Ex Vivo Antibody-Dependent Cellular Cytotoxicity Inducibility Predicts Efficacy of Cetuximab


Abstract

We conducted in vitro studies and a clinical trial for patients with squamous cell carcinoma of the head and neck (SCCHN) to study the relationship between FcyRIIa polymorphisms and antibody-dependent cellular cytotoxicity (ADCC). In vitro, FcyRIIa genotype was correlated with ADCC and innate cytotoxicity using natural killer (NK) cells harvested from healthy donors. In the phase II study, patients with recurrent or metastatic SCCHN were treated with cetuximab (500 mg/m² i.v. every 2 weeks) and lenalidomide with median PFS of 7.2 weeks and OS of 16.4 weeks. Thirty-six patients had FcyRIIa genotype: VF (2), VF (20), and FF (14), and 25 patients had sufficient NK-cell yield to perform ex vivo ADCC. FcyRIIa genotype was not associated with any clinical outcomes. Patients mounting ex vivo ADCC response had a higher likelihood of stable disease (P = 0.01) and showed a trend toward increased PFS: 14 weeks versus 6.8 weeks, respectively (P = 0.13). Enhanced ex vivo ADCC and innate immunity responses were more predictive of clinical response than FcyRIIa and may offer a functional assay to select patients suitable for cetuximab therapy.

Introduction

Cetuximab has established benefit in squamous cell carcinoma of the head and neck (SCCHN) and colorectal cancer and, by virtue of being an IgG1 antibody, can activate antibody-dependent cellular cytotoxicity (ADCC). In ADCC, the constant (Fc) region of IgG1-based mAbs that are bound to cell surface targets can engage and crosslink low-affinity canonical Fc receptors (FcγRIIa) expressed on natural killer (NK) cells, resulting in NK activation, degranulation, and lysis of the target cell (1, 2). There has been considerable effort to identify biomarkers that reflect the capacity to mount ADCC. Presently, the most studied biomarkers are single amino acid polymorphisms at the 158 position of FcγRIIa. Substitution of phenylalanine [F] by valine [V] increases FcγRIIa affinity for mAb Fc, which affects ADCC and tumor regression (3). As an example, it is generally accepted that non-Hodgkin lymphoma patients who are homozygous for the high-affinity FcγRIIa-158-V polymorphism have better response to rituximab, an IgG1 mAb directed against CD20, than do patients who carry low-affinity FcγRIIa-158-F polymorphisms.

However, the association between FcγRIIa-158 polymorphisms and ADCC response is less straightforward in other cancer types, including SCCHN, colorectal cancer, and breast cancer (4–9). Interestingly, in a previously published pilot study, we demonstrated that NK cells carrying the high-affinity FcγRIIa-158-V polymorphism were more effective in killing K562 leukemia cells, even in the absence of antibody (innate immunity), compared with NK cells homozygous for the low-affinity F polymorphism (10). These data suggest that FcγRIIa-158 polymorphisms may correlate not only with the FcγRIIa-binding affinity, but also with an NK phenotype that has a broader cytotoxicity profile.

In this study, we sought to more clearly define the link between FcγRIIa-158-V and F polymorphisms and ADCC response, both in vitro and in vivo. In addition, we studied colorectal cancer cells to expand our study to include another solid tumor system, in which cetuximab is used therapeutically. Based on our previous data, we hypothesized that NK cells carrying the FcγRIIa-158-V polymorphism would both have a higher level of innate cytotoxicity against colon cancer cell lines and induce more potent cetuximab-mediated ADCC in vitro. We now report that the
FcyRIIa-158-V polymorphism is not the sole determinant of the magnitude of cetuximab-mediated ADCC in vitro. We observe NK cells with a phenotype of broad cytotoxic capacity that simultaneously share high innate cytotoxicity and enhanced ADCC independent of FcyRIIa-158 genotype. We sought to validate these findings clinically without the potentially confounding effects of cytotoxic chemotherapy in SCCHN because cetuximab is rarely administered as a single agent in colorectal cancer. Therefore, we initiated a multi-institutional phase II clinical trial evaluating the efficacy of cetuximab in patients with recurrent or metastatic SCCHN in combination with lenalidomide. The rationale for choosing lenalidomide in combination with cetuximab is that there is strong evidence that lenalidomide enhances ADCC in combination with IgG1 antibodies, including cetuximab, and the mechanism of action is through ADCC (11–15). Our clinical trial data also suggest that FcyRIIa-158 polymorphisms alone are inadequate to predict clinical response in cetuximab and lenalidomide-treated patients. We obtained NK cells from enrolled patients to perform ex vivo ADCC; when NK cells from patients were able to effectively initiate ADCC ex vivo, they were described as 'ADCC inducible.' Moreover, our data suggest that the capacity of a patient’s NK cells to mount ADCC ex vivo best predicted improved clinical response, regardless of FcyRIIa polymorphisms. In concert, these data support that innate NK-cell cytotoxicity and the capacity to mount ADCC are more important than FcyRIIa polymorphisms in determining clinical response to cetuximab.

