Research Article

Cancer Immunology Research

Prospective Evaluation of Cetuximab-Mediated Antibody-Dependent Cell Cytotoxicity in Metastatic Colorectal Cancer Patients Predicts Treatment Efficacy

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Abstract

Cetuximab is a monoclonal antibody to the EGFR that induces antibody-dependent cell cytotoxicity (ADCC) through Fcγ receptors on immune cells. Although SNPs in genes encoding Fcγ receptors are functionally relevant to cetuximab-mediated ADCC in colorectal cancer, a direct correlation between *in vitro* ADCC and clinical response to cetuximab is not defined. We therefore enrolled 96 consecutive metastatic colorectal cancer (mCRC) patients at diagnosis in a study that assessed FcγR status and cetuximab-mediated ADCC. Patients carrying the FcγRIIa *H* alleles 131*H*/*H* and 131*H*/*R* had significantly higher ADCC compared with patients with the 131*R*/*R* alleles ($P = 0.013$). Patients carrying FcγRIIIa genotypes with the *V* alleles 158*V*/*V* and 158*V*/*F* displayed higher ADCC compared with patients carrying the 158*F*/*F* genotype ($P = 0.001$). Progression-free survival of patients with an FcγRIIIa 158*V* allele was significantly longer compared with patients carrying 158*F*/*F* ($P = 0.05$), whereas no significant difference was observed for overall survival. Twenty-eight of 50 mCRC patients with wild-type KRAS received cetuximab. The average ADCC-mediated killing was 30% of assay targets for patients who experienced cetuximab complete or partial response, 21% in patients with stable disease and 9% in patients with progressive disease. To characterize basal natural killer (NK) activity, cytotoxicity was evaluated in 39 of 96 mCRC patients. Patients who responded to first-line treatment had higher NK-cell cytotoxicity. Thus, although limited to this cohort of patients, *in vitro* cetuximab-mediated ADCC correlated with FcγR polymorphisms and predicted cetuximab responsiveness. *Cancer Immunol Res; 4(4); 366–74. ©2016 AACR.*

Introduction

Cetuximab (Erbitux; Merck) is a chimeric IgG1 monoclonal antibody with high affinity for the EGFR. It has now been approved in combination with chemotherapy as first- and second-line therapy in metastatic colorectal cancer (mCRC) patients with the wild-type (WT) version of the oncogene KRAS (1–3), and in monotherapy as a third-line treatment (4). Its principal mechanism of action is inhibition of EFGR signaling, resulting in reduced cell proliferation, cell survival, and angiogenesis. Also, cetuximab may induce antibody-dependent cell cytotoxicity (ADCC) by recruitment of immune effector cells (5–13) Allelic variation in the FcγRIIA and FcγRIIIa genes [FcγRIIa-*H*131*R* (rs1801274) and FcγRIIIa-*V*158*F* (rs396991), respectively] is reported to affect ADCC effectiveness (9, 14, 15), although the prognostic usefulness of FcγRIIa and IIIa genotypes is still controversial (16). Bibeau and colleagues showed that patients treated with cetuximab and irinotecan that carry FcγRIIa-131*H*/*H* and/or FcγIIIa-158*V*/*V* genotypes have longer progression-free survival (PFS) compared with patients carrying 131*R* and 158*F* alleles (5.5 vs 3.0 months; $P = 0.005$) independent of KRAS status (17). In addition, in mCRC patients with mutations downstream of the EGFR (such as mutated KRAS), those harboring FcγRIIa *H*/*H* alleles had a higher disease control rate than alternative genotypes (67% vs. 33%, $P = 0.017$). By multivariate analysis, FcγRIIa-131*H*/*H* was significantly correlated with disease control rate ($P = 0.008$). These data suggest that the mechanism of cetuximab effectiveness in KRAS-mutated patients is in part immune based (18–20). However, conflicting evidence has been reported on these issues (21, 22). Negri and colleagues found that patients with the FcγRIIIa-158*V*/*V* genotype have a significantly higher cetuximab-mediated ADCC, but could not establish a correlation between FcγR polymorphisms and response rate or time to progression after cetuximab-based therapy (23). As previously demonstrated, FcγRIIIa polymorphisms were significantly associated with response to anti–EGFR-based therapy in patients with KRAS-WT tumors; prognosis was unfavorable for patients carrying the FcγRIIIa-158*F*/*F* genotype, whereas prognosis was not affected by FcγRIIa polymorphisms (24). Natural killer (NK) cells exert an antibody-independent cytotoxic effect against cancer cells through NK receptors, including NKG2D,

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and killer inhibitory receptors (KIR; refs. 25–28). NKG2D is a receptor for different activating ligands overexpressed on cancer cells, whereas KIRs recognize MHC class I molecules; NK cells are also activated by the decrease in MHC class I molecules reported on cancer cells. These two mechanisms activate NK cells against tumor cells. In colorectal cancer, an extensive intratumoral infiltration of NK cells has been associated with a better prognosis (29). To investigate the predictive factors of clinical response in primary diagnosed mCRC, we performed a prospective evaluation of basal NK activity and *in vitro* cetuximab-mediated ADCC versus FcγRIIa-*H*131*R* and FcγRIIIa-*V*158*F* polymorphisms.

