c-Abl Modulates Tumor Cell Sensitivity to Antibody-Dependent Cellular Cytotoxicity

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Abstract

Monoclonal antibodies (mAb) can modulate cancer cell signal transduction and recruit antitumor immune effector mechanisms—including antibody-dependent cellular cytotoxicity (ADCC). Although several clinically effective antibodies can promote ADCC, therapeutic resistance is common. We hypothesized that oncogenic signaling networks within tumor cells affect their sensitivity to ADCC. We developed a screening platform and targeted 60 genes derived from an EGFR gene network using RNAi in an in vitro ADCC model system. Knockdown of GRB7, PRKCE, and ABL1 enhanced ADCC by primary and secondary screens. ABL1 knockdown also reduced cell proliferation, independent of its ADCC enhancement effects. c-Abl overexpression decreased ADCC sensitivity and rescued the effects of ABL1 knockdown. Imatinib inhibition of c-Abl kinase activity also enhanced ADCC—phenocopying ABL1 knockdown—against several EGFR-expressing head-and-neck squamous cell carcinoma cell lines by ex vivo primary natural killer cells. Our findings suggest that combining c-Abl inhibition with ADCC-promoting antibodies, such as cetuximab, could translate into increased therapeutic efficacy of mAbs. Cancer Immunol Res; 2(12); 1186–98. ©2014 AACR.

Introduction

Monoclonal antibodies (mAb) have proven useful in the targeted therapy of cancer, due to their specificity, versatility, and efficacy (1). Approved antibody therapies have multiple mechanisms of action, including perturbation of growth factor signaling, induction of apoptotic activity, and direct cytotoxicity. Antibodies can also recruit immune effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC; ref. 2).

ADCC involves the engagement of antibody, bound to the surface of a target cell, by activating Fc receptors (FcR) on immune effector cells. The low-affinity FcγR, FcγRIIA (FCGR3A), recognizes IgG1 and IgG3 antibody isotypes and is the predominant receptor involved in natural killer (NK)-cell–mediated ADCC (3). NK cells are well-studied effectors of ADCC, but other innate immune cells also contribute (1, 3, 4). The engagement of antibodies and FcRs activates cytoxic degranulation and cytokine release by NK cells (5). Perforin and granzyme released from cytoxic granules initiate apoptosis in targeted cells (3, 6).

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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doi: 10.1158/2326-6066.CIR-14-0083
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translate into enhanced ADCC and increase the clinical utility of mAb therapy.

Materials and Methods

Cell lines, primary cells, and culture

A431, A253, FaDu, HNSCC 1483, SCC-4, SCC-9, and SCC-25 cell lines were obtained from the Georgetown Lombardi Tissue Culture Shared Resource (TCSR). The SCC-61 cell line was provided by Igor Astsaturov (Fox Chase Cancer Center, FCCC). The UM-SCC-11a cell line was provided by John Deeken (Georgetown Lombardi Comprehensive Cancer Center). These cell lines were cultured in high-glucose DMEM (HyClone) supplemented with 10% FBS (Omega Scientific) and 2 mM of l-glutamine (Gibco). NK92-CD16V cells were provided by Kerry S. Campbell (FCCC) and maintained as previously described (1, 3, 4, 19). Cell lines were confirmed by short tandem repeat (STR) analysis (TCSR). Frozen primary peripheral blood mononuclear cells (PBMC) from three individual donors (AllCells) were enriched for NK cells (Human NK Cell Enrichment Kit; STEMCELL Technologies), yielding 3.6% to 6.7% of NK cells. The NK92-CD16V cells were maintained in RPMI-1640 with 10% FBS and were mycoplasma free and verified by short tandem repeat analysis (TCSR). Frozen primary peripheral blood mononuclear cells (PBMC) were maintained in RPMI-1640 with 10% FBS and 2 mM of l-glutamine, and stimulated with 500 units/mL of recombinant human IL2 (Life Technologies). All cells were cultured at 37°C and 5% CO2.

Antibody-independent natural cytotoxicity and ADCC assays

Target cell were seeded or reverse transfected in 96-well white-walled, clear-bottom tissue culture plates (Corning Costar). Pretreatments were added as indicated. At the time of assay, four treatments were added: vehicle (growth media); antibody; effector cells; and antibody with effector cells. Antibody was added at concentrations and effecter cells were added at effector-to-target ratios (E:T) indicated and incubated for 4 hours. CytoTox-Glo (Promega) was used to assess initial and total cytotoxicity signal per manufacturer’s instructions. Specific lysis was determined for antibody-independent natural cytotoxicity (NK92-CD16V cells only) as

\[
\text{Specific lysis} = \frac{\text{Cytotoxicity}_{\text{tar}} - \text{cytotoxicity}_{\text{eff}}}{\text{Total cytotoxicity}_{\text{tar}} - \text{cytotoxicity}_{\text{eff}}} \times 100
\]

and ADCC (effector cells with antibody) as

\[
\text{Specific lysis} = \frac{\text{Cytotoxicity}_{\text{tar}+\text{eff}+\text{mAb}} - \text{cytotoxicity}_{\text{tar}+\text{eff}}}{\text{Total cytotoxicity}_{\text{tar}+\text{eff}+\text{mAb}} - \text{cytotoxicity}_{\text{tar}+\text{eff}}} \times 100
\]

where tar refers to the plated target cells and eff refers to effector cells.

siRNA reverse transfection

All siRNAs, including AllStars Negative Control (siNEG) and Hs Death Control (siDEATH) siRNAs, were from Qiagen. The ERG (siERG) siRNA (ERG_10) target sequence was TAC-GAATATTAAACACTTCAA. Supplementary Tables S1 and S2 contain the target sequence for siRNAs used in screens. siRNAs working stocks were 1 mM in siRNA Suspension Buffer (Qiagen). Lipofectamine RNAiMAX (Invitrogen) was diluted in OptiMEM, and 10 mM of siRNA or v/v Suspension Buffer was added. Transfection mixtures were incubated for 10 minutes, plated, and overlaid with cells.

