Intrinsic Resistance to Cixutumumab Is Conferred by Distinct Isoforms of the Insulin Receptor

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Abstract

Despite a recent shift away from anti-insulin-like growth factor I receptor (IGF-IR) therapy, this target has been identified as a key player in the resistance mechanisms to various conventional and targeted agents, emphasizing its value as a therapy, provided that it is used in the right patient population. Molecular markers predictive of antitumor activity of IGF-IR inhibitors remain largely unidentified. The aim of this study is to evaluate the impact of insulin receptor (IR) isoforms on the antitumor efficacy of cixutumumab, a humanized mAb against IGF-IR, and to correlate their expression with therapeutic outcome. The data demonstrate that expression of total IR rather than individual IR isoforms inversely correlates with single-agent cixutumumab efficacy in pediatric solid tumor models in vivo. Total IR, IR-A, and IR-B expression adversely affects the outcome of cixutumumab in combination with chemotherapy in patient-derived xenograft models of lung adenocarcinoma. IR-A overexpression in tumor cells confers complete resistance to cixutumumab in vitro and in vivo, whereas IR-B results in a partial resistance. Resistance in IR-B-overexpressing cells is fully reversed by anti-IGF-II antibodies, suggesting that IGF-II is a driver of cixutumumab resistance in this setting. The present study links IR isoforms, IGF-II, and cixutumumab efficacy mechanistically and identifies total IR as a biomarker predictive of intrinsic resistance to anti–IGF-IR antibody.

Implications: This study identifies total IR as a biomarker predictive of primary resistance to IGF-IR antibodies and provides a rationale for new clinical trials enriched for patients whose tumors display low IR expression. Mol Cancer Res; 13(12): 1615–26. ©2015 AACR.

Introduction

For more than two decades, the insulin-like growth factor (IGF) system, which includes receptors (IGF-IR, IGF-IIR, and insulin receptor), ligands (IGF-I and IGF-II), and high-affinity IGF-binding proteins (IGFBP1-6), has been studied with great interest in cancer biology. Although this highly regulated pathway plays a crucial role in the normal development and growth of tissues, its deregulation contributes to tumor initiation, proliferation, and survival (1). Elevated circulating IGF-I levels have been associated with increased cancer risk (2). Conversely, individuals with genetic disorders resulting in low circulating levels of IGF-I and IGF-II are resistant to cancer development (3). In vitro studies have demonstrated the importance of functional IGF-IR for cell transformation induced by viral and cellular oncogenes (4). IGF-IR upregulation was observed in a variety of tumor types, including prostate, breast, colon, and lung cancer and melanoma (5, 6). Moreover, the IGF-IR pathway has also been implicated in the development of resistance to other antitumor modalities, including radiotherapy, chemotherapeutic agents, and targeted therapies (1). Therefore, targeting the IGF-IR pathway represents an attractive strategy for the treatment of various tumor types.

Over the last decade, a number of mAb and small-molecule tyrosine kinase inhibitors (TKI) directed against IGF-IR have made their way into clinical trials (www.clinicaltrials.gov). Anti–IGF-IR mAbs, including cixutumumab, a fully human mAb against IGF-IR, are currently the most clinically advanced molecules. Despite very promising results in preclinical and early phase clinical studies, results from phase III trials have failed to meet expectations (7). It is important to note, that although no significant clinical benefit was observed in the intention-to-treat population, a distinct subset of patients seems to benefit from IGF-IR targeting (8–11). Elucidating molecular markers predictive of antitumor efficacy of anti–IGF-IR therapy, however, is an important and ongoing challenge.