Materials and Methods

Patients

Ninety-six consecutive mCRC patients referred to the Division of Abdominal Medical Oncology of the National Cancer Institute (Naples, Italy) were enrolled into the study. Patient characteristics are shown in Table 1. Informed consent from each patient was sought. The research protocol number 52/09 was approved by the Human Ethical Committee of our institute. All patients underwent sequential standard chemotherapy and/or biologic therapies (bevacizumab, cetuximab, and panitumumab). Tumor response was evaluated every 3 months with computerized tomography scan and carcinoembryonic antigen according to the RECIST criteria and classified as complete response, partial response, stable disease, and progressive disease. The overall response rate was defined as complete response + partial response.

Cell culture

EGFR-expressing cells; HT29, a colon cancer cell line; and K562, an erythroleukemia cell line, were obtained from the National Cancer Institute's Developmental Therapeutics program (NCI DTP) approximately in 2009. Cell lines were grown in complete RPMI-1640 (BioWhittaker; Lonza) medium with the addition of 10% heat-inactivated FBS, 1% L-glutamine, and penicillin/streptomycin and cultured at 37°C in 5% CO_2 humidified atmosphere. Cell line identities were confirmed by short tandem repeat DNA typing at IDEXX BioResearch.

Human mononuclear cell cultures

Peripheral blood mononuclear cells (PBMC) from 96 mCRC patients were isolated at diagnosis by Ficoll-Paque Plus gradient centrifugation (GE Healthcare). Lymphokine-activated killer (LAK) cells were generated by culturing PBMCs in complete RPMI-1640 enriched with human IL2 (10 ng/mL) for 18 hours.

Characterization of FcγR polymorphisms

Genotypes of 148 unrelated healthy donors and 96 mCRC patients were evaluated. Genomic DNA was extracted from PBMCs using a Qiagen DNA extraction kit (Qiagen) according to the manufacturer's recommendations. Genotyping of FcγRIIa-*H*131*R* and the FcγRIIIa-*V*158*F* was done on genomic DNA by PCR in both forward and reverse directions. The primers to analyze FcγRIIa-*H*131*R* polymorphisms are forward 5′-GGAGAAACCATCATGCTGAG-3′ and reverse primer 5′-CAATTTTGCTGCTATGGGC-3′. The annealing temperature was 56°C. Purified PCR products (277 bp) were sequenced using the Big Dye terminators version 3.1 cycle sequencing Kit (Applied Biosystems) and the 3130 Genetic Analyzer (Applied Biosystems; refs. 30, 31).

To analyze the FcγRIIIa genomic sequence, the part of exon 4 that contains the polymorphic site at nt559 was amplified by PCR using the M13 forward primer 5′-TGTAAAACGACGGCCAG TTCATCATAATTCTGTCTTCT-3′ and the M13 reverse primer 5′-CAGGAAACAGCTATGA CCCTTGAGTGATGGTGATGTTCA-3′ (32, 33).

FcγRIIIa gene expression

FcγRIIIa gene expression was determined by quantitative real-time PCR. RNA (200 ng) extracted from PBMCs from 34 mCRC patients, carrying FcγRIIIa-158*V*/*V* ($n = 12$), *V*/*F* ($n = 14$), and *F*/*F* ($n = 8$), were reverse transcribed. The quantitative PCR reaction was conducted with 2 μL of cDNA in a 13.5 μL final volume mixture containing SYBR Green (Applied biosystems) and FcγRIIIa primers (sense, 5′-CCAAAAGCCACACTCAAAGAC-3′; antisense, 5′ACCCAGGTGGAAAGAA TGATG-3′). The quantity of FcγRIIIa mRNA in each sample was normalized to the relative quantity of Beta-glucuronidase (34).

ADCC assay

ADCC of LAK cells was evaluated by sulforhodamine B (SRB) assay (35). Target cells (HT29) were plated in a 96-well plate at 1×10^4 cells/well. Twenty-four hours later, human IL2-activated PBMCs (effectors) were added at a 10:1 effector:target (E:T) ratio in fresh medium, in the presence of cetuximab (10 μg/mL), or the rituximab (anti-CD20, 10 μg/mL), as negative control, or in the presence of staphylococcal enterotoxin B (SEB) as positive control. The specific cytolysis percentage was calculated using the following formula: Cytotoxicity (%) = [1 − (mean test optical density/mean optical density target)] × 100 (36, 37). Cetuximab-mediated ADCC is given by $\text{cytotoxicity}_{\text{with cetuximab}} - \text{cytotoxicity}_{\text{without cetuximab}}$. All experiments were performed in triplicate, and results were expressed as mean values ± SE. For 15 mCRC patients, ADCC was also conducted with the conventional 24-hour lactate dehydrogenase release experiment (CytoTox 96-Non-Radioactive Cytotoxicity Assay; Promega).

NK cytotoxicity assay

Direct NK-cell cytotoxicity was measured in 39 mCRC patients using K562 as target cells. Briefly, carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled K562 cells were incubated with LAK cells at 5:1 E:T ratios. After 4 hours, target cells were identified by 7-aminoactinomycin D uptake. All experiments were performed in triplicate, and results were adjusted for the rate of cell death in the absence of effector cells and for the NK-cell frequency in thawed PBMCs (38). The lymphocyte populations $CD3^-CD16^+CD56^+$ (NK) were determined by anti-CD3 PerCP-Cy5, anti-CD56 PE-Cy7, and anti-CD16 PE. Data acquisition was performed with a BD FACSCanto II Flow Cytometer, and data were analyzed using BD FacsDiva 6.1.3 (BD Biosciences).