Materials and Methods

ADCC assays

Whole blood was obtained from enrolled SCCHN patients for the isolation of NK cells to perform ex vivo ADCC assays, which in this study is distinguished from ADCC assays involving healthy blood donors utilized for in vitro colorectal cancer studies. From each enrolled patient, 150 mL of whole blood was processed and centrifuged; the buffy coat layer was isolated to harvest peripheral blood mononuclear cells (PBMC) using the Ficoll–Hypaque centrifugation method; NK cells were negatively selected using a MACS human NK cell isolation kit (Miltenyi Biotec). ADCC assays were performed using SCCHN cells (TU167) as targets, and purified NK cells as effectors from enrolled SCCHN patients. ADCC assays were also performed using a leukemia cell line (K562) for positive controls, two colon cancer cell lines (HT29, SW480) as target cells, and purified NK cells from healthy donors. Target cells were incubated with 150 μCi of chromium-51 (51Cr; Amersham) at 37°C for 1 hour, mixing thoroughly every 15 minutes, and washed twice with media. Cells were subsequently incubated with 10 μg/mL of cetuximab, 10 μg/mL of control human IgG1 isotype, or with media alone for another 30 minutes at 37°C, and washed twice with media to remove unbound antibodies. The concentration of cetuximab was established based upon our prior work and also on physiologic serum concentrations ranges: 4–8 μg/mL to 16–23 μg/mL for peak and trough, respectively, according to packet insert. Effector and target cells were plated at 50:1, 25:1, and 12.5:1 in 96-well plates and incubated for 14 to 16 hours. Each assay was performed in triplicate. Cell lysis supernatant was collected and mixed with the Optiphase Supermix scintillation fluid (Perkin Elmer) and counted in a MicroBeta 1450 scintillation counter (Wallac). Activity against NK-sensitive K562 tumor cell line was used as a positive control for all ADCC experiments and provided the measure for innate cytotoxicity. Results were expressed as the percentage of specific lysis according to the following formula:

\[
\frac{(\text{Experimental cpm} - \text{spontaneous cpm}) \times 100}{(\text{maximum cpm} - \text{spontaneous cpm})}
\]

Determination of FcyRIIa polymorphisms

Genomic DNA from PBMCs obtained from enrolled SCCHN patients was isolated using the Qiagen DNA extraction Kit (Qiagen) and stored at −20°C. The FcyRIIa valine, V, or phenylalanine, F, at position 158 was determined by PCR. Briefly, the PCR reaction was optimized using 250 ng of DNA, 0.5 mmol/L dNTPs, 1 unit GoTag polymerase (Promega), and corresponding 1× buffer containing 1.5 mmol/L MgSO4 to a final volume of 50 μL. For PCR amplification, samples were subjected to an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles at 94°C for 40 seconds, 52°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Amplified PCR samples were cleaned using phenol extraction and ethanol precipitation before restriction digestion. Two digestions per sample were performed to distinguish the FcyRIIa polymorphisms. First, 10 μL of the PCR product was incubated at 37°C overnight with 10 units of Rsal with the appropriate 1× buffer (Promega) to a final volume of 20 μL. For the second digestion, 10 μL of the RsaI-digested sample was subsequently incubated at 37°C overnight with 40 units of Eco130I with the appropriate 1× buffer (Fermentas) to a final volume of 30 μL. Both RsaI- and RsaI/Eco130I-digested products were separated on a 3% agarose gel with ethidium bromide and visualized under UV light.

Cell culture

SCCHN cell line TU167 was obtained from The University of Texas MD Anderson Cancer Center (Houston, TX). Cells were cultured in RPMI 1640 complete media containing 10% heat-inactivated FBS (Atlanta Biologics), 1% c-glutamine (Gibco), 1% penicillin–streptomycin (Gibco), and HEPES buffer (Mediatech, Inc.). The K562, HT29, and SW480 cell lines purchased from the ATCC, which characterizes the cell lines by various methods. No other authentication assays were performed.