Somatic genetic aberrations are frequently the major determinants of oncogenic and pharmacologic dependence in cancer (12, 13). In most tumors, however, IGF-IR pathway is not altered genetically suggesting that additional nongenomic factors may mediate sensitivity or resistance to IGF-IR–targeted therapies. Intrinsic or acquired resistance to targeted agents frequently results from the activation of alternative receptor tyrosine kinases (RTK), including ERBB, MET, FGFR, and AXL family members (14–18).
Insulin receptor (INSR or IR), which shares up to 70% homology with IGF-IR and is commonly expressed in neoplasms and tumor cells, might be implicated in the resistance to anti–IGF-IR therapy. Alternative splicing of INSR transcript results in two isoforms, IR-A and IR-B, which differ by the exclusion of exon 11 encoding 12 amino acids (19). Although IR-B isoform binds primarily insulin, IR-A is capable of binding both insulin and IGF-II (20). IGF-II upregulation has been reported in numerous tumor types (5, 6) and frequently results from the loss of imprinting (LOI) of the IGF2 gene (21). Additionally, inactivating mutations or loss of heterozygosity of the gene encoding IGF-IR, thought to act as a scavenger for IGF-II, can also contribute to increased IGF-II bioavailability (22, 23). This provides yet another alternate route for IGF-II signaling via the IR and results in mitogenic and antiapoptotic signals in tumors. Deregulated IGF-II expression in tumors and the ability of this ligand to signal through the IR-A in addition to the IGF-IR suggest that endogenous IR expression may be an important determinant of sensitivity to IGF-IR mAbs.

In the present study, we provide evidence that IR, irrespective of isoform type, mediates primary resistance to IGF-IR–targeted therapy and can be used as a potential biomarker for patient selection.

Materials and Methods

Materials

Chemicals and materials were obtained from the following sources: CHAPS (Affymetrix); Cisplatin (Medac GmbH); Linsitinib (OSI-906; Selleckchem); Matrigel (BD Bioscience); Ham’s F12 Nutrient Mix (F-12 HAM); RPMI 1640 medium, Improved Minimum Essential Medium (IMEM), NuPage 4% to 12% Bis-Tris, iBlot Gel Transfer Stacks Nitrocellulose, and puromycin (Invitrogen); plasmids Lv-105-IGF-I, Lv-105-IGF-II, and Lv-105-IGF-IR (GeneCopoeia); recombinant human IGF-I and IGF-II (R&D Systems); Complete Protease Inhibitor Cocktail, PhosSTOP Phosphatase Inhibitor Cocktail, and recombinant human insulin (Roche); Xba-I, Spe-I, Pierce Protein Assay, and Spectra Multicolor Gel LMunescent Cell Viability Assay (Promega); pemetrexed disodium heptahydrate (Eli Lilly).

Antibodies

The following antibodies were purchased from commercial sources as indicated: mouse mAbs against Akt (#2920) and p42/p44 MAPK (Erk1/2; #9101), rabbit mAbs against phospho-AktSer473 (#4060) and phospho-IGF-IR beta (Thermo Scientific); Insulin Receptor beta (Ab35175; Biolegend); rabbit polyclonal antibody against phospho-p42/p44 MAPK (Erk1/2; #9101; Cell Signaling Technology); mouse mAb against IGF-IR (#MS-641-P) and Insulin Receptor (#MS-632-P; Thermo Fisher Scientific); rabbit polyclonal Insulin Receptor (#241) and (#251); Santa Cruz Biotechnology; mouse mAb against IGF-II (MAB292) and goat F(ab’)2 anti-mouse IgG-phycoerythrin (#F01028; R&D Systems); goat polyclonal anti-mouse IRDye 680 conjugated (#926-32220) and anti-rabbit IRDye 800 conjugated (#926-32211; LI-COR Biosciences). Cixutumumab was supplied by Eli Lilly and Company. Control human IgG was purchased from Equitech-Bio Inc.

IGF-IR, IR-A, and IR-B mRNA analysis in The Cancer Genome Atlas data sets

Receptor isoform expression was quantified using RNA-seq v2 (level 3) data downloaded from The Cancer Genome Atlas (TCGA) Data Portal. mRNA expression levels were estimated by RSEM (http://deweylab.biostat.wisc.edu/rsem/) and then RNA-Seq by Expectation-Maximization (RSEM) expression estimates were normalized to set the upper quartile count at 300. IGF-IR (combined expression of two major IGF-IR isoforms, uc101bon.2 and uc002bul.2), IR-A (uc002mge.1), and IR-B (uc002mgd.1) expression levels were calculated for 6,943 malignant samples representing 21 tumor types.