Statistical analysis

Statistical analyses were performed using the MedCalc 9.3.7.0 and Excel software. A χ^2 test and one-way ANOVA were used. Progression-free survival (PFS) and overall survival (OS) were estimated using the Kaplan–Meier method. PFS and

OS were defined as the interval between the beginning of treatment and clinical progression, death, or last follow-up if disease had not progressed. The comparison between SRB assay and CytoTox 96 was performed using the Bland-Altman plot (39). The distributions of the FcγRIIa and FcγRIIIa genotypes were tested for the Hardy–Weinberg equilibrium. Differences were considered to be statistically significant at P values below 0.05.

Results

Study population features

Ninety-six consecutive mCRC patients referred to the Division of Abdominal Medical Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione "G. Pascale"-IRCCS-Napoli, were enrolled in the study (Table 1). The genotypic distributions and allelic frequencies of *H*131*R* for FcγRIIa and *V*158*F* for FcγRIIIa gene polymorphisms were analyzed in 96 patients and 148 control subjects (Table 2). Genotype frequencies were compatible with the Hardy–Weinberg equilibrium. FcγRIIa-*H*131*R* and FcγRIIIa-*V*158*F* were the most common genotypes. The *H*131*R* and *V*158*F* frequencies did not significantly differ between the control and study groups ($P = 0.207$ and $P = 0.970$, respectively). Although the *H*131 allele was more frequent in mCRC patients compared with healthy controls, the difference was not statistically significant ($P = 0.193$). FcγRIIIa polymorphism did not significantly differ between the controls and mCRC cases ($P = 0.665$).

Table 1. Patient characteristics ($N = 96$)

Sex	
Male	55 (57%)
Female	41 (43%)
Age, years	
Median	62
Range	(28–81)
Primary tumor location	
Colon	70 (73%)
Rectum	26 (27%)
Number of metastatic sites	
1	74 (77%)
2	17 (18%)
>2	5 (5%)
Pattern of metastatic disease	
Liver	68 (63%)
Lung	24 (22%)
Bone	3 (3%)
Peritoneum	6 (6%)
Other	6 (6%)
KRAS status	
WT	50 (52%)
MUT	43 (45%)
Missing	3 (3%)
First-line therapy	
CT	17 (18%)
CT + anti-VEGF	69 (72%)
CT + anti-EGFR	10 (10%)
Response rate to first line	
CR	8 (8%)
PR	32 (33%)
SD	43 (45%)
PD	8 (8%)
Missing	5 (5%)

Abbreviations: CR, complete response; CT; chemotherapy; MUT, mutant; PD, progressive disease; PR, partial response; SD, stable disease.

Table 2. Patient characteristics: genotypic distributions and allelic frequencies of FcγRIIa-H131R/FcγRIIIa-V158F

	mCRC *N* (%)	Controls *N* (%)	*P* value
FcγRIIa-H131R			
H/H	39 (40%)	47 (32%)	
H/R	41 (43%)	74 (50%)	
R/R	16 (17%)	27 (18%)	*0.207*
Allele			
H131	119 (62%)	168 (57%)	
R131	75 (38%)	128 (43%)	*0.193*
FcγRIIIa-V158F			
V/V	26 (27%)	38 (26%)	
V/F	47 (49%)	74 (50%)	
F/F	23 (24%)	36 (24%)	*0.970*
Allele			
V158	99 (51%)	150 (50%)	
F158	93 (49%)	146 (50%)	*0.665*

NOTE: *P* values are for the χ^2 test comparing mCRC and control groups.
Abbreviations: F, phenylalanine allele; FcγR, fragment c-γ receptor; H, histidine allele; R, arginine allele; V, valine allele.

Cetuximab-ADCC correlates with FcγR polymorphisms

IL2-activated PBMC (LAK) cells isolated from 96 consecutive mCRC patients were evaluated *in vitro* for cetuximab-mediated ADCC induced against HT29, an EGFR-positive human colon cancer cell line. A representative assay is shown in Fig. 1A–E. A progressive increase in ADCC is shown in HT29 cells in the presence of LAKs derived from patients carrying *H131R/F158F*, *H131R/V158F*, and *H131R/V158V* plus cetuximab (10 μg/mL; Fig. 1C). Patients carrying the V allele displayed higher ADCC activity.