Clinical trial design

The phase II multi-institutional trial enrolled patients with recurrent/metastatic SCCHN recruited at three cancer centers: University of Chicago Comprehensive Cancer Center, University of Maryland Greenebaum Cancer Center, and Medical College of Wisconsin Cancer Center. The primary endpoint was objective response rate (RR). Secondary endpoints included estimating progression-free survival (PFS), overall survival (OS), as well as the plausible association of ex vivo ADCC response (NK cells harvested from enrolled patients) with PFS, OS, and FcyRIIa polymorphisms.

Patient eligibility

Patients with recurrent or metastatic squamous cell or undifferentiated carcinoma of the head and neck not amenable to curative therapy were screened for eligibility. Patients were eligible if they were at least 18 years of age, able to understand and provide voluntary consent, had an Eastern Cooperative Oncology Group (ECOG) status of at least 2, serum chemistry reflecting normal kidney, liver, and hematologic function, and had not received...
therapy (including radiation, hormonal therapy, and surgery) for at least 4 weeks before enrollment. In addition, patients had to have measurable disease as defined by RECIST (16). Patients were excluded if they had a secondary malignancy within 3 years of enrollment, with the exception of treated basal cell or squamous cell carcinoma of the skin, or carcinoma in situ of the cervix or breast. This study was conducted in accordance with the Institutional Review Board approval from each participating institution.

**Treatment administration**

Patients received cetuximab (500 mg/m²) i.v. infusions via infusion pump or gravity drip every 2 weeks. Lenalidomide, 25 mg, orally or via feeding tube once daily was provided in accordance with the RevAssist program of Celgene Corporation. Per standard RevAssist requirements, all physicians who prescribed lenalidomide for research subjects enrolled into this trial were registered in and complied with all requirements of the RevAssist program.

**Evaluation during study and response assessment**

Clinical response was evaluated with CT every 2 cycles (8 weeks). RECIST was used to determine response. At treatment discontinuation, a safety assessment was done approximately (8 weeks). RECIST was used to determine response. At treatment discontinuation, a safety assessment was done approximately (8 weeks). RECIST was used to determine response. At treatment discontinuation, a safety assessment was done approximately 30 days after the last dose of protocol therapy.

**p16 determination for oropharyngeal SCCHN patients**

After deparaffinization and rehydration, tissue sections were treated with antigen retrieval buffer (S1699; DAKO) and heated in a steamer for 20 minutes. Anti-p16 antibody (Santa Cruz Biotechnology; Cat#sc-56330, mouse IgG, JC8, 1:100) was applied on tissue sections for 1-hour incubation at room temperature in a humidity chamber. Following TBS wash, the antigen–antibody binding was detected with Envision system (DAKO; K4001) and DAB+ chromogen (DAKO; K3468). Tissue sections were briefly immersed in hematoxylin for counterstaining and were covered with cover glasses to determine p16 positivity.

**Statistical analysis**

Formal power calculations, and resulting adequate sample size, were obtained based on our preliminary in vitro FcyRIIa polymorphism ADCC studies. However, there are no published data regarding differential clinical response for FcyRIIa polymorphic genotype in SCCHN patients treated with cetuximab. Power of the statistical test and a corresponding sample size were constructed to detect an improvement in median OS of 8 weeks for patients with a favorable FcyRIIa polymorphic genotype. Thus, the total required accrual was 40 to 45 patients.

OS was defined as time from diagnosis to date of death from any cause, censored at the date the patient was last known to be alive. PFS was defined as time from the date of the first treatment dose administered to the earlier of either disease progression or death from any cause.

OS and PFS functions were estimated by the Kaplan–Meier method. OS and PFS medians with the corresponding confidence intervals (CI) were used to summarize the time-to-event distributions. Patients were grouped for the analyses according to their FcyRIIa polymorphism status. The log-rank test was used to test whether distinct categories of patients had different OS and/or PFS experience. The multivariable Cox regression model was utilized to estimate HRs. The following risk factors were assessed in the regression model, FcyRIIa genotype, ADCC inducibility, human papilloma virus (HPV) status, prior treatment, and tumor site. However, modeling options were very limited due to rather small number of events in the dataset. The general liner model approach was applied to estimate plausible differences (between patients’ categories) in continuous variables, and exact tests for rxc contingency tables were appropriate to use for the categorical ones. All hypothesis tests were conducted at the 0.05 level of statistical significance, were two-sided, and exact where appropriate. Statistical analyses were performed using SAS statistical software (SAS version 9.1; SAS Institute) and S-plus (TIBCO, v.8.2).