Patient specimens

Snap-frozen non–small cell lung cancer (NSCLC) and colorectal carcinoma tumor samples were provided by CureLine, Indi- vumed, and Tissue Solutions. Specimens were collected in compliance with all applicable regulations.

Cell cultures

Cell lines used in this study were obtained from the ATCC. A549, NCI-H1299, and MCF-7 cells were cultured in F-12 HAM, RPMI 1640, and IMEM medium, respectively, supplemented with 1× GlutaMAX supplement (Life Technologies) and 10% FBS at 37°C in 5% CO2 atmosphere and 95% humidity.

Generation of stable cell lines that overexpress insulin receptor isoforms

Plasmids with cDNA encoding the IR-A and IR-B sequence were generated at Eli Lilly. CDNAS encoding IR isoforms were amplified with 5’TATACGTATGCGCCACCGGGGAAGAAGGG3’ and 5’-TATCTAGACTAAGAAGGATTGGACCGAGTATGGCCACCGGGGGGAAGAAGGG3’ primers, digested with Spe-I and Xba-I and ligated into pLVX-IRES-puro HIV-1-based lentiviral expression vector (Clontech). Lentiviruses were engineered by transfecting plasmids into HEK-293T cells using reagents and protocols provided with the Lenti-X Lentiviral Expression Systems (Clontech). A549, NCI-H1299, and MCF-7 cells were transduced with the respective virus for 48 hours and then cultured in the presence of puromycin (1 μg/mL) for selection of stably transduced A549 (A549-Mock, A549-IR-A, and A549-IR-B), NCI-H1299 (NCI-H1299-Mock, NCI-H1299-IR-A, and NCI-H1299-IR-B), and MCF-7 (MCF-7-Mock, MCF-7-IR-A, and MCF-7-IR-B) variants.

Colonization assay

Colonization assay was performed as described by Llanet and colleagues (24) with some minor modifications. Tested on control (T/C) values below and above 50% were considered sensitive and resistant, respectively.

Animal models

Experimental studies in xenograft models of pediatric solid tumors were conducted through Pediatric Preclinical Testing Program (PPTP) and have been described previously (25). In the in vivo studies including xenograft A549 model with overexpression of IR-A and IR-B isoforms and patient-derived xenograft (PDX) models of lung adenocarcinoma, 5- to 8-week-old female nude mice (nu/nu) were used (The Jackson Laboratory; Harlan Laboratories). To evaluate pharmacodynamics effects of cixutumumab, C57Bl/6 male mice were used (Charles River Laboratories). Animals were maintained under barrier conditions, and experiments were performed according to the institutional protocols (Eli Lilly, Oncotest) in compliance with the NIH Guide for the Care and Use of Laboratory Animals and German Animal
To study the antitumor efficacy of cixutumumab in xenograft A549 model with overexpression of IR isoforms, mice (n = 30 for each variant) were subcutaneously injected with 2 \times 10^7 A549-Mock, A549-IR-A, or A549-IR-B cells resuspended in 100% Matrigel. When tumors reached approximately 250 to 300 mm\(^3\), animals were randomized into two treatment groups (0.9\% USP Saline and cixutumumab; n = 12 for each group). USP Saline [10 \mu L/g of body weight (BW)] and cixutumumab (60 mg/kg BW) were administered i.p. twice weekly for 5 weeks. To evaluate the efficacy of cixutumumab in combination with chemotherapy, nine PDX models of lung adenocarcinoma were employed. LXEA-289, LXEA-297, LXEA-526, LXEA-623, LXEA-629, LXEA-737, LXEA-749, LXEA-923, and LXEA-983 models were derived from tumor specimens of patients treated at the University Hospital in Freiburg, Germany, and directly implanted into nude mice according to the procedure established at Oncotest GmbH. Additional information on the PDX models including clinical annotations and mutational status of genes commonly mutated in lung adenocarcinoma is provided in Supplementary Table S2. The aforementioned models were used to evaluate the antitumor activity of cixutumumab in monotherapy and in combination with cisplatin and pemetrexed, a current standard of care in patients with metastatic nonsquamous NSCLC. Mice were randomized into four treatment groups – cisplatin, pemetrexed, cixutumumab/pemetrexed/cisplatin) hIgG and cixutumumab were used to evaluate the antitumor activity of cixutumumab in patients with metastatic nonsquamous NSCLC. Mice were randomized into four treat-
tment groups (6–7 animals per group) when tumors reach 50 to 250 mm\(^3\) (control, cixutumumab, pemetrexed/cisplatin, cixutumumab/pemetrexed/cisplatin) hIgG and cixutumumab were administered i.p. at 40 mg/kg of BW three times a week for a duration of 3 to 4 weeks. Pemetrexed and its vehicle [0.9\% (w/v) NaCl] were given i.p. at 100 mg/kg of BW, daily except weekends, for 2 to 4 weeks. Cisplatin (3.2 mg/kg BW) and its vehicle (PBS) were administered subcutaneously once weekly for 3 weeks.