Cetuximab-mediated ADCC results from all 96 studied patients are shown in Fig. 2. Patients carrying the 131*H* allele of FcγRIIa (131*H*/*H* and 131*H*/*R*) displayed significantly greater average ADCC scores (percentage of cetuximab-mediated ADCC value) of 25% (0%–76%) and 17% (0%–55%), respectively, compared with patients carrying FcγRIIa 131*R*/*R*

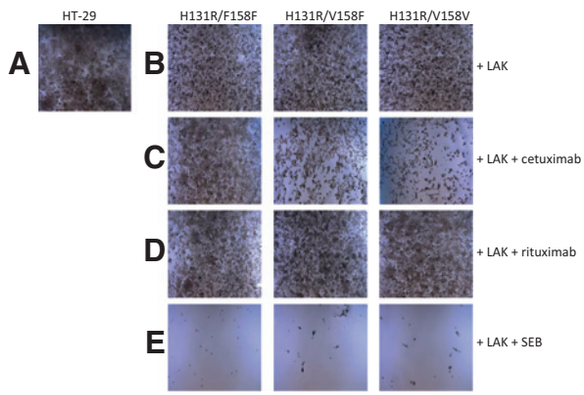

Figure 1.
Representative cetuximab-mediated ADCC assay on HT29 human colon cancer cells. The ADCC tests were performed at a 10:1 E:T ratio in the presence of cetuximab (10 μg/mL); rituximab, an unrelated monoclonal antibody (10 μg/mL); and SEB (staphylococcus toxin B), an inducer of ADCC. A, HT29 human colon cancer cells alone. B–E, HT29 cells in the presence of LAK cells derived from 3 patients carrying different genotypes, cultured alone (B) or with (C) cetuximab, (D) rituximab, or (E) SEB. Photographs of the SRB assay were taken using an optical microscope at ×40 magnification.

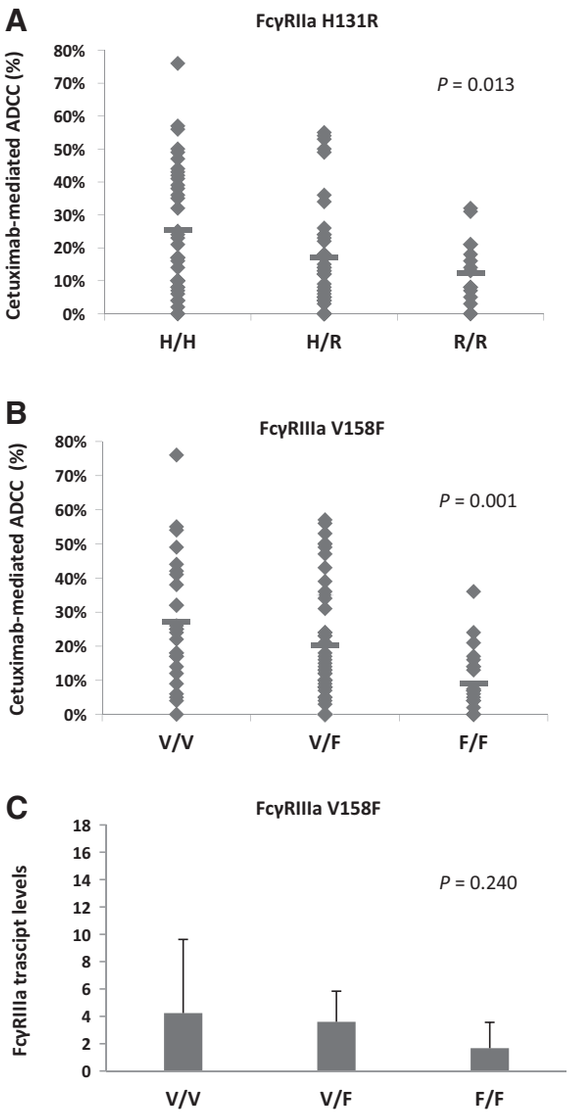

Figure 2.
Higher cetuximab-mediated ADCC in mCRC patients carrying H allele/FcγRII and V allele/FcγRIIIA. A, percentage of cetuximab-mediated ADCC by LAK cells relative to FcγRII. B, percentage of cetuximab-mediated ADCC by LAK cells relative to FcγRIII genotypes. C, FcγRIIIa mRNA expression.

genotype, with 12% (0%–32%; $P = 0.013$; Fig. 2A). The FcγRIIIa 158*V*/*V* and 158*V*/*F* genotypes were associated with higher cetuximab-mediated ADCC compared with 158*F*/*F*, 27% (0%–76%) and 20% (0%–57%) versus 9% (0%–36%), respectively ($P = 0.001$; Fig. 2B). Expression of different amino acid 158 alleles of FcγRIIIa was examined by mRNA transcript in 34 mCRC patients, which showed FcγRIIIa-158 *V*/*V* in 12, *V*/*F* in 14, and *F*/*F* in 8 patients. Though the FcγRIIIa transcript level was higher in patients with FcγRIIIa-158 *V*/*V* or *V*/*F*, compared with *F*/*F*, analysis of variance showed no significant difference ($P = 0.240$; Fig. 2C). SRB-mediated ADCC was compared with a standard assay, CytoTox96, in 15 patients and the concordance quantified through Bland–Altman plot. The data are within the limits of agreement (Supplementary Fig. S1).

FcγR polymorphisms and cetuximab-ADCC predict clinical response

Patients with mutant KRAS comprise almost 55% of the total population. Anti–EGFR-based therapies are indicated in patients with WT KRAS and comprise cetuximab or panitumumab. Thirty-two of 50 patients with WT KRAS were treated with anti–EGFR-based therapy. Twenty-eight patients were treated with cetuximab and four with panitumumab. In cetuximab-treated patients, the correlation between FcγR polymorphisms, *in vitro* cetuximab-mediated ADCC response, and their clinical response was evaluated. The characteristics of patients receiving cetuximab are shown in Table 3.