**Results**

**Phase II trial of cetuximab and lenalidomide in SCCHN**

Based on these in vitro data, we decided to further evaluate the relationship between FcyRIIa polymorphisms, ex vivo ADCC response, and clinical response in cetuximab-treated patients with recurrent or metastatic SCCHN in the context of the immune modulator, lenalidomide. Lenalidomide was selected for combination therapy with cetuximab because of evidence that it enhances ADCC in the presence of cetuximab. Forty-two patients were enrolled (Table 1). Two patients were removed from the study because their clinical status declined substantially during the interval between registration and initiation of therapy. For all patients enrolled, the median OS and PFS were 16.4 and 7.2 weeks, respectively (Fig. 1). In addition, no patients demonstrated complete or partial response to treatment, whereas 14 (33%) showed stable disease and 22 (51%) had progressive disease at first assessment.

**FcyRIIa genotype does not correlate with cetuximab-associated ADCC inducibility and clinical outcome**

 Determination of FcyRIIa polymorphic genotype at position 158 was available for 36 patients: VV, VF, and FF. A total of 6 patients did not have genotyping performed (two of the shipped samples were not available within 24 hours, technical difficulties were encountered during the processing of two samples, and for 2 patients, adequate blood sample was not obtained during enrollment). Patients who were both homozygous and heterozygous for the high-affinity V allele at position 158 were grouped together and compared with those who were homozygous at the F allele because our previous data demonstrated an ADCC advantage if at least 1 V allele is present (10). When NK cells from trial patients were able to effectively initiate ADCC ex vivo, they were described as “ADCC inducible.” No association between FcyRIIa genotype and OS (HR for FF 0.9; 95% CI, 0.4–1.9, \( P = 0.82 \)) or PFS (HR for FF 1.0; 95% CI, 0.5–2.1, \( P = 0.9 \)), or RR (\( P = 0.1 \)) was observed (Tables 2, 3, and 4). In addition, there was no association with ADCC inducibility and FcyRIIa genotype. These data suggest that FcyRIIa polymorphisms are not adequate to predict clinical outcome in cetuximab/lenalidomide-treated SCCHN patients.

**Ex vivo ADCC predicts clinical outcome**

Twenty-five patients had adequate harvest of NK cells to perform ex vivo ADCC against SCCHN cell lines (Table 5). It was our
goal to determine if patients who were able to initiate effective ADCC \textit{ex vivo} would also have improved clinical outcomes, such as clinical response, OS, and PFS. Seventeen patients did not have \textit{ex vivo} ADCC performed, with the most common reason being inadequate NK-cell yield to perform ADCC from 13 patients; shipped samples from 2 patients were not available within 24 hours; and blood sample was not taken from 2 patients during enrollment. In order to determine if any bias existed for the patients for whom we were able to perform \textit{ex vivo} ADCC versus those who were not, we estimated and compared the OS and PFS experience for the two groups of patients, and demonstrated that no difference existed in OS or PFS between groups (Supplementary Fig. S1).

Among the 25 patients who had adequate NK-cell yield to perform ADCC, 8 showed enhanced cytotoxic activity (>30% cytotoxicity) and were termed "ADCC inducible." Interestingly, ADCC inducibility was the most predictive marker for clinical effectiveness. Compared with the 17 patients who did not mount an \textit{ex vivo} ADCC response, those who were ADCC inducible showed a trend toward increased PFS (3.5 vs. 1.7 months; HR = 0.6; 95% CI, 0.6–1.3, \(P = 0.13\); Table 2). Furthermore, there was a significantly greater likelihood of having stable disease versus progressive disease for patients who were ADCC inducible compared with those who did not mount an \textit{ex vivo} ADCC response (\(P = 0.01\), exact and two-sided). No association was found with OS.

These data suggest that the capacity to mount ADCC \textit{ex vivo} may correlate with improved clinical outcome. This, together with the finding that Fc\(\gamma\)RIIIa genotype did not correlate with ADCC response, provides further rationale to identify biomarkers