Tumor size was measured twice weekly via calipers. Antitumor activity was evaluated as maximum tumor volume inhibition compared with the vehicle control group (optimal T/C values calculated based on median values). Prior to euthanasia, tumors were carefully excised and immediately snap-frozen in liquid nitrogen for further analysis.

RNA extraction and cDNA preparation
Total RNA was isolated from snap-frozen tumor tissue using the TissueLyser with stainless steel beads (5 mm) and the AllPrep DNA/RNA Mini kit from QIAGEN or the MagMAX 96 Total RNA isolation kit from Life Technologies. RNA concentration was determined spectrophotometrically with the OD\(_{260}\) (260/280 > 1.9) and purity was verified with the Agilent Bioanalyzer. RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Life Technologies) and random primers according to the manufacturer's instructions. cDNA was then diluted 1:5 (20 ng/µL) for qPCR use.

PCR primers and probe sets
Commercial TaqMan Gene Expression assays were purchased from Life Technologies for quantification of total IR (INSR, Assay ID: Hs00981655_m1), IGF-IR (IGF-IR, Assay ID: Hs00569566_m1), IGF-I (IGF-I, Assay ID: Hs01547656_m1), IGF-II (IGF-II, Assay ID: Hs01005963_m1), and EGFR (EGFR, Assay ID: Hs01076078_m1). Full-length mRNA transcript sequences for IR-A (NM_01079817) and IR-B (NM_000200) isoforms were retrieved from the NCBI Reference Sequence database. The IR-B TaqMan Gene Expression assay was designed using the Custom TaqMan Assay Design Tool from Life Technologies. Program input specified exon 11 inclusion; final positioning of the probe spanned the exon 10/11 junction. All probes incorporated a minor groove binding moiety and were labeled with a fluorescent dye (FAM) for detection and a nonfluorescent quencher. Custom-made primers/probe sequence for IR-B includes: probe sequence: 5’-TCCCAGA-AAAACCTC-3’, forward primer: 5’-CTGCAAGTGGCTTTTGCGAA-3’. To confirm specificity of the IR-B probe, spiking experiments were performed using plasmids containing cDNA encoding IR-A or IR-B. It was not possible to identify completely specific TaqMan probes to IR-A isoforms, therefore, calculated by subtracting IR-B values from the total IR values.

