



Fc gamma receptor polymorphisms as predictive markers of Cetuximab efficacy in epidermal growth factor receptor downstream-mutated metastatic colorectal cancer

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Abstract *Background:* The immunoglobulin G1 (IgG₁) monoclonal antibody (MoAb) Cetuximab is active in metastatic colorectal cancer (mCRC) as first or subsequent lines of therapy. Efficacy seems restricted to KRAS wild-type tumours. IgG₁ may also induce antibody dependent cell mediated cytotoxicity (ADCC) by recruitment of immune effector cells. ADCC is influenced by Fc gamma receptor (FcγR) polymorphisms. We investigated the association of FcγR polymorphisms and disease control rate (DCR) in mCRC patients treated with chemotherapy plus Cetuximab.

Patients and methods: Tumour tissues from 106 patients were screened for KRAS codon 12 and 13 mutations using a sensitive multiplex assay (DxS, Manchester, United Kingdom). NRAS (codons: 12, 13 and 61), PI3K (exon 20) and BRAF (exon 15) were analysed by direct sequencing. Fcγ RIIa and Fcγ RIIIa polymorphisms were genotyped by TaqMan assays.

Results: DCR was significantly higher in KRAS wild-type tumours (61% versus 39%, $p = 0.049$). In epidermal growth factor receptor (EGFR) downstream-mutated mCRC patients, those harbouring an FcγRIIa H/H genotype had a higher DCR than alternative genotypes (67% versus 33%, $p = 0.017$). By multivariate analysis, FcγRIIa-131H/H remained significantly correlated with DCR ($p = 0.008$).

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Conclusion: Fc γ R polymorphisms may play a role in the clinical efficacy of Cetuximab in EGFR downstream mutated mCRC patients. Further research into Cetuximab immune-based mechanisms in KRAS-mutated patients seems warranted.

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1. Introduction

Cetuximab, a chimeric IgG₁ monoclonal antibody (MoAb) that binds to the extracellular domain of epidermal growth factor receptor (EGFR), leads to inhibition of its downstream signalling, mainly the *RAS-RAF-MAPK* axis and the *PI3K-PTEN-AKT* pathway. Although Cetuximab has been extensively used in a wide variety of scenarios in metastatic colorectal cancer (mCRC)¹ only a small fraction of patients derive clinical benefit. Since the establishment of KRAS mutations as a major negative predictor of efficacy, additional biomarkers have emerged in an attempt to further empower the selection of patients eligible for this therapy.² Indeed, recent work has suggested that a comprehensive integrated analysis of several components of the pathway triggered by the EGFR is likely to enhance the prediction ability of a single marker individually.^{3–6}

The occurrence of KRAS mutations only accounts for about 30–40% of non-responsive patients, and some patients carrying KRAS mutated tumours have been reported to respond to Cetuximab.⁷ These findings strongly suggest that an oncogenic activation of EGFR downstream effectors do not completely accounts for the lack of benefit from anti-EGFR MoAbs. Alternative mechanisms involved in Cetuximab mediated antitumour efficacy, such as antibody-dependent cell mediated cytotoxicity (ADCC) have been suggested. ADCC is induced through the interaction of the Fc region of the MoAb with the Fc gamma receptor (Fc γ R), surface receptors for immunoglobulin G located on immune effector cells such as natural killer lymphocytes and macrophages.⁸ Cetuximab coats EGFR and binds to Fc γ R and this interaction triggers the activation and degranulation of the effector cells, resulting in the lysis of antibody coated cells. Specific polymorphic variants of genes encoding for the activating receptors Fc γ RIIa and Fc γ RIIIa provide different binding affinities to IgG₁ MoAbs and appear to be correlated with patients' outcome in a variety of diseases.^{9–11} However, conflicting data have been published in mCRC patients treated with Cetuximab for the allele with the best predictive ability for clinical response.^{9,12,13} In addition, few clinical data are so far available regarding the role of these polymorphisms in KRAS mutated patients.⁹ Moreover there is a growing body of preclinical evidence suggesting that KRAS mutations are not sufficient to render tumour cells resistant to ADCC and, importantly, that Fc-engineered EGFR MoAbs are able to induce lysis of KRAS mutated tumour cells via this immunological mechanism.^{14,15}

For these reasons, we attempted to elucidate the role of ADCC in a subset of mCRC patients with a downstream EGFR mutated phenotype, including mutations other than KRAS codon 12 and 13 with a previously reported predictive value in Cetuximab efficacy.