\begin{table}[h]
\begin{center}
\begin{tabular}{ll}
\hline
Patient characteristics & Frequency (\%) \\
\hline
Gender & \\
Female & 10 (23.8) \\
Male & 32 (76.2) \\
Race & \\
White & 30 (71.4) \\
African American or Black & 7 (16.7) \\
Asian & 1 (2.4) \\
American Indian or Alaska native & 1 (2.4) \\
Unknown & 3 (7.3) \\
Age range (years) & \\
40–49 & 6 (14.3) \\
50–59 & 17 (40.5) \\
60–69 & 14 (33.3) \\
\geq 79 & 2 (4.8) \\
Unknown & 1 (2.4) \\
Age (years) & \\
Median & 59 \\
Range & 42–82 \\
Status & \\
Alive & 4 (9.5) \\
Dead & 36 (85.7) \\
Unknown & 2 (4.8) \\
Clinical measures & \\
Primary tumor site & \\
OP (oropharynx) & 16 (38.1) \\
Non-OP (all other sites) & 24 (57.1) \\
Not available & 2 (4.8) \\
Genotype & \\
FF & 14 (33.3) \\
VF & 19 (45.2) \\
VV & 3 (7.1) \\
Not available & 6 (14.3) \\
ADCC performed & \\
No & 17 (40.0) \\
Yes & 25 (60.0) \\
ADCC & \\
Noninducible & 17 (39.2) \\
Inducible & 8 (30.8) \\
Prior chemoradiation & \\
No & 3 (7.1) \\
Yes & 36 (85.7) \\
Unknown & 3 (7.1) \\
Prior surgery & \\
No & 14 (33.3) \\
Yes & 25 (59.5) \\
Unknown & 3 (7.1) \\
p16 staining & \\
Negative & 7 (16.7) \\
Positive & 6 (14.3) \\
Not tested & 29 (69.0) \\
ECOG status & \\
0 & 13 (30.9) \\
1 & 27 (64.3) \\
Unknown & 2 (4.8) \\
Disease location & \\
Local only & 5 (11.9) \\
Metastatic only & 21 (50.0) \\
Local and metastatic & 14 (33.3) \\
Unknown & 2 (4.8) \\
\hline
\end{tabular}
\end{center}
\caption{Distribution of phase II trial clinical, molecular, and demographic characteristics (\(N = 42\)).}
\end{table}
Table 2. Results of the univariate Cox regression model for PFS* (N = 42)

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>N</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31</td>
<td>1.0</td>
<td>0.68</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>0.8 (0.4-1.9)</td>
<td>0.13</td>
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<tr>
<td>Race</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>White</td>
<td>30</td>
<td>1.0</td>
<td>0.73</td>
</tr>
<tr>
<td>Black</td>
<td>6</td>
<td>0.9 (0.4-2.1)</td>
<td>0.77</td>
</tr>
<tr>
<td>Other</td>
<td>10</td>
<td>0.8 (0.4-1.6)</td>
<td>0.77</td>
</tr>
<tr>
<td>Age</td>
<td>40</td>
<td>1.0 (0.9-1.0)</td>
<td>0.18</td>
</tr>
</tbody>
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Clinical measures

<table>
<thead>
<tr>
<th>Primary tumor site</th>
<th>N</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Non-OP</td>
<td>24</td>
<td>1.0</td>
<td>0.53</td>
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<td>OP</td>
<td>16</td>
<td>1.2 (0.6-2.3)</td>
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<tr>
<td>Polymorphism</td>
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<tr>
<td>FF</td>
<td>14</td>
<td>1.0</td>
<td>0.90</td>
</tr>
<tr>
<td>FV or VV</td>
<td>21</td>
<td>1.0 (0.5-2.1)</td>
<td>0.42</td>
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<td>Surgery (in addition to treatment)</td>
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<td>1.0</td>
<td>0.81</td>
</tr>
<tr>
<td>Surgery</td>
<td>25</td>
<td>0.9 (0.5-1.8)</td>
<td>0.90</td>
</tr>
<tr>
<td>PI6 staining</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>1.0</td>
<td>0.71</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>0.9 (0.3-2.6)</td>
<td>0.71</td>
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<tr>
<td>ADCC response</td>
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<tr>
<td>No response</td>
<td>18</td>
<td>1.0</td>
<td>0.13</td>
</tr>
<tr>
<td>Response</td>
<td>8</td>
<td>0.6 (0.2-1.3)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Abbreviation: OP, oropharynx.

*PFS is defined as time from the start of treatment until progression or death. For cases without progression, follow-up was censored at date patient was last known to be progression free.

Beyond the FcRIIIa genotype that define innate NK-cell cytotoxicity and potential ADCC response.

HPV status and outcome

In this study, 16 of the 42 patients enrolled had primary tumors originating in the oropharynx. Because HPV is associated with oropharyngeal SCCCHN and has been established to correlate with better survival, we determined the HPV status of the patients with oropharyngeal cancers by staining for the presence of p16. We were able to determine HPV status for 13 of the 16 patients with oropharyngeal SCCCHN. Six patients were p16 positive, and these patients showed a trend toward increased OS (HR, 0.3; P = 0.09; Table 3). There was no association between HPV status and FcRIIIa genotype or objective RR. Table 4 provides a contingency table for clinical RR by HPV status, ADCC inducibility, and FcRIIIa genotype.