Gene expression analysis
qPCR was performed in a 96-well format on 100 ng of cDNA (20 ng/µL) with 1 µL of TaqMan Gene Expression Assay (20×) and 10 µL of TaqMan Gene Expression Master Mix (2×) in a final volume of 20 µL. Each sample was tested in triplicate. All assays were run on Applied Biosystems 7500 Fast detection system using standard setting (10 minutes incubation at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute). Absolute values (mRNA copies/µg of cDNA) were derived from a standard curve generated with a serial dilution of plasmid containing the cloned sequence of the target gene. Each target standard curve demonstrated linearity with regression coefficients (r\(^2\) values) above 0.997. All assays had efficiency between 88% and 100% with a standard curve slope between −3.3 and −3.6. Data were extracted with the 7500 Software V2.0.5 (Life Technologies).

Statistical analysis
Results are expressed as the mean ± SEM. Student t test, one-way ANOVA, two-factor ANOVA, ANOVA on repeated measurement (RM-ANOVA) followed by Tukey post-test and mixed model were used for statistical analysis. Mixed model analysis for repeated measures was used for the evaluation of the statistical significance of tumor inhibition. The term mixed model refers to the use of both random and fixed effects in the same analysis, which is ideal for analyzing “unbalanced” data sets (due to death of mice, etc.).

Results
IGF-IR, IR-A, and IR-B expression in human malignancies
To understand the prevalence of IGF-IR, IR-A, and IR-B mRNA expression in patient tumor samples, we evaluated mRNA levels of IGF-IR and IR isoforms using TCGA RNA-seq data from 6,943 samples representing 21 tumor types. These results suggest that expression of all three receptors significantly varies between tumor types and individual tumors (Fig. 1A).

High levels of IGF-IR expression were observed in breast, ovarian, prostate, head and neck, and squamous lung cancer and melanoma. IR-A expression was observed in virtually all tumor types, and was particularly high in clear cell renal cell
carcinoma. Many tumor samples also displayed significant IR-B expression, with highest levels observed in clear cell renal cell carcinoma and hepatocellular carcinoma. We further verified RNA-seq data by a more accurate qPCR analysis of clinical NSCLC and colon cancer samples. Median levels of IGF-IR transcripts were 392, 680, and 328 copies/ng cDNA in lung adenocarcinoma, squamous cell carcinoma of the lung, and colorectal carcinoma, respectively (Fig. 1B). Median levels of IR-A and IR-B mRNA were 230 and 120 copies/ng cDNA in lung adenocarcinoma, 256 and 106 copies/ng cDNA in squamous cell carcinoma of the lung, and 328 and 85 copies/ng cDNA in colorectal carcinoma. Furthermore, in colorectal carcinoma samples IR-A and IR-B expression was several fold higher compared with expression levels of EGFR, a clinically validated target in this tumor type. Taken together, these data indicate that both IR isoforms are broadly expressed in human tumors.
IGF-IR protein levels and antitumor efficacy of cixutumumab monotherapy in preclinical models of pediatric solid tumors in vivo.

A, total IR and IGF-IR protein levels in tumor lysates were measured by Meso Scale Discovery Electrochemiluminescence assay. B, correlation analysis of IR and IGF-IR protein levels and antitumor efficacy of cixutumumab expressed as T/C% values. Correlation coefficient (Pearson r) and P value (P) are indicated.

Total IR expression inversely correlates with antitumor efficacy of cixutumumab monotherapy in preclinical models of pediatric solid tumors

As previously demonstrated (25), single agent cixutumumab exhibited antitumor activity in vivo (T/C < 50%) in 16 of 34 xenograft models of pediatric solid tumors (rhabdoid, Ewing’s sarcoma, rhabdomyosarcoma, glioblastoma, neuroblastoma, and osteosarcoma) tested through PPTP. It is important to state that cixutumumab exhibits equipotent binding to both human and mouse IGF-IR and is therefore suitable for xenograft studies in mice. We observed elevated levels of growth hormone, IGF-I, and insulin in the circulation of mice treated with cixutumumab (Supplementary Fig. S1). Therefore, mouse models properly mimic the pharmacodynamic changes seen in humans (Eli Lilly, data on file; refs. 10, 11).