Screening

Arrayed 96-well siRNA library plates were used with the 60 inner wells containing two pooled siRNAs per gene (Supplementary Table S1). Control siRNAs and treatment control wells were included in the 36 outer wells for quality control and normalization.

A431 cells were reverse transfected with library plate siRNAs into assay plates using fluidic instrumentation. First, 10 mM of diluted Lipofectamine RNAiMAX transfection reagent in OptiMEM (Invitrogen)—0.3 µL of RNAiMAX per 10 µL of OptiMEM—was plated by Combi-nl (Thermo Scientific). Then, 10 µL of 100 mM/L siRNAs were aliquoted from library plates into the diluted transfection reagent using a CyBi-Well vario (CyBio) and incubated at room temperature for 10 minutes. A431 cells (5000) in 80 µL were overlaid using a WellMate (Thermo Scientific), resulting in 10 mM/L of siRNA. Duplicate plates were reverse transfected for each treatment.

At 48 hours, the reverse-transfected cells were treated with cetuximab, NK92-CD16V effector cells, or cetuximab and NK92-CD16V effector cells. Cetuximab was used at 1 µg/mL. NK92-CD16V effector cells (20 000) were added to approximately 1:1 E:T. Treatments were added to duplicate plates using the Combi-nl.

Cytotoxicity values were normalized per plate by the within-plate median of the vehicle-treated negative control siRNA cytotoxicity. ADCC-specific lysis and fold changes were calculated relative to negative controls. Differential cytotoxicity was

\[
\text{Differential cytotoxicity} = \frac{(\text{siRNA cytotoxicity}_{\text{tar}+\text{eff}+\text{mAb}} - \text{siNEG cytotoxicity}_{\text{tar}+\text{eff}+\text{mAb}}) - (\text{siRNA cytotoxicity}_{\text{tar}+\text{eff}} - \text{siNEG cytotoxicity}_{\text{tar}+\text{eff}})}{(\text{siRNA cytotoxicity}_{\text{tar}+\text{mAb}} - \text{siNEG cytotoxicity}_{\text{tar}+\text{mAb}})}
\]

ANOVA was conducted with Dunnett multiple comparison correction. Secondary screens are described in Supplementary Methods.

Quantitative reverse transcription PCR

Reverse-transfected cells were collected at 48 hours and processed using the Fast SYBR Green Cells-to-Ct Kit (Ambion) per manufacturer’s instructions. QuantiTect Primer Assays (Qiagen) were used (Supplementary Table S3). Quantitative PCR was conducted on a 7900HT in 96-well MicroAmp plates (Applied Biosystems). Relative quantitation was assessed by the comparative C\(_t\) (2\(^{-\Delta\Delta\,C_t}\)) method calibrated to GAPDH (GAPDH_1_SG) level.

Real-time cell assay

The xCELLigence real-time cell assay (RTCA) with 96-well E-plates (Roche) was used per manufacturer’s instructions.
E-plates with media or reverse-transfection components were equilibrated in an RTCA system at 37°C in 5% CO₂. Background measurements were obtained, cells were added, and the assay was started. Cell index was measured every 10 minutes. Treatments were added by pausing, aliquoting, and restarting the assay.

**Viability and proliferation assays**

CellTiter-Blue (Promega) assays were conducted in 96-well format per manufacturer’s instructions.

**c-Abl overexpression and rescue**

Wild-type c-Abl in the pCEFL-AU5 plasmid was kindly provided by Anna Riegel (Georgetown Lombardi). Cells were seeded overnight to 50% to 60% confluence. Forward transfection of c-Abl plasmid and/or 10 nmol/L siRNA was conducted using Lipofectamine 2000 (Invitrogen) per manufacturer’s instructions.

**Kinase inhibitors and treatments**

Imatinib mesylate (Selleck Chemicals) and dasatinib, ponatinib, and nilotinib (kindly provided by John Deeken) were solubilized in DMSO. Cells seeded overnight were treated with inhibitors diluted in growth media for 48 hours. Vehicle treatment (DMSO) was used at the highest equivalent v/v.

**Western blotting**

Cells were lysed in RIPA buffer with EDTA (Boston BioProducts) supplemented with protease (Roche) and phosphatase inhibitors (Pierce). Cleared lysate concentrations were obtained by DC Protein Assay (BioRad). Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Western blot analyses were conducted using rabbit anti-Abl [Cell Signaling Technology (CST); #2862], rabbit anti-pCrkl (CST; #3181), mouse anti-CrkL (CST; #3182), rabbit anti-EGFR (Abcam; Clone EP38Y), and mouse anti-β-actin (Sigma; Clone AC-74). Goat anti-rabbit or donkey anti-mouse IgG horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) were used with chemiluminescence substrates (Pierce). Densitometry was assessed by ImageJ (5, 20).

**Data analysis**

Data analysis was conducted in R with the reshape and multcomp packages (3, 6, 21–23). Multiple comparison test correction was used to obtain adjusted P values by the single-step method (7, 23). Otherwise, two-tailed t tests were conducted. Statistical significance was obtained when P < 0.05.