Toxicities

Of the 40 patients that received cetuximab/lenalidomide, all had at least one adverse event (AE), and of these, 39 (99%) patients had an AE related to cetuximab/lenalidomide (Table 6). Six grade 4 AEs were observed. One patient developed an oropharyngeal hemorrhage due to a pseudoaneurysm of the left internal maxillary artery, which was not attributed to treatment. Seventeen patients (43%) had serious AEs (SAEs) that did not result in death, and 5 of these SAEs (13%) were deemed related to cetuximab/lenalidomide. The most frequent SAE was neutropenia (2/40, 5%). Other SAEs related to cetuximab/lenalidomide included an infusion-related reaction (1/40, 3%), muscle weakness (1/40, 3%), and deep vein thrombosis (1/40, 3%). A total of 9 patients (23%) died during the study, and one of the deaths was associated with multiple deep vein thrombosis and pulmonary thromboembolism was considered possibly related to cetuximab/lenalidomide. Causes of death included pulmonary edema, aspiration, and progressive disease; 5 patients died of unspecified causes.

In vitro ADCC and innate cytotoxicity

Our previously published work showed that NK cells carrying at least one FcRIIIa-158-V allele are more effective at killing (positive control) NK-sensitive K562 leukemia cells than FF genotype NK cells. More specifically, even in the absence of tumor antigen–targeted mAbs, cytotoxic activity of VV and VF NK cells was 41% compared with 21% for FF (P = 0.04), suggesting that FcRIIIa polymorphisms may be associated with enhanced NK-cell cytotoxicity through mechanisms unrelated to FcRIIIa engagement (10). To test this concept further, we evaluated the relationship between FcRIIIa polymorphisms and cytotoxicity in the absence of antibody (innate cytotoxicity) against K562 leukemia cells, as well as cetuximab-induced ADCC using a larger cohort of NK-cell healthy donors against two separate colon cancer cell lines, HT29 (N = 27 FF and 35 VV/VF) and SW480 (N = 23 FF and 30 VV/VF). Consistent with our previous data, the FcRIIIa-158-V polymorphism in vitro was associated with higher innate cytotoxicity against the highly sensitive K562 cells compared with the FcRIIIa-158-F polymorphism in this larger cohort (FF 40%; VF and VV = 47%; P = 0.054). Similarly, we found that FcRIIIa-158-V–expressing NK cells had a higher cytotoxic effect against the examined colon cancer cell lines compared with the FF genotype (HT29 mean cytotoxicity: FF 16.1%, VV/VF = 24.3% (P = 0.015) and SW480: FF 11.7%, VV/VF = 21.0% (P = 0.006)). Thus, when

Table 3. Results of the univariate Cox regression model for OS* (N = 42)

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>N</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
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<tr>
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<td>White</td>
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<td>0.77</td>
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<tr>
<td>Black</td>
<td>6</td>
<td>0.9 (0.3-2.3)</td>
<td>0.77</td>
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<tr>
<td>Other</td>
<td>10</td>
<td>1.0 (0.5-2.2)</td>
<td>0.77</td>
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<tr>
<td>Age</td>
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<td>0.28</td>
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Clinical measures

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<tbody>
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<td>OP</td>
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<td>7</td>
<td>1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>0.3 (0.1-1.2)</td>
<td>0.09</td>
</tr>
<tr>
<td>ADCC response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>17</td>
<td>1.0</td>
<td>0.85</td>
</tr>
<tr>
<td>Response</td>
<td>8</td>
<td>1.1 (0.4-2.9)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Abbreviation: OP, oropharynx.

*OS is defined from the date of start of treatment till the date of death or censored at the date last known alive.

we examined the relationship between FcγRIIIa genotype and cetuximab-induced ADCC, we observed that donor NK cells carrying the FcγRIIIa-158-V polymorphism were generally more effective at inducing ADCC against colorectal cancer cell lines than those carrying only the FcγRIIIa-158-F polymorphism.

Interestingly, we also found that innate cytotoxicity (against K562) positively correlated with the magnitude of cetuximab-induced ADCC. Figure 2 shows the linear relationship between innate cytotoxicity (without antibody) and the magnitude of cetuximab-induced ADCC. Innate cytotoxicity rather than RIIIA-158 polymorphism demonstrated both high innate cytotoxicity and high cetuximab-induced ADCC against colorectal cancer cell lines in vitro, the observed cytotoxicity was highly variable between the individual donors, regardless of their genotype. Specifically, many donors who were homozygous for the FcγRIIIa-158-F polymorphism demonstrated both high innate cytotoxicity and high cetuximab-induced ADCC. Figure 2 shows the linear relationship between innate cytotoxicity (without antibody) and the magnitude of cetuximab-induced ADCC. Innate cytotoxicity rather than FcγRIIIa polymorphisms had the strongest relationship for predicting the magnitude of cetuximab-mediated ADCC response.