2. Patients and methods

2.1. Eligible patients

All patients in this study had histologically confirmed diagnosis of metastatic colon or rectum adenocarcinoma, with a performance status of 0–2 according to the WHO scale. Patients were treated with Cetuximab administered on an every-second week schedule at a dose of 500 mg/m² combined with standard chemotherapy. Tumour response was evaluated by CT-scan every three cycles of treatment according to RECIST criteria, and it was classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). Disease control rate (DCR) included patients with CR, PR or SD lasting >6 months. Relevant clinical data were obtained by retrospective chart review (gender, age, performance status, Köhne risk index, consolidative procedures). The local ethics committee approved the pharmacogenetic study protocol and all subjects signed an informed consent before participating in the study. All patients with tumour sample available were included and anonymised.

2.2. DNA extraction

Appropriate surgical specimens (paraffin blocks) were selected for each patient and 5 μ m thick sections were cut for molecular analysis, at least with 80–90% of tumour cells. Depending on the sample amount, three or more tissue sections were transferred into a micro centrifuge tube and 600 μ l of mineral oil was added and boiled for 2 min. After centrifugation at 12,000 rpm/min for 3 min at room temperature (RT), the supernatant was removed. This step was repeated once and then lyses buffer was added. After the last centrifugation, three layers were obtained, with the first two layers of supernatant being discarded.

DNA was extracted from the deparaffinised samples using the DNA extraction and purification kit, NucleoSpin[®] Tissue (Macherey-Nagel, Germany). The samples were processed according to the provided protocol. The purified DNA was finally eluted in a total

volume of 80–100 µl of elution buffer (according to tissue size processed initially). Yielded DNA was quantified by NanoDrop 3.0.0 (Nucliber, Wilmington, DE, United States of America). About 7–509 ng/µl of DNA was extracted and 40–200 ng of genomic DNA was used as template.

For the germline polymorphism detection DNA was isolated from peripheral white blood cells by the QIA-amp DNA Mini Kit (Qiagen-IZASA- Barcelona Spain), following manufacturer's instructions.

2.3. Somatic mutation analysis

Polymerase chain reaction (PCR)-amplified NRAS exon 2 and 3, BRAF exon 15 and PI3K exon 20 were processed. The purified PCR products were analysed by direct sequencing, BigDye v1.1 (ABI PRISM™ 3130XL DNA Sequencer for Applied Biosystems, Foster City, CA, United States of America). Primer sequences are provided in [Supplementary Table 1](#).

KRAS codon 12 (six possible changes) and 13 (one change) mutations were tested using a sensitive multiplex assay (DxS, Manchester, United Kingdom).

2.4. FcγRIIa-H131R and FcγRIIIa-V158F polymorphisms

The FcγRIIa and FcγRIIIa genotypes were determined using a TaqMan Allelic Discrimination Assay (Applied Biosystems code: C_9077561_20 and C_25815666_10, respectively) according to the manufacturer's instructions. Briefly, 40 ng of DNA and 6.25 µl of Taqman Universal PCR Master Mix were added to 12.5 µl of reaction containing forward and reverse primers along with two allele-specific labelled probes. After thermal cycling, the 7500 Applied Biosystems instrument determined the allelic content of each sample in the plate by reading the generated fluorescence using the v2.0.1 software. Samples with a known genotype were put on each reaction plate as a quality control.

2.5. Statistical analysis

The Chi-square test (χ^2) test was used to assess Hardy–Weinberg equilibrium. Fisher's exact and χ^2 tests as appropriate assessed the association between categorical parameters and polymorphism status.