**Results**

**Development of a functional genomics screen for ADCC**

Several methods used to assess ADCC, such as chromium-release and flow-based apoptosis detection assays, are low throughput and not easily scaled for screening. Therefore, we developed a high-throughput screening platform for assessing the effect of RNAi on tumor cell sensitivity to ADCC. The platform used siRNAs arrayed in 96-well format for reverse transfection of adherent target cells. Our model system included A431 squamous cell carcinoma cells, which overexpress EGFR; cetuximab, an IgG1 anti-EGFR antibody capable of engaging FcRs on NK cells; and NK92-CD16V effector cells, an NK-like cell expressing an FCGR3A (CD16) variant that induces ADCC (8–12, 19).

We adapted a luminescence-based cytotoxicity assay for screening. This assay used protease activity released from dying cells, exhibited greater sensitivity with similar background versus a lactate dehydrogenase release assay (data not shown), and provided a two-step cytotoxicity measurement from each well. The assay was capable of assessing cytotoxicity across a range of both E:T ratios and cetuximab concentrations (Fig. 1A). Specific lysis, a measurement of NK-cell–mediated antibody-independent natural cytotoxicity and ADCC, was readily derived (Fig. 1B). Antibody-independent natural cytotoxicity was low (0%–15%) across the range of E:T (1:1–4:1; Fig. 1B and Supplementary Fig. S1A).

We assessed two additional antibodies to verify that ADCC was specific to antigen-bound antibody engagement of NK cells. Rituximab (anti-CD20) does not bind A431 target cells due to lack of antigen expression, and panitumumab (anti-EGFR) binds A431 cells, but has limited capacity to engage NK92-CD16V FcγRIIa. Neither rituximab (Supplementary Fig. S1B and S1C) nor panitumumab (Supplementary Fig. S1D and S1E) promoted ADCC.

Next, we identified conditions for siRNA reverse transfection wherein modulation of ADCC could be detected. Surface expression of EGFR has been correlated with magnitude of cetuximab-mediated ADCC (24). We confirmed that siRNA knockdown of EGF significantly reduced EGFR surface expression (Fig. 1C) and ADCC (Fig. 1D).

Because we aimed to identify genes whose knockdown enhanced sensitivity to ADCC, we sought assay conditions with modest levels of ADCC. At an E:T of 1:1 and using 1 µg/mL of cetuximab, ADCC-specific lysis plateaued at approximately 30% (Fig. 1B). We anticipated that siRNA knockdown could enhance this level of ADCC, outside of the antibody concentration-dependent range of ADCC (Fig. 1B; 1–100 ng/µL). Z-factor, a measure of dynamic range and signal-to-noise, was assessed to determine robustness for screening (25). Under our prescribed conditions, a Z'-factor ≥ 0.5 indicated that assay conditions were suitable for screening (Fig. 1E; Z’-factor = 0.51).

**Screening for sensitizers of ADCC**

In a prior screening study of an EGFR-centered siRNA library targeting 638 genes, knockdown of 61 genes enhanced the effectiveness of EGFR-targeted therapeutics, independent of ADCC (26). We assessed whether knockdown of any of those 61 genes could also enhance cetuximab-mediated ADCC, aiming to find gene targets wherein two antitumor effects—synthetic lethality and ADCC sensitivity—would be enhanced. An arrayed library of pooled siRNAs...
was prepared, with the exception of one target gene, RPL10P16, because it was reannotated as a pseudogene (Supplementary Table S1).

In two primary screens, A431 cells were reverse transfected with two pooled siRNAs per target gene. Two days later, three treatments were added to the transfected cells: cetuximab; NK92-CD16V cells; and the combination of cetuximab and NK92-CD16V treatments. ADCC sensitivity was assessed after 4 hours and normalized by total A431 cytotoxicity (see Materials and Methods). ADCC-specific lysis was determined for the absence (−cetuximab) or presence of 1 μg/mL of cetuximab. Specific lysis of A431 cells by NK92-CD16V cells following knockdown of EGFR (siEGFR, black bars) was compared with negative control siRNA (siNEG, gray bars) in the absence and presence of cetuximab. **; P < 0.001 from two-tailed t test. E, A431 cells seeded overnight in 96-well plates were treated with 1:1 E:T of NK92-CD16V cells in the absence (“natural cytotoxicity,” squares) or presence (“ADCC,” triangles) of 1 μg/mL of cetuximab. Cytotoxicity was assessed 4 hours later. Z'-factor, which measures the suitability of an assay for screening, was assessed between the ADCC vs. natural cytotoxicity treatments. Dashed lines, mean of sample replicates, and shaded area, ±3 SD from the mean. The Z'-factor incorporates both sample mean and ±3 SD. For all panels, error bars represent SD of at least three independent experiments (n = 3).