Some NK cells expressing FcγRIIIa-158-F/F demonstrated high innate cytotoxicity could also mount ADCC more effectively than FF or VF NK cells with low innate cytotoxicity. These in vitro data suggest that FcγRIIIa-158 polymorphisms alone are insufficient to predict the magnitude of ADCC response for colorectal cancer cell lines and provide rationale to identify biomarkers independent of FcγRIIIa polymorphisms that reflect the level of innate NK-cell cytotoxicity.

### Discussion

Previous work has demonstrated that cetuximab can induce ADCC in vitro; however, it remained uncertain whether ADCC effects translated into clinically meaningful tumor regression in patients. Moreover, although FcγRIIIa-158-V and F polymorphisms have been associated with clinically relevant ADCC in lymphoma patients, their predictive value in solid organ malignancies remains less defined (7, 17, 18). Here, we demonstrate, in colon cancer cell lines, that FcγRIIIa polymorphisms alone are insufficient to predict ADCC response and that innate NK-cell cytotoxicity, a property for which there are no validated biomarkers, may be more important. In fact, NK cells expressing low-affinity FcγRIIIa-158-F receptors could mount robust ADCC if they had high innate cytotoxicity. Furthermore, in a phase II clinical trial using cetuximab and lenalidomide in SCCHN patients, our findings support that it is possible that the capacity of NK cells to induce ADCC ex vivo independently correlates with improved clinical outcomes irrespective of FcγRIIIa polymorphisms. Taken together, these data provide clinical evidence of the relevance of ADCC to the mechanism of action of cetuximab and lenalidomide.

---

**Table 5. Ex vivo ADCC assays from patients enrolled in phase II trial**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Media (−)</th>
<th>IgG (−)</th>
<th>Cetuximab</th>
<th>K562 (−)</th>
</tr>
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<tbody>
<tr>
<td>VF</td>
<td>11%</td>
<td>9%</td>
<td>25%</td>
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<tr>
<td>FF</td>
<td>6%</td>
<td>5%</td>
<td>8%</td>
<td>4%</td>
</tr>
<tr>
<td>VF</td>
<td>7%</td>
<td>5%</td>
<td>11%</td>
<td>7%</td>
</tr>
<tr>
<td>VF</td>
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<tr>
<td>FF</td>
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<td>54%</td>
<td>32%</td>
</tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>VF</td>
<td>9%</td>
<td>5%</td>
<td>31%</td>
<td>51%</td>
</tr>
<tr>
<td>VF</td>
<td>6%</td>
<td>7%</td>
<td>41%</td>
<td>51%</td>
</tr>
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</table>

**Table 6. Summary of AEs**

<table>
<thead>
<tr>
<th>Cetuximab &amp; lenalidomide (N = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Grades</strong></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Fatigue</td>
</tr>
<tr>
<td>Rash</td>
</tr>
<tr>
<td>Anemia</td>
</tr>
<tr>
<td>Constipation</td>
</tr>
<tr>
<td>Anorexia</td>
</tr>
<tr>
<td>Nausea</td>
</tr>
<tr>
<td>Mucositis</td>
</tr>
<tr>
<td>Hypoalbuminemia</td>
</tr>
<tr>
<td>Leukopenia</td>
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<tr>
<td>Lymphopenia</td>
</tr>
<tr>
<td>Neutropenia</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td>Vomiting</td>
</tr>
<tr>
<td>Hypoanemia</td>
</tr>
<tr>
<td>Diarrhea</td>
</tr>
<tr>
<td>Xerostomia</td>
</tr>
<tr>
<td>Hypomagnesemia</td>
</tr>
<tr>
<td>Infusion reaction</td>
</tr>
<tr>
<td>Thromboembolic events</td>
</tr>
</tbody>
</table>

**NOTE:** Results from ex vivo chromium-51 ADCC assays performed for 25 patients from phase II trial when adequate sample was available. First column indicates FcγRIIIa genotype, subsequent columns represent percentage of specific cell lysis for negative controls (media and IgG), cetuximab, and positive controls (K562), respectively. Bold rows indicate ADCC-inducible ex vivo assays.
Figure 2.
ADCC versus innate cytotoxicity (K562) against colorectal cancer cell lines SW480 and HT29.

suggest that functional measures of ADCC and NK-cell innate cytotoxicity, rather than FcγRIIIa polymorphisms, may provide a more reliable means of selecting patients who will benefit from this drug.