The objective response was significantly different between patients carrying the FcγRIIa 131*H* allele (*H*/*H* and *H*/*R* genotypes) compared with those with the *R*/*R* genotype ($P = 0.035$) and between patients carrying the FcγRIIIa 158 *V* allele (*V*/*V* and *V*/*F* genotypes) compared with the *F*/*F* genotype ($P = 0.025$; Fig. 3A and B). The PFS of patients with FcγRIIIa 158*V*/*V* and *V*/*F* was significantly longer than that of patients with 158*F*/*F* (10.8 vs. 5.1 months respectively, $P = 0.05$, log-rank test; Supplementary Fig. S2), whereas OS was not affected. FcγRIIa alleles did not correlate with either PFS or OS ($P = 0.55$ for PFS; $P = 0.15$ for OS, log-rank test; data not shown). In Fig. 3C, the average value of *in vitro* cetuximab-mediated ADCC is shown: 30.6% (9%–55%) in patients with complete or partial responses, 21% (0%–57%) in patients with stable disease, and 8.3% (0%–23%) in patients with progressive disease, respectively ($P = 0.020$; ANOVA test). As previously reported (40), patients were classified as ADCC not inducible (<30% cetuximab-mediated cytotoxicity) and ADCC inducible (>30% cetuximab-mediated cytotoxicity). Although *in vitro* ADCC significantly correlated with cetuximab clinical response, no impact on prognosis was detected.

NK-cell cytotoxicity and response to first-line therapy

To evaluate basal NK activity in mCRC patients, NK cytotoxicity was determined in 39 of the patients previously characterized for *in vitro* ADCC. First-line treatment of these patients was distributed as follows: 10 (25%) received chemotherapy alone, 24 (62%) received chemotherapy + anti-VEGF therapy, and 5 (13%) received chemotherapy + cetuximab-based therapy (Table 4). NK-cell cytotoxicity is shown in Fig. 4A. Specific killing is expressed as a function of percent lysis at a 5:1 ratio (ET), normalized for NK-cell frequency within PBMCs for each subject. Significant correlations were detected between NK-cell cytotoxicity and patient response to first-line therapy. Patients displaying complete or partial responses showed a higher percent cytotoxicity/percent NK-cell index compared with patients with stable or progressive disease (3.7, 1.3, and 0.26, respectively, $P = 0.046$; Fig. 4B). The patients with a percent cytotoxicity/percent NK-cell index > 0.6 (median value) were arbitrarily considered patients with high NK-cell cytotoxicity (against K562). A prognostic effect was detected on PFS but not on OS (11.23 vs. 7.11 months; $P = 0.048$; Fig. 4C).

Discussion

In this study, 96 patients newly diagnosed with mCRC were prospectively characterized for FcγR polymorphisms, and the relation to *in vitro* cetuximab-mediated ADCC was determined. Twenty-eight of 96 patients enrolled in the study underwent

Table 3. Detailed characteristics of 28 KRAS-WT patients receiving cetuximab therapy

Patient	Gender	Age (years)	Primary tumor	Site of metastatic disease	FcγRIIIa-V158F	FcγRIIa-H131R	Cetuximab-mediated ADCC vs. HT29 cells (%)	Anti-EGFR therapy	Response to anti-EGFR therapy
1	Female	63	Colon	Liver	VV	HH	38.1 ± 6.3	FU+IRI+CET	SD
2	Male	64	Colon	Liver + lung	VF	HR	9.3 ± 0.3	FU+IRI+CET	PR
3	Female	62	Colon	Liver	VF	HH	23.0 ± 2.5	IRI+CET	PD
4	Male	68	Colon	Liver	FF	HR	0.0 ± 0.5	IRI+CET	PD
5	Female	57	Colon	Liver	FF	HR	4.0 ± 1.4	FU+IRI+CET	PD
6	Male	55	Colon	Liver	VF	HR	34.3 ± 1.5	FU+IRI+CET	PR
7	Male	69	Colon	Liver + lung	FF	HR	5.3 ± 1.9	IRI+CET	SD
8	Male	71	Colon	Lung	FF	HH	4.2 ± 0.8	FU+IRI+CET	PD
9	Female	58	Colon	Liver	VF	HH	10.0 ± 0.2	FU+IRI+CET	SD
10	Female	52	Colon	Liver	FF	RR	0.0 ± 0.1	IRI+CET	PD
11	Female	66	Rectum	Lung	FF	HH	36.0 ± 3.5	FU+IRI+CET	SD
12	Female	60	Colon	Liver	VV	HH	41.2 ± 0.3	FU+IRI+CET	PR
13	Male	68	Rectum	Liver	VV	RR	18.0 ± 3.7	IRI+CET	PD
14	Male	28	Rectum	Liver	VF	HH	57.3 ± 1.3	FU+IRI+CET	SD
15	Male	62	Colon	Liver+ lung	VF	HR	15.8 ± 0.1	IRI+CET	SD
16	Male	66	Colon	Peritoneum	VF	RR	15.7 ± 0.9	IRI+CET	PD
17	Female	70	Rectum	Peritoneum	VF	HH	7.3 ± 1.0	FU+IRI+CET	PD
18	Male	47	Colon	Liver	VF	HR	13.1 ± 3.7	FU+IRI+CET	PR
19	Female	66	Colon	Peritoneum	VF	RR	5.1 ± 1.0	FU+IRI+CET	SD
20	Female	70	Colon	Lung	VF	HR	5.0 ± 1.7	IRI+CET	SD
21	Male	57	Rectum	Lung	VF	RR	3.7 ± 1.4	FU+IRI+CET	PD
22	Male	69	Colon	Liver	VV	HH	6.8 ± 1.8	IRI+CET	SD
23	Male	60	Rectum	Lung	VV	HR	55.0 ± 0.1	IRI+CET	PR
24	Male	69	Colon	Liver + lung	VF	HR	12.2 ± 0.8	IRI+CET	CR
25	Female	40	Rectum	Liver	VV	RR	31.7 ± 1.0	CET	SD
26	Female	50	Rectum	Liver	VV	HH	48.6 ± 0.9	CET	PR
27	Male	62	Colon	Liver	VV	HH	32.7 ± 0.4	FU+OXA+CET	PR
28	Male	61	Colon	Liver	VF	RR	25.0 ± 2.3	FU+OXA+CET	SD