**Figure 1.** Development and characterization of a functional genomics screening assay for assessment of ADCC. A, A431 cells were seeded overnight in 96-well plates and then treated with vehicle (media), cetuximab only, NK92-CD16V only, and combined cetuximab and NK92-CD16V treatments. Cytotoxicity signal (relative luminescence units, RLU) was assessed 4 hours later across a range of concentrations of cetuximab (0–10 μg/mL) and E:T ratios (0:1–4:1). B, specific lysis was calculated from the cytotoxicity signal values in A by subtraction of initial A431 cytotoxicity and NK92-CD16V spontaneous cytotoxicity from combined treatment-induced cytotoxicity, before normalization by total A431 cytotoxicity (see Materials and Methods for details). C, A431 cells were reverse transfected with 10 nmoL EGFR siRNA (siEGFR) or negative control siRNA (siNEG) and collected 48 hours later. Cells were stained with cetuximab and anti-human IgG(H + L)-PE–conjugated secondary antibody to assess EGFR surface expression or with secondary antibody alone. Secondary Ab-only staining was similar to unstained controls (not shown) for all transfected cell lines. D, A431 cells were reverse transfected as in C and assessed for ADCC 48 hours later. Specific lysis was assessed using a 4:1 E:T using NK92-CD16V effector cells in the absence (−cetuximab) or presence of 1 μg/mL of cetuximab. Specific lysis of A431 cells by NK92-CD16V cells following knockdown of EGFR (siEGFR, black bars) was compared with negative control siRNA (siNEG, gray bars) in the absence and presence of cetuximab. **; P < 0.001 from two-tailed t test. E, A431 cells seeded overnight in 96-well plates were treated with 1:1 E:T of NK92-CD16V cells in the absence (“natural cytotoxicity,” squares) or presence (“ADCC,” triangles) of 1 μg/mL of cetuximab. Cytotoxicity was assessed 4 hours later. Z'-factor, which measures the suitability of an assay for screening, was assessed between the ADCC vs. natural cytotoxicity treatments. Dashed lines, mean of sample replicates, and shaded area, ±3 SD from the mean. The Z'-factor incorporates both sample mean and ±3 SD. For all panels, error bars represent SD of at least three independent experiments (n = 3).
secondary screens were conducted and analyzed similarly to primary screens (Supplementary Fig. S2C and S2D). These secondary screens identified three of the primary hits—GRB7, PRKCE, and ABL1—whose knockdown by at least two independent siRNAs significantly enhanced ADCC (Supplementary Fig. S2C and S2D and Table 1, adjusted \( P < 0.05 \)). Knockdown of these three genes, along with all screened genes, was confirmed at the transcript level by real-time quantitative reverse-transcription PCR (qRT-PCR; Fig. 2B). Relative expression of transcripts after knockdown ranged from 1% to 60% and did not correlate with ADCC-specific lysis (Supplementary Fig. S2E).

**Characterization of the effects of ABL1 knockdown on proliferation and sensitivity to ADCC**

We focused on ABL1 as several clinically useful inhibitors target c-Abl kinase activity. After identifying ABL1 as a putative modulator of ADCC, we further characterized the effects of its knockdown. An RTCA system was used to assess cell index, a surrogate measurement of adhesion, viability, and proliferation. By cell index, NK-cell and ADCC responses were detected three of the primary screens (Supplementary Fig. S2C and S2D). Secondary screens identifi ed three of the primary screens (Supplementary Fig. S2C and S2D). Secondary screens were conducted and analyzed similarly to primary screens (Supplementary Fig. S2C and S2D). These secondary screens identified three of the primary hits—GRB7, PRKCE, and ABL1—whose knockdown by at least two independent siRNAs significantly enhanced ADCC (Supplementary Fig. S2C and S2D and Table 1, adjusted \( P < 0.05 \)). Knockdown of these three genes, along with all screened genes, was confirmed at the transcript level by real-time quantitative reverse-transcription PCR (qRT-PCR; Fig. 2B). Relative expression of transcripts after knockdown ranged from 1% to 60% and did not correlate with ADCC-specific lysis (Supplementary Fig. S2E).
Reduced cell index relative to control siRNA (Fig. 3A, top left). Viability assays across a comparable time series demonstrated similar effects of ABL1 knockdown on proliferation (Fig. 3B).

Seven days after transfection, A431 cells recovered from the effects of transient ABL1 knockdown by cell index (Fig. 3A, top left) and viability (Fig. 3B) assessments.

### Table 1. Validated gene targets whose knockdown by at least two independent siRNAs significantly enhanced ADCC

| Gene target | Specific lysis, P < 0.05 | Differential cytotoxicity, P < 0.05 |
|-------------|--------------------------|-----------------------------------|
| GRB7        | siGRB7_7, siGRB7_3       | siGRB7_7, siGRB7_3                |
| PRKCE       | siPRKCE_5, siPRKCE_2     | siPRKCE_5                         |
| ABL1        | siABL1_9 ("siABL1 #1"), siABL1_10 ("siABL1 #2") | -                                  |

NOTE: Validation was confirmed by enhanced specific lysis (Fig. 2A) and/or differential cytotoxicity (Supplementary Fig. S2B) by at least two independent siRNAs in secondary screens. siRNA target sequences can be found in Supplementary Table S2.

*aUsed in subsequent characterization studies (Fig. 3C and D).