The primary end-point of this pharmacogenetic study was to investigate the association between FcγR and DCR. Multivariate binary logistic regression analysis was used to determine the independent association between genetic markers and DCR. Age, sex, previous lines of chemotherapy and Köhne risk index as clinically significant variables were included in the regression analysis and the adjusted odds ratio (OR) with their 95% confidence interval (CI) were calculated.

In order to specifically investigate the role of ADCC in our patient's population we distinguish a mutated group consisting of all patients with an EGFR pathway downstream mutation. All of the mutations studied have been consistently correlated with a lack of response to anti-EGFR-MoAb.³ For statistical analysis, patients harbouring activating mutations in KRAS and other downstream components of the EGFR pathway (BRAF, NRAS and PI3K exon 20) were jointly analysed as the mutated group. Patients with a quadruple negative phenotype (lack of mutations in any of the above-mentioned genes) were termed as the wild-type group. The FcγRIIa-H131R and FcγRIIIa-V158F polymorphisms were only determined in the mutated group.

Statistical tests were conducted by SPSS software 15.0 version for Windows (SPSS Inc., Chicago). All *P* values were two-sided and were considered statistically significant when ≤ 0.05 was.

3. Results

One hundred and six mCRC patients (59 males, 47 females; median age, 59 years) treated in the pre-KRAS selection era (June 2001 to February 2007) with Cetuximab combined with standard chemotherapy in either first (31%) or second (69%) line therapy were retrospectively analysed ([Table 1](#)).

Forty-one per cent of the 106 patients ($n = 44$) harboured a KRAS mutation in codon 12 or 13 and, as expected, had a significantly lower DCR (39% versus 61%, $p = 0.049$) than patients with a wild-type KRAS ([Table 2](#)). Among the 62 wild-type KRAS patients, other EGFR pathway downstream mutations were detected in 3 patients (2 in BRAF exon 15 and 1 in NRAS exon 2). These 3 patients were subsequently included in the mutated group. Some samples were noted as 'unknown' because they could not be amplified due to a relatively frequent occurrence of PCR inhibitory substances in the samples as well as low DNA quality and quantity extracted from paraffin source. Nevertheless, sequencing analysis was available for more than 85% of the patients.

In accordance with recent reports,³ additional genotyping of BRAF, NRAS and PI3K exon 20 mutations in the KRAS wild type population further refined response prediction. DCR was 53% in the unselected population ($n = 106$), 61% in the KRAS wild-type codon 12 or 13 patients ($p = 0.049$) and 65% in the KRAS, BRAF, NRAS and PI3K exon 20 wild-type group ($p = 0.03$) ([Table 2](#)).

When the 47 patients of the mutated group were considered, DCR was 67%, 50% and 17% for those with FcγRIIa 131H/H, 131H/R and 131R/R genotypes, respectively ($p = 0.017$), ([Table 2](#)). Patients harbouring an FcγRIIa H-containing genotype had a DCR of 55.2% compared to 17.6% in those homozygous for the 131R allele ($p = 0.016$), data not shown. A tendency

Table 1
Patients characteristics (n = 106).

Median age (years; range)	59.5 (33–81)
Sex	n (%)
Female	47 (44)
Male	59 (56)
ECOG	
0	23 (22)
1	64 (60)
2	19 (18)
Köhne risk index	
Low	40 (38)
Intermediate	39 (37)
High	27 (25)
Number of metastatic sites	
1–2	85 (80)
>2	21 (20)
Line of therapy	
First line	34 (32)
Others lines	72 (68)
Chemotherapy scheme	
Irinotecan-based	25 (24)
Oxaliplatin-based	81 (76)

for a higher DCR was also observed for patients with the FcγRIIIa V-containing genotype (p = 0.08). When FcγRIIa and FcγRIIIa polymorphisms were combined, patients with any 131H and/or 158V allele had a