Abbreviations: BEV, bevacizumab; CAPE, capecitabine; CET, cetuximab; CR, complete response; FU, 5-fluorouracil; IRI, irinotecan; OXA, oxaliplatin; PD, progressive disease; PR, partial response; SD, stable disease.

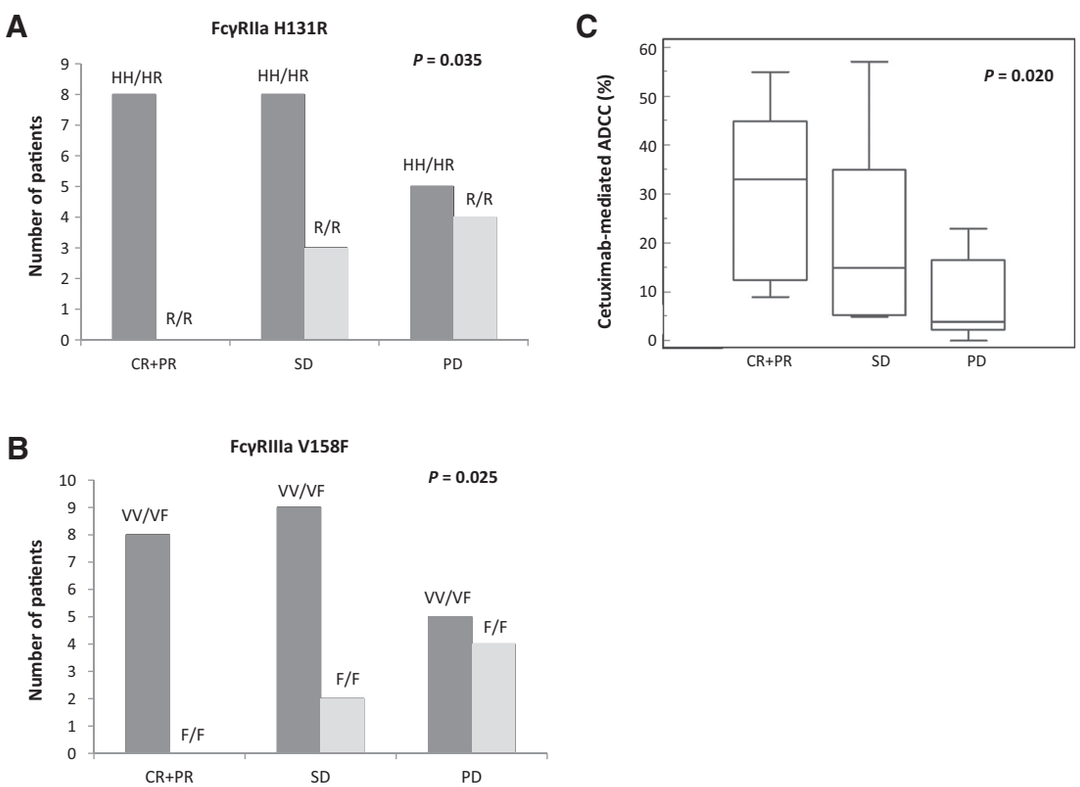

Figure 3.
Cetuximab clinical response correlates with FcγR polymorphisms and cetuximab-mediated ADCC activity. A and B, clinical response to cetuximab according to FcγR polymorphisms. C, clinical response to cetuximab according to *in vitro* cetuximab-mediated ADCC activity.