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Figure 3. Characterizing the effect of ABL1 knockdown on proliferation, viability, and sensitivity to ADCC in A431 cells. A, A431 cells were reverse transfected with 10 nmol/L ABL1 (siABL1, gray) or negative control siRNA (siNEG, black) in a 96-well RTCA plate. Cell index was measured by RTCA every 10 minutes over the entire time course of the experiment (9 days). Cell index was derived from the impedance of adhered cells in the individual wells of the RTCA plate. Following reverse transfection, treatments were added at 48 hours (dashed line) as follows: top left, vehicle (media); top right, 1 μg/mL of cetuximab; bottom left, 20,000 NK92-CD16V effector cells; bottom right, 1 μg/mL of cetuximab and 20,000 NK92-CD16V effector cells. Each cell index line represents the mean of three independent assessments (n = 3) from one representative of three experiments. B, A431 cells were reverse transfected with 10 nmol/L of ABL1 siRNA (siABL1, gray) or negative control siRNA (siNEG, black) in 96-well format. Fluorometric viability assays were conducted at the indicated time points. Each viability measurement represents the mean of three independent measurements (n = 3) from one representative of four experiments. C, A431 cells were reverse transfected in six-well plates with 10 nmol/L of ABL1 siRNA (siABL1 #1 or #2, gray bars) or negative control siRNA (siNEG, black bars). siABL1 #1 is the same ABL1 siRNA used in A. Reverse-transfected cells were collected at 48 hours and replated at 20,000 viable cells per well in a 96-well plate. After a brief incubation, the following treatments were added: vehicle (media); cetuximab (0.01, 0.1, and 1 μg/mL); 20,000 NK92-CD16V effector cells; and 20,000 NK92-CD16V effector cells and cetuximab (0.01, 0.1, and 1 μg/mL). Cytotoxicity was assessed 4 hours later, and specific lysis by NK92-CD16V cells was determined in the absence or presence of cetuximab. Each ABL1 siRNA was compared with the negative control siRNA within each subpanel. ∗P < 0.05; ∗∗P < 0.01; and ∗∗∗P < 0.001 from two-tailed t tests. Results are from three independent experiments (n = 3). D, A431 cells were reverse transfected without siRNA (–) or with 10 nmol/L of negative control (siNEG) or ABL1 siRNA (siABL1 #1 or #2 as used in C). Cell lysates were collected, applied to SDS-PAGE, and transferred to a membrane, and blotted for c-Abl. c-Abl expression was undetectable for the two ABL1 siRNAs transfections, although faint bands were visible upon overexposure (data not shown). The same membrane was blotted for β-actin as a loading control. Results are representative of two experiments. For B and C, error bars represent SD of the mean.
We also used RTCA to assess the effects of ABL1 knockdown on ADCC. As evident in Fig. 3, cetuximab (top right) or NK92-CD16V cell (bottom left) modestly enhanced the effects of ABL1 knockdown alone (top left). However, the combined ADCC treatment dramatically reduced cell index in ABL1 knockdown cells (Fig. 3A, bottom right). By the end of the assay, the negative control siRNA-transfected cells had recovered from the ADCC treatment, whereas ABL1 knockdown cells were at background (Fig. 3A, bottom right). Endpoint endpoint assay showed that c-Abl expression was assessed by flow cytometry following cotransfection partially rescued endogenous c-Abl knockdown by ABL1 siRNA to approximately 80% of the endogenous c-Abl levels (Fig. 4D).

Because ABL1 knockdown reduced the proliferation of A431 cells, it was plausible that specific lysis was enhanced due to differences in relative ET at the time of ADCC assay. ABL1 knockdown resulted in 50% to 60% reduction of A431 cells at 48 hours, when assay treatments were added (Fig. 3A, top left, and and B). Because NK92-CD16V cells were added at the same density to A431 cells in the negative control and ABL1 knockdown conditions, relative ET was approximately 2-fold higher in the latter, which alone could account for an increase in ADCC-specific lysis (Fig. 1B).

To account for these effects of ABL1 knockdown, we used a modified ADCC assay. Negative control and ABL1 knockdown A431 cells were collected 48 hours after transfection, replated at equivalent density, and assayed. With these modifications and using two independent siRNAs, ABL1 knockdown significantly enhanced ADCC, with a minor effect on cetuximab-independent NK-cell natural cytotoxicity (Fig. 3C). ABL1 knockdown by the two independent siRNAs resulted in c-Abl levels undetectable by Western blot (Fig. 3D).

EGFR expression is correlated with magnitude of ADCC, which we (Fig. 1C and D) and others have demonstrated (24). Enhanced ADCC following ABL1 knockdown could be due to increased EGFR surface expression, as c-Abl activity has been shown to regulate EGFR endocytosis (27). Therefore, EGFR surface expression was assessed by flow cytometry following ABL1 knockdown. In A431 cells reverse transfected with ABL1 siRNA, EGFR surface expression was unchanged compared with controls (Supplementary Fig. 5A and S4B).

c-Abl expression and sensitivity to ADCC

Knockdown of c-Abl by siRNA enhanced the sensitivity of A431 cells to ADCC, independent of its effects on proliferation. We further investigated whether the level of c-Abl could regulate ADCC responsiveness. A wild-type c-Abl plasmid expression construct was forward transfected into A431 cells, and ADCC sensitivity was assessed. Because c-Abl overexpression has been shown to disrupt the cell cycle (28), minimal quantities (1–50 ng) of the plasmid were used in short-term (24 hours) transfections. Increasing quantities of transfected c-Abl plasmid were associated with concomitant decreases in ADCC sensitivity, without altering antibody-independent natural cytotoxicity (Fig. 4A). We confirmed that c-Abl expression was proportional to the amount of plasmid transfected by Western blot (Fig. 4B).

We hypothesized that c-Abl expression could rescue the effects of ABL1 knockdown. We selected an ABL1 siRNA that targets the 3’-untranslated region of endogenous c-Abl transcript (siABL1 #1 from Fig. 3D and Supplementary Table S2), but not the c-Abl plasmid, which contains only the ABL1 coding sequence. In short-term forward transfections, siRNA knockdown of ABL1 enhanced ADCC (Fig. 4C) while reducing c-Abl expression by approximately 50% (Fig. 4D). c-Abl plasmid cotransfected with a negative control siRNA reduced ADCC sensitivity (Fig. 4C) with a modest increase in c-Abl expression of approximately 20% (Fig. 4D).

