 95% confidence intervals (95% CI) and P values were calculated from the logistic regression model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor

Discussion

We demonstrate that the *FCGR3A* 818C-allele and the *EGFR*≥20 genotype were associated with a decreased PFS in a large group of *KRAS* wild-type mCRC patients treated with cetuximab, bevacizumab and chemotherapy in a randomized trial, compared with patients with the *FCGR3A* 818AA or *EGFR*<20 genotype, respectively. Moreover, the predictive role of these polymorphisms appears to be independent of *KRAS* mutation status. *KRAS* mutant patients who carried the *EGF* 61G-allele had shorter PFS when treated with cetuximab, bevacizumab and chemotherapy, and longer PFS when treated with bevacizumab and chemotherapy alone, compared with patients with the *EGF* 61AA genotype. Patients who carried the *FCGR3A* 818C-allele had decreased risk of cetuximab related skin toxicity, compared with patients with the *FCGR3A* 818AA genotype.

Bibeau and colleagues recently also reported that the *FCGR3A* polymorphism is independent of *KRAS* mutation status. However, in their study patients who were homozygous for the C-allele had longer PFS compared with carriers of the A-allele[14], which is not in agreement with our data. In one other previous study, the *FCGR3A* C-allele was also associated with decreased PFS in previously pretreated mCRC patients who were treated with cetuximab as a single agent[12], though this was not confirmed in an extended analysis of this study with more patients.[27] This indicates that the earlier association could have been a false positive finding, making it not suitable for comparison with our study. Another study with 110 patients who received cetuximab monotherapy as salvage treatment for mCRC did also not find a significant association between the *FCGR3A* polymorphism and the efficacy of cetuximab.[13]

A possible mechanism for the opposite association of the *FCGR3A* polymorphism could be that the high affinity C-allele[28-30] results in increased activation of tumor associated macrophages (TAMs) by cetuximab through cross-linking of the Fc gamma receptor[31], instead of increasing ADCC in our study. As a result of TAM activation, pro-angiogenic mediators are released in the tumor microenvironment, such as VEGF and matrix metalloproteinases (MMPs).[32,33] In our study, patients had not received palliative chemotherapy before, whereas patients in the other studies had been exposed to irinotecan and/or other lines of chemotherapy prior to cetuximab[12-14,27], which could have altered the infiltration of cells of the myeloid lineage, such as TAMs.[34] However, it must be noted that the *FCGR3A* C-allele was associated with increased efficacy of the IgG_1-type monoclonal antibodies rituximab in lymphoma[35,36] and trastuzumab in advanced breast cancer.[37] Therefore, fundamental research should be performed to support our highly speculative hypothesis. However, because this is an extracellular mechanism, and therefore independent of intracellular KRAS signaling, it explains why the effect of the *FCGR3A* polymorphism was independent of *KRAS* mutation status.

Our finding that patients with a lower number of CA-repeats for the *EGFR* polymorphism experience longer PFS is in line with the study by Graziano and colleagues[13], even though the categorization of genotypes was different. However, another study did not find a significant association between this EGFR polymorphism and PFS in cetuximab treated mCRC patients[27].

The biological mechanism for the association of the *EGFR* polymorphism is concordant with the finding that patients with the *EGFR*≥20 genotype had shorter PFS. Transcription of the *EGFR* gene is lower for increased number of CA-repeats[38]. Although EGFR expression, as measured by immunohistochemistry, is not a predictor of the efficacy of cetuximab,[39,40] the number of *EGFR* gene copies is associated with the response to cetuximab treatment[41].

It would be expected that the *EGFR* CA-repeat polymorphism is only associated with PFS in *KRAS* wild-type patients, because EGFR is upstream of KRAS. Since VEGF expression is regulated by the EGFR pathway, a role of the *EGFR* polymorphism in the response to cetuximab in combination with bevacizumab cannot be excluded.

In our study, patients who carried the *EGF* G-allele had increased PFS. In two other studies, the G-allele was associated with decreased PFS in advanced colorectal cancer patients treated with cetuximab[13,27].

Skin toxicity is a major side effect of cetuximab treatment and the severity of skin toxicity is associated with the response to cetuximab,[5,42] but the underlying mechanism is not yet unraveled. Since we demonstrate a relationship between the *FCGR3A* polymorphism with the incidence of grade 2-3 skin toxicity, the involvement of immune effector cells is likely. Unexpectedly, we did not confirm previous findings that a lower number of CA-repeats is associated with increased incidence of skin toxicity during anti-EGFR therapy.[13,43] However, previous findings could have been biased by the correlation between the response to anti-EGFR therapy and the incidence of skin toxicity.

Even though the previous pharmacogenetic studies on cetuximab have used peripheral blood[12,13], normal tissue[14] or tumor tissue[27], this should not have influenced the results, because there is an almost perfect degree of concordance between germline genotype in tumor and normal tissue.[44]

Importantly, the polymorphisms in *FCGR3A* and *EGFR* are only predictive for the efficacy of cetuximab and do not influence the PFS in patients not treated with cetuximab.

Biomarker- and genetic association studies are hampered by divergent and inconsistent results.[45] Retrospective pharmacogenetic studies must therefore be interpreted as hypothesis generating that require confirmation in an independent cohort.

Although our large study was set up to confirm previously published associations and included a control group[11-14], the results are conflicting and therefore remain inconclusive. It is likely that heterogeneity among the different studies, such as the

stage and nature of the disease, previous treatment and concomitant medication may explain the discordance. These variables should therefore be carefully considered in retrospective biomarker studies, as these factors probably have large influence on the results.

Apart from rare examples such as *KRAS*, a biomarker usually identifies subsets of patients with relatively higher or lower risk of response. Therefore, a predictive model should be developed with genetic and other predictive factors, such as the model we present. Such models can be clinically applied only after confirmation in a prospective trial.[45,46]

In conclusion, we demonstrate that germline polymorphisms in *FCGR3A*, *EGFR* and *EGF* are associated with the efficacy of cetuximab. Due to inconsistent results among studies, our results require confirmation before they can be applied in clinical practice.

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