The observation that innate NK cytotoxicity is independently correlated with ADCC may partly explain why there are conflicting data regarding NK FcγRIIa polymorphisms and prognosis in cancer patients treated with tumor antigen–targeted mAbs. For example, although the FcγRIIa V allele is clearly associated with better prognosis in rituximab-treated patients with follicular lymphoma or with Waldenstrom macroglobulinemia, the same does not hold true in patients with lymphocytic leukemia (4, 5, 19–23). Similarly, two recent studies evaluating clinical responses in cetuximab-treated, KRAS-mutant colon cancer patients yielded conflicting results (6, 7). Ferris and colleagues (24) recently reported results from a phase III RTOG trial of SCCHN patients randomized to receive radiation and cisplatin or radiation, cisplatin, and cetuximab to evaluate the FcγRIIIa-H131R and FcγRIIIa-V158F polymorphisms as predictive biomarkers for cetuximab response. They found that polymorphisms in FcγRIIa and FcγRIIIa did not correlate with OS or PFS and concluded that other immune- and non–immune-mediated mechanisms are likely responsible for clinical activity following treatment with cetuximab (24). We postulate that individual specific variation in innate NK-cell cytotoxicity and tumor susceptibility to ADCC may play a role. In other words, although the FcγRIIIa-V polymorphism accurately characterizes FcγRIIIa-binding affinity, it does not reliably predict a phenotype with enhanced ADCC responsiveness against different tumor types. The significance of this finding is that to reliably identify patients who could benefit from ADCC-inducing antibodies, it may be necessary to characterize innate NK-cell cytotoxicity or ex vivo ADCC as well as the FcγRIIIa genotype.

There may be a variety of factors related to why no association was found between FcγRIIIa polymorphisms and clinical outcomes in this study. First, the relatively small sample of patients in our trial may have not been sufficient to detect a difference, if one exists; however, the HRs obtained did not even suggest a trend toward a difference based on polymorphism. Second, the patients in this study had significant tumor burden and a high rate of previous chemotherapy exposures, both likely resulting in dysfunction of their NK responses compared with those of healthy donors and newly diagnosed patients. Of course, the overall milieu of inflammatory cells within the tumor, which is difficult to measure, may influence FcγRIIIa polymorphism response to cetuximab, as well as NK-cell behavior. For instance, in one study, CD163-positive M2 macrophages with FcγRIIIa V allele had a pronounced tumor-promoting response in the presence of cetuximab (18). Although it is possible that the addition of lenalidomide might conceivably affect the influence of FcγRIIIa genotype, other studies suggest that a negative interaction is unlikely (25). In this study, we present data that suggest that other factors may be more influential and predictive for tumoricidal activity than NK FcγRIIIa polymorphisms.

We also explored the potential interaction of HPV and outcome in our patients with recurrent or metastatic oropharyngeal SCCHN receiving cetuximab and lenalidomide. There is emerging evidence that even among patients with current and metastatic oropharyngeal SCCHN, HPV status portends better OS (26, 27). In this trial, HPV status was the only clinical factor that demonstrated a trend for an association with OS (HR = 0.3; 95% CI, 0.1–1.2; P = 0.09). We did not observe a relationship between HPV status and ADCC inducibility. Our current data show that the strongest predictor of preclinical and clinical efficacy in the presence of cetuximab is ADCC inducibility. In SCCHN patients, an ex vivo functional assay establishing ADCC inducibility was linked with a greater likelihood of stable clinical disease and improved PFS. In addition, our measure of innate immunity correlated more closely with ADCC effectiveness in vitro than FcγRIIIa genotype, suggesting that other factors may be more influential in predicting ADCC response. Therefore, discovering functional assays or markers that predict ADCC inducibility may identify patients who are good candidates for cetuximab-based treatments.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Jain, O. Goloubeva
Development of methodology: A. Jain, M. Nagilla
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Jain, L. Silpino, J. de Souza, T. Seiwert, V. Villaflor
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Jain, O. Goloubeva, S. Kronberg, M. Nagilla, J. de Souza, T. Seiwert, V. Villaflor
Writing, review, and/or revision of the manuscript: A. Jain, O. Goloubeva, S. Kronberg, J. de Souza, T. Seiwert
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kronberg, M. Nagilla, L. Silpino
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References

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