Inhibition of c-Abl kinase activity and sensitivity to ADCC

c-Abl is a ubiquitously expressed protein with pleiotropic roles in growth factor response, cell cycle, DNA damage, apoptosis, and cytoskeletal dynamics reflected in its functional domains (29). The N-terminus of c-Abl contains regulatory Src Homology 3 (SH3) and SH2 domains and a kinase catalytic core, much like c-Src. To dissect the role of c-Abl on modulation of ADCC, we used imatinib to inhibit c-Abl tyrosine kinase activity. Imatinib was the first approved kinase inhibitor of the fusion gene product, Bcr-Abl, found in chronic myelogenous leukemia. Beyond inhibiting the constitutive kinase activity of the fused c-Abl kinase domain, imatinib also inhibits endogenous c-Abl, platelet-derived growth factor receptors, and c-Kit (30).

We pretreated A431 cells with imatinib to assess the effects of c-Abl kinase inhibition on ADCC sensitivity. Just before assessment, we replaced the imatinib treatments with fresh growth media to focus on target cell effects and limit potential off-target effects on NK92-CD16V effector function (31). Pretreatment of A431 cells with imatinib for 48 hours significantly enhanced ADCC (Fig. 5A, 10 \( \mu \)mol/L of imatinib with 0.1 or 1 \( \mu \)g/mL of cetuximab and NK92-CD16V cells). Higher doses of imatinib resulted in significant direct cytotoxicity to A431 cells independent of ADCC (data not shown). Imatinib pretreatment of A431 cells also significantly enhanced ex vivo IL-2-stimulated human NK-cell–mediated ADCC (Fig. 5B).

We next assessed phospho-CrkL (p-CrkL), an endogenous surrogate for c-Abl kinase activity (32). A concentration-dependent reduction in p-CrkL versus total CrkL was observed by Western blot, with the most reduction at 10 \( \mu \)mol/L imatinib (Fig. 5C, 20% of vehicle). Because ABL1 knockdown inhibited proliferation of A431 cells (Fig. 3B), we assessed the effect of imatinib on proliferation. Imatinib pre-exposure did not affect the proliferation of A431 cells over 48 hours (Fig. 5D). Similar to ABL1 knockdown, 10 \( \mu \)mol/L imatinib did not affect EGFR surface expression (Supplementary Fig. S4C and S4D).

We assessed additional tyrosine kinase inhibitors, including dasatinib, nilotinib, and ponatinib, that have been developed to treat Bcr-Abl–positive cancers. These inhibitors have been shown to inhibit c-Abl as well as several other kinases (33). Dasatinib (Sprycel) is a second-generation dual Src/Abl tyrosine kinase inhibitor with a pan-tyrosine kinase inhibitor
profile (33). Dasatinib pretreatment of A431 cells demonstrated concentration-dependent inhibition and complete abrogation of ADCC (Supplementary Fig. S5A; 100 nmol/L).

Nilotinib (Tasigna) is a second-generation Abl inhibitor with a kinase profile similar to imatinib (33). Nilotinib pretreatment enhanced ADCC in a concentration-dependent manner with a modest, but significant, enhancement at the highest concentration assessed (Supplementary Fig. S5B; 1 nmol/L). Ponatinib (AP24534) is a third-generation c-Abl inhibitor with dual Src/Abl tyrosine kinase activity like dasatinib, but with the capacity to inhibit Bcr-Abl mutations associated with resistance (34). Ponatinib significantly enhanced ADCC at subnanomolar concentrations (Supplementary Fig. S5C; 0.01 and 0.1 nmol/L), but inhibited ADCC of A431 cells at higher concentrations (Supplementary Fig. S5C; 10 and 100 nmol/L). Ponatinib also had significant, but inconsistent, effects on antibody-independent natural cytotoxicity (Supplementary Fig. S5C; 0.1 and 100 nmol/L).

As c-Src and Src family kinases (SFK) are important for NK-cell activity, we hypothesized that inhibition of ADCC with dasatinib and ponatinib was due to c-Src inhibition of NK-cell function (35, 36). Even with pretreatment and replacement before ADCC assessment, abrogation of ADCC still occurred (Supplementary Fig. S5A and S5C). However, it was plausible that inhibition of c-Src or other SFKs in targeted cells protected them from ADCC. To clarify the role of c-Src on target cell susceptibility to ADCC, we assessed the effects of SRC knockdown in A431 cells. SRC knockdown in A431 cells did not change their sensitivity to ADCC (Supplementary Fig. S5D).

**Imatinib inhibition of c-Abl kinase activity in EGFR-expressing HNSCC lines and sensitivity to ADCC**

Cetuximab-mediated ADCC has been reported against HNSCC cell lines and is a purported mechanism of therapeutic activity (14, 37). To examine the translational potential of imatinib on cetuximab-mediated ADCC, we assessed a panel of eight HNSCC cell lines: A253, FaDu, HNSCC 1483, SCC-4, SCC-9, SCC-25, SCC-61, and UM-SCC-11a. Using our NK92-CD16V model system, imatinib pretreatment of A253, FaDu, HNSCC 1483, and UM-SCC-11a...
cells significantly enhanced cetuximab-mediated ADCC (Fig. 6A). Imatinib also significantly enhanced antibody-independent natural cytotoxicity of HNSCC 1483 and UM-SCC-11a cells (Fig. 6A). SCC-9 and SCC-25 demonstrated no change, and SCC-4 and SCC-61 cell lines had significantly reduced ADCC after imatinib treatment (Supplementary Fig. S6A). Using ex vivo IL2-stimulated NK cells, we found that imatinib pretreatment enhanced ADCC of A253, FaDu, HNSCC 1483, and UM-SCC-11a cells (Fig. 6B). As was demonstrated with NK92-CD16V cells (Fig. 6A), imatinib pretreatment also enhanced natural cytotoxicity of UM-SCC-11a cells by ex vivo NK cells (Fig. 6B). In the cell lines where imatinib enhanced ADCC, we assessed whether imatinib also affected target cell viability. Imatinib treatment was associated with significant reduction in UM-SCC-11a viability, whereas A253, FaDu, and HNSCC 1483 cells were unaffected (Fig. 6C).