To understand if baseline IR and IGF-IR expression in tumor tissue is predictive of cixutumumab efficacy in vivo, tissue samples collected from individual tumor models were analyzed for IR and IGF-IR by ECL assay and qPCR. The amount of IR and IGF-IR protein and mRNA varied significantly across different tumor models (Fig. 2). However, a significant correlation was observed between mRNA and protein levels of both IR and IGF-IR (r = 0.82 and 0.91, respectively, P < 0.0001) within individual tumor samples. The impact of the two receptors on the outcome of cixutumumab therapy, though, differed greatly. Although IGF-IR expression levels tended to directly correlate with sensitivity to the antibody, high IR was associated with higher T/C values indicative of de novo or intrinsic resistance (Figs. 2 and 3A and B). Expression of IR isoforms, IR-A and IR-B, was also evaluated by qPCR analysis. Although IR-A was the predominant isoform, ubiquitously and robustly expressed in various pediatric solid tumors (range: 0–406; mean, 157 copies/ng cDNA), expression of IR-B was rather weak (range: 0–210; mean, 12 copies/ng cDNA) and restricted to fewer tumor models. In contrast to total IR, however, expression of either IR isoform failed to correlate with cixutumumab efficacy suggesting that both IR isoforms can potentially contribute to cixutumumab resistance (Fig. 3C and D).

It is known that IGF-I primarily stimulates IGF-IR, whereas IGF-II is capable of activating both IGF-IR and IR-A. We aimed to elucidate which of the two ligands is expressed in pediatric solid tumors. As demonstrated by qPCR, IGF-I expression was barely detectable in most samples (range: 0–1,100; mean, 74 copies/ng cDNA), whereas IGF-II was expressed at markedly high levels (range: 0–107,723; mean, 23,657 copies/ng cDNA) in a significant number of tumor models (Supplementary Table S1). It is therefore plausible that in cixutumumab-treated tumors with high IR expression, IGF-II may be capable of promoting tumorigenesis, bypassing the IGF-IR.

Total IR, IR-A, and IR-B expression correlates with antitumor efficacy of cixutumumab in combination with cisplatin/pemetrexed in PDx models of NSCLC

Clinical data indicate that IGF-IR antibodies display rather weak single agent activity in epithelial tumors, and in most trials they were used in combination with other antitumor agents including chemotherapy. We therefore tried to understand if baseline IGF-IR and IR expression was associated with the antitumor efficacy of cixutumumab used in combination with pemetrexed and cisplatin, which is a standard of care in NSCLC with nonsquamous histology. When combined with chemotherapy, cixutumumab displayed significantly improved efficacy over chemotherapy alone in 3 out of 9 patient-derived models of lung adenocarcinoma (Fig. 4; Supplementary Table S2). To identify predictors of cixutumumab sensitivity or resistance, individual lung adenocarcinoma models were analyzed for baseline levels of total IGF-IR, total IR, IR-A, IR-B, IGF-I, and IGF-II expression by qPCR (Fig. 5A and Supplementary Fig. S2). In contrast to pediatric solid tumors, IGF-IR expression was not predictive of the efficacy of the IGF-IR mAb in lung adenocarcinoma models. Expression of total IR and, more importantly, IR-A and IR-B was associated with the outcome of cixutumumab therapy (Fig. 5B). Of note, although IR-A was the predominant isoform (range: 12–301; mean, 89 copies/ng cDNA), IR-B expression (range: 19–123; mean, 61 copies/ng cDNA) was significantly higher in lung
adenocarcinoma compared with the aforementioned pediatric solid tumors. In addition, IGF-I expression was negligible while IGF-II was expressed (range: 1–2,302; mean, 490 copies/ng cDNA) in most lung adenocarcinoma models at various levels (Supplementary Fig. S2). These results further support our initial observation suggesting that (1) IGF-II rather than IGF-I is expressed in most tumors and (2) both IR isoforms seem to contribute to de novo cixutumumab resistance.