Table 3
Multivariate analysis in the mutated phenotype population. N = 44.^a

Factor	Adjusted odds ratio (95% CI)	LRT p-Value
FcγRIIa (HH + HR versus RR; 28 versus 16)	8.6 (1.7–42)	0.008
FcγRIIIa (VV + VF versus FF; 31 versus 13)	3.8 (0.5–26)	0.16
Köhne risk index (low + intermediate versus high; 34 versus 10)	16 (1.65–158.5)	0.017
Treatment line (first versus others; 13 versus 31)	6.3 (0.71–55.9)	0.09

^a Three samples could not be amplified due to frequent PCR inhibitory substances in samples as well as low DNA quality and quantity extracted from paraffin source.

significantly higher DCR (65% versus 35%, p = 0.014) (Table 2). As previously reported, linkage disequilibrium between FcγRIIIa-158V/V and FcγRIIIa-131H/H polymorphisms was found.¹⁶

A multivariate logistic regression model, adjusted for baseline characteristics, showed that Köhne risk index and FcγRIIIa-H131R were independent predictors of DCR in this population (Table 3).

Table 2
Polymorphism and somatic mutation analysed. Correlation with DCR.

		N (%)	Disease control rate (DCR)		p-Value
			Responder (CR + PR + SD >6 months) n (%)	Non-responder (SD <6 months) n (%)	
KRAS (codon 12 or 13)	Mutated	106 (100)	56 (53)	50 (47)	0.049
	Wild-type	44 (41.5)	18 (41)	26 (59)	
		62 (58.5)	38 (61)	24 (39)	
KRAS, BRAF, NRAS and PI3K ^a	Mutated (any)	47 (44)	19 (40)	28 (60)	0.029
	Wild-type	37 (35)	24 (65)	13 (35)	
	Unknown ^b	22 (21)			
Mutated phenotype. N = 47	Fcγ RIIa	N = 46 (100)			0.017
	H/H	12 (26)	8 (67)	4 (33)	
	H/R	16 (35)	8 (50)	8 (50)	
	R/R	18 (39)	3 (17)	15 (83)	
	FCy RIIIa	N = 44 (100)			0.08
	V/V + VF	31 (70)	16 (52)	15 (48)	
	F/F	13 (29)	3 (23)	10 (77)	
Fcγ RIIa + FcγRIIIa	N = 44 (100)			0.014	
Any H or any V	20 (45)	13 (65)	7 (35)		
R/R or F/F	24 (55)	6 (25)	18 (75)		

^a Considered mutated only when all the four genes could be analysed.

^b Not included in statistical analysis.

4. Discussion

To date it is widely accepted that activating mutations in KRAS gene abrogates the therapeutic effect of anti-EGFR MoAbs. However, a minority of patients harbouring a KRAS mutation achieves an objective response, whereas stable disease is reported in almost 50% of them.^{3,17} This is of particular importance, since clinical benefit is not confined to objective responders. Indeed, delaying disease progression may improve clinical symptoms and the patients' quality of life.¹⁸ Moreover, KRAS mutational status has not been consistently correlated with Cetuximab efficacy in recent trials^{19,20} suggesting that alternative molecular determinants of response have yet to be identified.