Table 4. Characteristics of 39 patients tested for NK-cell cytotoxicity

Patient	Gender	Age (years)	Primary tumor	Site of metastatic disease	First-line therapy	Response to first-line therapy	% Cytotoxicity/ % NK cells
1	Male	54	Colon	Liver	FU+OXA	SD	1.15
2	Female	52	Colon	Liver	CAPE+OXA	SD	1.02
3	Female	72	Colon	Liver	FU+OXA+BEV	PD	0.60
4	Male	40	Colon	Liver	FU+OXA	PD	0.00
5	Male	28	Rectum	Liver	CAPE+OXA+BEV	SD	0.53
6	Female	40	Colon	Liver	FU+OXA+BEV	PR	3.00
7	Male	58	Colon	Liver	FU+OXA+BEV	PR	3.57
8	Female	61	Colon	Liver + lung	FU+OXA+BEV	PD	0.26
9	Female	64	Colon	Liver	CAPE+OXA+BEV	SD	1.02
10	Female	68	Colon	Liver	FU+OXA+BEV	PR	15.59
11	Male	57	Colon	Liver + lung	FU+OXA+BEV	SD	0.13
12	Male	64	Rectum	Lung	FU+OXA+BEV	SD	0.79
13	Male	40	Colon	Lymph nodes	FU+OXA+BEV	PR	0.18
14	Male	77	Rectum	Liver	CAPE+IRI	PD	0.55
15	Male	57	Rectum	Lung	FU+IRI+CET	PD	0.15
16	Female	55	Colon	Liver	FU+OXA+BEV	PR	0.63
17	Female	70	Rectum	Peritoneum	FU+IRI+CET	PD	0.00
18	Female	62	Colon	Liver	FU+OXA+BEV	PR	0.41
19	Male	53	Colon	Liver	FU+OXA+BEV	PR	0.57
20	Male	70	Colon	Liver	FU+OXA+BEV	SD	4.40
21	Male	69	Rectum	Liver	FU+IRI+BEV	PR	7.37
22	Female	63	Colon	Liver	CAPE+OXA	CR	2.33
23	Male	66	Colon	Liver	FU+OXA	SD	0.00
24	Female	48	Colon	Liver	FU+OXA	PR	6.25
25	Female	51	Rectum	Liver	CAPE+OXA	PR	10.77
26	Male	68	Colon	Liver	FU+OXA+BEV	SD	10.47
27	Male	47	Colon	Liver	FU+OXA+BEV	PR	0.05
28	Female	45	Colon	Liver + lung	FU+OXA+BEV	SD	0.53
29	Female	58	Colon	Liver	CAPE+OXA	SD	0.93
30	Female	66	Colon	Peritoneum	FU+IRI+CET	SD	0.69
31	Female	70	Colon	Lung	FU+IRI+BEV	SD	0.15
32	Male	57	Colon	Liver	FU+OXA+BEV	CR	1.57
33	Male	56	Colon	Liver	FU+OXA+BEV	SD	0.61
34	Male	71	Colon	Lung	FU+IRI+CET	PR	0.38
35	Male	57	Rectum	Liver	CAPE+OXA+BEV	SD	0.38
36	Male	69	Colon	Lung	FU+OXA+BEV	SD	0.04
37	Female	66	Rectum	Lung	FU+IRI+CET	SD	0.37
38	Male	70	Colon	Liver	CAPE+OXA	SD	0.81
39	Male	67	Colon	Liver	FU+OXA+BEV	PD	0.22

Abbreviations: BEV, bevacizumab; CAPE, capecitabine; CET, cetuximab; CR, complete response; FU, 5-fluorouracil; IRI, irinotecan; OXA, oxaliplatin; PD, progressive disease; PR, partial response; SD, stable disease.

cetuximab-based therapy. A significant correlation was detected between *in vitro* cetuximab-mediated ADCC and cetuximab clinical responses, suggesting that high lytic efficiency and the absence of KRAS mutations magnify the clinical response to cetuximab. Moreover, NK basal cell cytotoxicity, evaluated in 39 patients, was correlated with first-line treatment responses. The survival of patients affected by mCRC has improved over the past decade, mainly due to the use of effective targeted therapies, such as anti-EGFR agents. Large randomized multicenter phase III clinical trials demonstrated the predictive value of KRAS for anti-EGFR therapy (41, 42). A meta-analysis of 11 studies showed that KRAS status was closely associated with the response rate ($P < 0.001$) and PFS ($P = 0.005$; ref. 43). KRAS mutation is a predictive marker for the efficacy of anti-EGFR agents in the treatment of mCRC as stated in guidelines from the National Comprehensive Cancer Network, European Society for Medical Oncology, and Japanese Society for Cancer of the Colon and Rectum, which recommend the use of antibodies to EGFR only for mCRC patients with WT KRAS. However, the prediction of response to first-line anti-EGFR therapy is a much more complex issue. De Roock and colleagues reported that patients with KRAS codon 13 mutants (G13D) treated with cetuximab showed significantly longer PFS and OS as compared with KRAS codon 12 mutants, and several other gene alterations aside from KRAS have been identified as candidate biomarkers for predicting the efficacy of anti-EGFR treatment (e.g., BRAF, PI3K, AKT, PTEN, MET, EGFR ligands; refs. 44, 45). Seo and colleagues (46) reported a significant correlation between EGFR expression and ADCC activity but not with the mutational status of KRAS and BRAF in colorectal cancer. The immune phenomena underlying these differences are as yet unknown (46). In addition, rare KRAS alterations and NRAS mutations have been recently included, defining the "RAS status" as the new validated marker of response to antibodies to EGFR (47, 48). Thus, subsets of cetuximab-refractory patients treated as KRAS WT could have harbored NRAS mutations.