Two insulin receptor isoforms confer resistance to cixutumumab in NSCLC and breast cancer models

To understand if there is a mechanistic link between individual IR isoforms and cixutumumab resistance, we developed stably transduced variants of NSCLC and breast cancer cell lines (A549, NCI-H1299, and MCF-7) that overexpress either IR-A or IR-B. A549, NCI-H1299, and MCF-7 cells display sensitivity to cixutumumab when tested in vitro in anchorage-independent colony formation assay or anchorage-dependent cell viability assay. Furthermore, A549 cells exhibit sensitivity to cixutumumab in nude mice. IR expression was markedly increased in IR-A and IR-B-overexpressing A549, NCI-H1299, and MCF-7 cells as demonstrated by qPCR, Western blot, and/or flow cytometry. Cell surface IGF-IR expression, however, did not differ significantly between A549-Mock and A549-IR-overexpressing variants (Supplementary Fig. S3A). Furthermore, A549-Mock cells were responsive to stimulation with IGF-I, IGF-II, and, to a lesser extent, insulin as exemplified by increased phosphorylation of Akt and/or IGF-IR/IR. ERK1/2 phosphorylation was not significantly altered in mock-transduced A549 cells. Although IGF-I-induced signal transduction was not very different in mock- and IR-overexpressing cells, insulin potently stimulated downstream signaling in both IR-overexpressing variants as demonstrated by increased phosphorylation of IGF-IR/IR, Akt, and ERK1/2. In addition, treatment of A549-IR-A and A549-IR-B cells with IGF-II also resulted in increased phosphorylation of IGF-IR/IR and ERK1/2, which was barely seen in A549-Mock cells (Supplementary Fig. S3B). These results are in agreement with previously published data indicating that both insulin and IGF-II are capable of binding to IR, although IGF-II affinity for IR-B is several fold lower compared with IR-A (26, 27).

The efficacy of cixutumumab was then tested in A549 and NCI-H1299 variants using colony formation assay (Fig. 6A and B). As expected, in A549-Mock and NCI-H1299-Mock variants, the antibody inhibited colony formation by 70% to 80% (T/C 19.5% and 32.8%, respectively). However, IR-A overexpression fully abolished this effect (T/C 93.8% and 76.8% for A549 and NCI-H1299 cells, respectively). Ectopic expression of IR-B resulted in a partial cixutumumab resistance (T/C 45.4% and 57.6% for A549 and NCI-H1299 cells, respectively). Moreover, the efficacy of cixutumumab was tested in MCF-7 variants under anchorage-dependent conditions. In line with soft-agar assay data, the mock-transfected line exhibited cixutumumab
sensitivity (maximum inhibition 60.4%), while the IR-A overexpressing cells were completely resistant (maximum inhibition 0%) and the IR-B overexpressing cells were partially resistant (maximum inhibition 26.9%; Supplementary Fig. S4). To corroborate these findings in vitro, mice bearing A549-Mock, A549-IR-A, or A549-IR-B xenograft tumors were treated with cixutumumab for up to 33 days (Fig. 6C). A549-IR-A tumors grew more rapidly compared with A549-Mock and A549-IR-B xenografts (RM-ANOVA followed by Tukey multiple comparison test; \( P \leq 0.01 \)). More importantly, treatment with cixutumumab resulted in a statistically significant (\( P = 0.0014 \)) tumor growth inhibition in A549-Mock model only. Consistent with our in vitro findings, overexpression of IR-A and, to a lesser extent, IR-B led to cixutumumab resistance.