We next assessed imatinib-treated A253, FaDu, HNSCC 1483, and UM-SCC-11a cell lines for expression of EGFR, c-Abl, or total CrkL. (Fig. 6D; Supplementary Fig. S6B). EGFR expression was evident and varied, with the most expression in HNSCC 1483 cells. Imatinib treatment of the FaDu cell line was associated with a modest increase in EGFR (1.3-fold), c-Abl (1.4-fold), and CrkL (1.2-fold) expression compared with vehicle-treated cells (Supplementary Fig. S6B). HNSCC 1483 cells had a modest increase in c-Abl (1.2-fold) expression with imatinib treatment (Supplementary Fig. S6B). For A253 and UM-SCC-11a, imatinib did not result in apparent changes in expression of EGFR, c-Abl, or total CrkL. For all cell lines, imatinib treatment—regardless of changes in c-Abl expression—correlated with reduced CrkL phosphorylation (Fig. 6D; Supplementary Fig. S6B, p-CrkL vs. total CrkL ≤ 0.3-fold).

Because imatinib-treated FaDu cells had a slight increase in EGFR expression by Western blot, we assessed EGFR surface expression in A253, FaDu, HNSCC 1483, and UM-SCC-11a cells by flow cytometry. Relative EGFR surface expression across the cell lines was similar to levels detected by Western blot (Supplementary Fig. S6C). Imatinib treatment was not associated with a significant change in EGFR surface expression across all cell lines assessed (Supplementary Fig. S6D).

Discussion

The anti-EGFR mAb cetuximab, which is capable of inducing ADCC, plays an important role in the treatment of colorectal and HNSCC cancers. Although KRAS mutations have been associated with cetuximab resistance, FcγRI polymorphisms were independent of KRAS status in predicting clinical
Figure 6. Inhibition of c-Abl kinase activity by imatinib and sensitivity of HNSCC cell lines to ADCC. A and B, A253, FaDu, HNSCC 1483, and UM-SCC-11a cells were seeded overnight in 96-well plates and treated for 48 hours with vehicle (0, DMSO) or imatinib (10 μmol/L). Treatments were aspirated and replaced with fresh growth media just before addition of effector cells in the absence or presence of cetuximab. Cytotoxicity was assessed 4 hours later, and specific lysis was determined. For A, 40,000 NK92-CD16V cells were used in the absence or presence of 1 μg/mL of cetuximab. For B, 50,000 IL2 negatively selected, IL2-stimulated NK effector cells were used in the absence or presence of 10 μg/mL of cetuximab. Percent change in specific lysis was quantified to account for varying levels of donor-specific lysis against target cells. Results represent one of two independent experiments using three independent donors (n = 3). C, A253, FaDu, HNSCC 1483, and UM-SCC-11a cells were seeded overnight in 96-well plates and treated for 48 hours with vehicle (0, DMSO) or imatinib (10 μmol/L). Viability was assessed by fluorometric assay. For A, B, and C, imatinib pretreatment was compared with vehicle control within each subpanel. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 by two-tailed t test. Results are from three independent experiments (n = 3) for each cell line. Error bars represent SD of the mean. D, A253, FaDu, HNSCC 1483, and UM-SCC-11a cells were seeded in six-well plates overnight and treated for 48 hours with vehicle (0, DMSO) or imatinib (10 μmol/L). Cell lysates were collected, and Western blot analyses were conducted. c-Abl was blotted before stripping and reblotting for EGFR. p-CrkL was blotted before reblotting for both total CrkL and then β-actin as a loading control. Densitometry was conducted, and relative expression was assessed within each cell line for vehicle and imatinib treatments (Supplementary Fig. S6C). Results are representative of two independent experiments.
outcomes in colorectal cancer, and ADCC is independent of KRAS status in vitro (12, 13, 17). The Cancer Genome Atlas data suggest that KRAS is altered in more than 40% of colorectal cancer cases; however, less than 2% of HNSCCs have alterations in KRAS (6 of 302 cases), in which cetuximab resistance also occurs (38, 39). Therefore, we hypothesized that other components of EGFR oncogenic signaling networks could modulate ADCC response. We targeted a set of EGFR-related genes, assessing how their knockdown could enhance ADCC sensitivity, in addition to ADCC-independent synthetic lethal responses previously reported (26). Screening revealed and stringently validated three genes—GRB7, PRKCE, and ABL1—whose knockdown enhanced ADCC. Silencing of ABL1 expression was phenocopied by imatinib, suggesting a new approach for augmenting the ADCC-mediated antitumor effects of cetuximab.

Previous studies have defined a role for c-Abl in cell-cycle regulation and proliferation (28, 40). We also found that c-Abl silencing affected proliferation. However, imatinib inhibition of c-Abl kinase activity did not affect proliferation in most cell lines assessed. Considering that ABL1 knockdown and imatinib treatment enhanced ADCC, these results suggest a kinase-independent role of c-Abl in proliferation, but a kinase-dependent role of c-Abl in ADCC sensitization.