To evaluate the basal NK status, NK cytotoxicity was evaluated in 39 patients (treated with different first-line therapies) and correlated to response to first-line therapy. Correale and colleagues found that a cytotoxic lymphocyte antitumor response was stimulated by dendritic cell (DC)–mediated

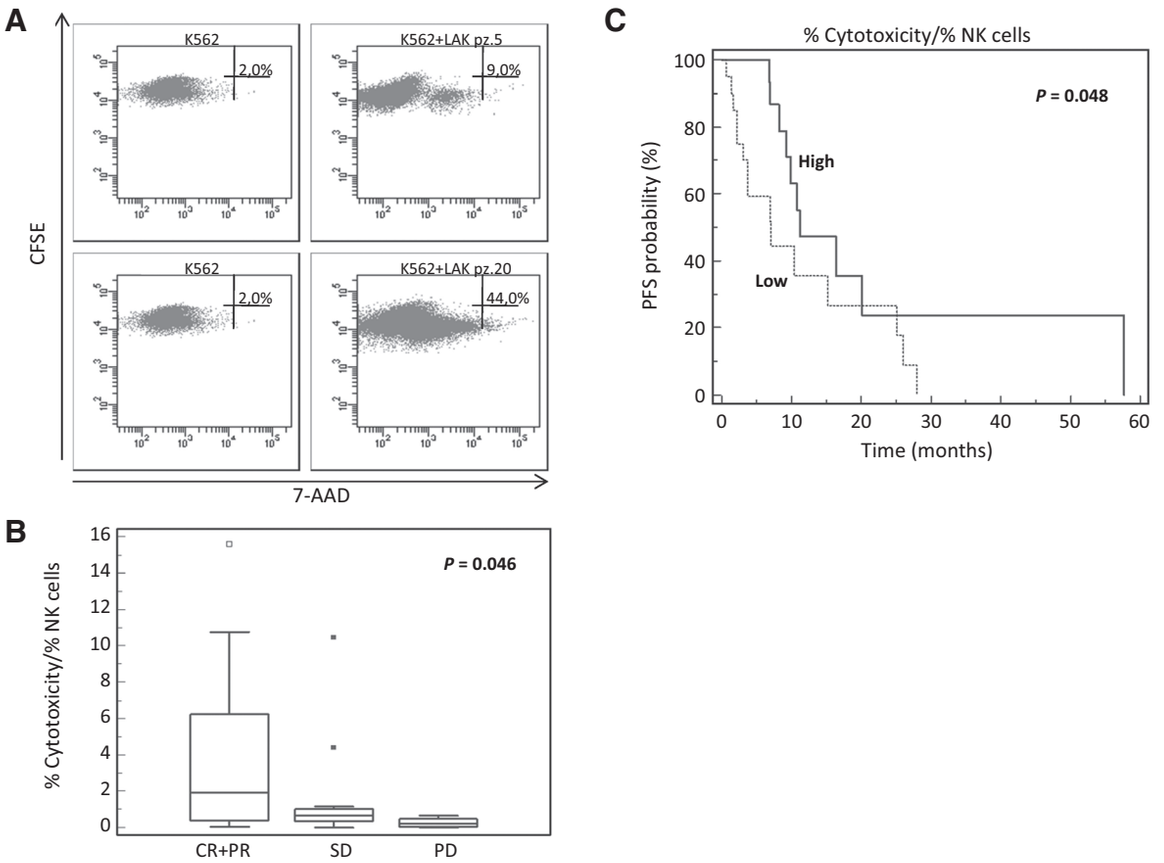

Figure 4.
NK-cell cytotoxicity significantly correlated with response to first-line therapy. A, representative NK-cell cytotoxicity in mCRC patient, 7-AAD uptake by CFSE-K562 target cells in the presence of LAK cells. B, correlation between NK-cell cytotoxicity and response to first-line treatment. C, Kaplan–Meier for PFS. Patients with high NK-cell cytotoxicity (19 patients, 10 events) showed a longer PFS compared with patients with low NK-cell cytotoxicity (20 patients, 15 events); log-rank test for two curves: $P = 0.048$; HR, 0.46; CI, 0.19–0.99. 7-AAD, 7-aminoactinomycin D.

cross-priming of antigens derived from cetuximab-covered cancer cells (49). The antitumor function can also be enhanced by NK-cell–DC cross-talk, which ensues after the recruitment of both NK cells and DCs to the inflamed areas caused by cancer, decreasing the activity and the number of immunosuppressive regulatory T cells. The resulting effective signaling can shape not only the innate immune response in inflamed peripheral tissues but also the adaptive immune response within secondary lymphoid organs (50). Our data strongly suggest that immune interactions between host and tumor are important in chemotherapy or chemobiotherapy-induced response beyond molecular alterations. In conclusion, with the limits relative to the number of patients evaluated, prospective evaluation of the *in vitro* cetuximab-mediated ADCC was correlated with the FcγR polymorphisms and may predict cetuximab responsiveness in primarily diagnosed mCRC patients. Also, NK-cell basal activity can contribute to the evaluation of first-line therapy response. Thus, the *in vitro* evaluation of basal and ADCC-induced NK activity may help predict the therapeutic responses of mCRC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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