Several studies have attempted to correlate clinical outcome with FcγR polymorphisms known to modulate antibody-receptor engagement, with conflicting results. FcγRIIIa-158V/V wild type confers a higher binding affinity for IgG₁ and patients harbouring this genotype have shown superior responses to rituximab and herceptin.^{10,11} In contrast, FcγRIIIa-158F/F has been correlated with response and a longer progression free survival (PFS) in mCRC treated with Cetuximab^{12,13} whereas improved clinical outcomes have been reported by other authors in FcγRIIIa-158V/V carriers.⁹ In agreement with preclinical work,²¹ our results also point to a favourable role of FcγRIIIa-158V/V genotype. Interestingly, in our study, FcγRIIIa-131H/H emerges as an independent predictor of Cetuximab efficacy. The role of this polymorphism remains unclear, since it does not show differential IgG₁ affinity. Nonetheless, previous work suggested a higher response rate (29% versus 9%) with irinotecan plus Cetuximab in mCRC patients harbouring the FcγRIIIa-131H/H genotype, independent from KRAS status,⁹ although the difference was not statistically significant. The relatively small sample sizes, and the retrospective nature of most of the published trials, the use of different primary end-points (Overall Response Rate-ORR- or PFS) and the known linkage disequilibrium between FcγRIIIa-158V/V and FcγRIIIa-131H/H polymorphisms¹⁶ may contribute to the observed discrepancies. Finally, as recently reported, tumour infiltrating macrophages, where FcγRIIIa are preferentially expressed, seem to play a key role in restoring tumour immune surveillance in preclinical models.²² Although FcγR polymorphisms might shed light into the biological basis of clinical responses in patients carrying KRAS mutated tumours, an integrated molecular dissection of the patients' immune system may more precisely define the role of ADCC-mediated cytotoxicity in the efficacy of Cetuximab. Prospective analysis of the different types of FcγR and their expression profiles, SNPs in the gene and promoter region of the inhibitory FcγRIIb²³ expression of non-classical MHC antigens capable of inhibiting effectors lymphocytes, such as

HLA-G and HLA-E²⁴ or the functional status of NK cells, T cells, regulatory cell subsets or peripheral chemokines, among others, seem thus warranted.

Although ADCC induction appears convincing in *in vitro* models, some drawbacks have been raised regarding its applicability to cancer patients. ADCC has been shown to be markedly impaired due to the frequently observed NK cell dysfunction in patients with cancer,²⁵ especially those with advanced disease and a high tumour burden. In addition, metastatic cancer patients have a suppressed immune function owing to an increased production of immunosuppressive soluble mediators or to the myeloablative effects of chemotherapy.²⁶ Since CD56+ cells, mainly NK cells,²⁷ are involved in Cetuximab induced ADCC, this variable may influence the therapeutic effect independently of FcγR polymorphisms. This issue remains, however, controversial. NK cells may not necessarily be the most important effector cell population involved in ADCC activity. Indeed, recent work²⁸ has suggested that T cells are crucial for the antitumour effect of anti-EGFR MoAb, whereas NK cells function was irrelevant. In addition, although neutropenia is a frequent side-effect of chemotherapy, lymphopenia is rarely observed. Indeed, some chemotherapeutics may exhibit beneficial effects on the immune system, such as depletion of suppressive regulatory T cells²⁹ and induction of immunogenic cell death.³⁰ Finally, preclinical work has demonstrated that some cytotoxic drugs up-regulate EGFR expression and enhances Cetuximab-mediated ADCC in colon cancer cell lines independent from KRAS status.¹⁴ The DCR achieved in our cohort of mutated patients with the 131H/H genotype is higher than the one expected (49%) for patients harbouring an EGFR downstream signalling pathway mutation³ and seems to reinforce preclinical data suggesting that expression of an activating KRAS mutation in colon cancer cell lines correlates with a higher susceptibility to Cetuximab-mediated ADCC.¹⁴ If present data are confirmed, clinical enhancement of Cetuximab-mediated ADCC by NK cell stimulatory cytokines such as IL-2, IL-12 or IL-21 might be explored.³¹ Nevertheless, our results should be interpreted as hypothesis generating. Indeed, a recent international consortium trial in 472 mCRC patients found a lack of correlation between FcγR polymorphisms, ORR and survival times. Several issues should however be taken into account that may partly explain these different results, such as the KRAS mutational status (WT in 100% of the patients in the Geva et al. study³²), the use of ORR instead of DCR as a primary end-point and the inclusion of a more heavily pre-treated and chemorefractory population.

In conclusion, our results suggest a potential role for immune activation in this subset of patients that may partly explain variability of Cetuximab-mediated efficacy.

Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ejca.2012.01.007](https://doi.org/10.1016/j.ejca.2012.01.007).

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