We investigated the mechanism of enhanced ADCC by ABL1 knockdown or c-Abl kinase inhibition. Although c-Abl has been shown to modulate EGFR endocytosis, and EGFR surface expression positively correlates with ADCC (24, 27), EGFR surface expression was unchanged by ABL1 knockdown or imatinib exposure. c-Abl could regulate apoptosis, which is a critical mechanism of NK-cell–mediated cytotoxicity (6, 29). We assessed apoptosis sensitivity independent of ADCC following ABL1 knockdown in A431 cells, but did not detect differential caspase-3/7 response (data not shown). Fluctuations in target cell membrane cytoskeletal organization can modify ADCC responsiveness (41). Subtle morphologic changes observed following ABL1 knockdown and imatinib treatment (data not shown) have spurred us to investigate the role of cytoskeletal dynamics in ADCC responsiveness. We speculate that c-Abl, perhaps through actin modulation, may modify how targeted cells engage NK cells at the immune synapse (29, 42). Although preliminary flow cytometry studies following ABL1 knockdown in A431 cells have not shown significant differences in conjugation (data not shown), further studies are needed.

Imatinib has complex effects on human NK cells (31, 43), and has been shown to indirectly enhance NK-cell activity, independent of the effects we demonstrate on tumor cell sensitivity to ADCC. Imatinib inhibition of c-Kit in dendritic cells (DC) has been shown to enhance NK-cell activity in vivo (43, 44). Two phase I studies have combined imatinib and IL2 to enhance NK-cell antitumor activity (45, 46). In our studies, imatinib enhanced ADCC in some but not all HNSCC cell lines assessed, suggesting that imatinib also exerts an NK-cell–autonomous effect on tumor cells. Furthermore, our studies of ABL1 knockdown, overexpression, and rescue demonstrate that the effects of imatinib were not attributable to c-Abl inhibition in NK cells. Irrespective of the mechanism by which imatinib promotes ADCC, our findings and the reported effects on NK-cell activity warrant further assessment.

While investigating the effects of c-Abl inhibition on tumor cells, we found that two Src/Abl inhibitors, dasatinib and ponatinib, abrogated ADCC, possibly due to the inhibition of c-Src and/or other SFKs, which are critical for NK-cell activation (5, 36). Although SRC knockdown in target cells did not affect their sensitivity to ADCC, other SFKs inhibited by dasatinib may modulate ADCC sensitivity (33). Our results corroborate prior studies showing dasatinib inhibition of ex vivo NK-cell activity (47, 48). Considering all the evidence at hand, inhibition of c-Abl in tumor cells and c-Kit in DCs—without SFK inhibition in NK cells—could enhance antitumor ADCC.

Primary screening studies were facilitated by the use of A431, an ADCC-sensitive squamous carcinoma cell line, and the NK-like cell line, NK92-CD16, to provide a consistent pool of effector and target cells for large-scale ADCC assays. NK92-CD16 cells constitutively express the activating CD16 necessary for ADCC, but also lack inhibitory coreceptors commonly found on NK cells in vivo. Importantly, the findings of our screening studies were confirmed in a panel of HNSCC cell lines, using ex vivo NK cells from multiple donors. It remains to be seen whether imatinib can also enhance ADCC of colorectal cancer cells and, furthermore, if c-Abl kinase activity is a mechanism of resistance to cetuximab or other ADCC-mediating antibody therapies in patients. Future studies may reveal how c-Abl modulates ADCC and identify the molecular determinants that impart the heterogeneity in imatinib responsiveness we observed in our in vitro and ex vivo studies. It is clear that the functions of c-Abl are diverse and still being unraveled (49). Lastly, additional studies examining how the other genes identified in our screens—GRB7 and PRKCE—mediate ADCC have yielded different mechanisms of action—including sensitivity to apoptosis and regulation of EGFR expression (data not shown)—that are being pursued independently.

Our findings justify further examination of the combination of imatinib and an ADCC-promoting antibody. A distinct challenge in the assessment of ADCC in vivo is the lack of well-characterized xenograft-based animal models that can appropriately recapitulate the anticipated therapeutic effects in patients. Although humanized tumor mouse models do exist, they have not been well characterized to date (50). Instead, we have used this work as a basis for an ongoing phase I trial combining cetuximab with the c-Abl kinase inhibitor, nilotinib, in patients with HNSCC or colorectal cancer (http://clinicaltrials.gov/show/NCT01871311). We anticipate that this trial—and others combining targeted therapies—will reveal efficacious strategies for enhancing antibody-based immunotherapy in cancer.

Disclosure of Potential Conflicts of Interest

L.M. Weiner reports receiving a commercial research grant from Symphogen, is a consultant/advisory board member for Merrimack Pharmaceuticals,

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and has provided expert testimony for Novartis and Abbvie. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.C. Murray, S. Wang  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.C. Murray, L.M. Weiner  
**Writing, review, and/or revision of the manuscript:** J.C. Murray, L.M. Weiner  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.C. Murray, D. Aldeghather, S. Wang, S.A. Jablonski  
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**Acknowledgments**

The authors thank Kerry Campbell, Rishi Surana, and Casey Shuptrine for their critiques and feedback and the Georgetown Lombardi Shared Resources (NCI P30-CA051008) for their expertise.

**Grant Support**

J.C. Murray was supported by a MD-PhD NRSA fellowship, NCI F30-CA165474. L.M. Weiner was supported by NCI R01-CA050633 and P30-CA051008.

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Received May 1, 2014; revised September 8, 2014; accepted October 2, 2014; published OnlineFirst October 9, 2014.
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c-Abl Modulates Tumor Cell Sensitivity to Antibody-Dependent Cellular Cytotoxicity

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Cancer Immunol Res 2014;2:1186-1198. Published OnlineFirst October 9, 2014.