**IGF-II mediates resistance to cixutumumab in NSCLC cells with IR-B overexpression**

A previous study has shown that IGF-II acting through IR-A could be accountable for the limited efficacy of anti-IGF-IR therapy (24). To understand if IR-B–mediated resistance to cixutumumab is driven by IGF-II, we examined the effect of cixutumumab in the presence or absence of neutralizing anti–IGF-II mAb (clone 75015) in A549-IR-B cells. Both cixutumumab and anti–IGF-II mAb only partially inhibited clonogenic potential of A549 cells with ectopic IR-B overexpression. Combination of the two antibodies, however, completely reversed drug-resistant phenotype suggesting that IGF-II is implicated in the development of cixutumumab resistance in tumor cells with IR-B overexpression (Fig. 7A). We also confirmed that treatment with IGF-II at the concentration capable of binding to IR-B (>20 nmol/L; ref. 28) resulted in marked phosphorylation of IR, Akt, and ERK1/2 in cixutumumab-pretreated A549-IR-B cells (Fig. 7B). A similar approach was employed with lurbinib, a dual IGF-IR/IR inhibitor. In contrast to cixutumumab, lurbinib abrogated IGF-II-mediated Akt and ERK1/2 phosphorylation in A549-IR-B cells (Fig. 7C). Furthermore, lurbinib was equipotent in inhibiting A549 and MCF-7 tumor cells irrespective of insulin receptor isofrom expression (Fig. 7D). Collectively, these data indicate that IGF-II is capable of transducing downstream signal via IR-B in tumor cells with antibody-mediated blockade of IGF-IR.
Discussion

IGF-IR mAbs have been tested in multiple clinical trials (www.clinicaltrials.gov). Although overall results in intention-to-treat population were generally negative, in some clinical trials subsets of patients seem to benefit from IGF-IR therapy (8–11). Robust molecular markers predictive of the efficacy of this class of agents remain largely unidentiﬁed.

The objective of our study was to explore the role of the insulin receptor as a potential negative predictive biomarker for IGF-IR–targeted therapy. To our knowledge, this study is the ﬁrst report demonstrating the adverse impact of both IR isoforms on the efﬁcacy of IGF-IR mAb, cixutumumab.

The role of insulin signaling in cancer biology has recently received a considerable amount of attention. Based on epidemiologic studies, obesity and type II diabetes were found to be associated with increased cancer risk and mortality (29). Compensatory hyperinsulinemia and/or insulin therapy utilized to treat type II diabetes can activate the IR signaling pathway in tumor cells leading to enhanced tumorigenesis (30). Moreover, a number of studies described the role of IR in different tumor types (28). A compensatory mechanism between IGF-IR and closely related IR has been demonstrated: genetic, shRNA-mediated, or pharmacologic inactivation of IGF-IR can result in IR upregulation (31–34). In line with these observations, we found that baseline IR expression in tumors could negatively affect the efﬁcacy of anti–IGF-IR therapy. In our proof-of-concept experiments, we demonstrated that ectopic IR expression is indeed sufﬁcient to alleviate the effect of cixutumumab in otherwise sensitive cell lines. We also analyzed IR expression levels in a large panel of tumor models and noted a negative correlation between IR and the efﬁcacy of cixutumumab.

In previously published studies, the role of individual IR isoforms as a potential mechanism of intrinsic resistance to IGF-IR targeting had not been dissected, although studies in Ewing’s sarcoma cell lines with almost exclusive expression of IR-A (≥100% of total IR) support a role of this isoform in the development of drug resistance (34). The role of IR-B in this process, however, remained poorly understood.

In normal tissue, the two IR isoforms mediate very distinct functions. IR-B, which primarily is expressed in the liver, muscle, and fat tissue, regulates metabolic functions, whereas IR-A,
highly expressed in various tissues during prenatal life, mediates proliferative and antiapoptotic effects (20). It is currently believed that IR-A rather than IR-B is implicated in tumor growth because it is frequently upregulated in tumor tissue (28) and has a greater affinity for IGF-II, a growth factor also known to be markedly overexpressed in some tumors as a result of LOI (35). However, several lines of evidence also indicate that expression levels of the B isoform in tumor cells are quite significant, representing up to 50% of the total IR pool in breast, lung, and colorectal tumors (28). We therefore aimed to understand the roles of the two IR isoforms in the resistance to IGF-IR mAbs. Our results demonstrate that ectopic expression of either IR-A or IR-B in tumor cells resulted in the resistance to cixutumumab in vitro and in vivo, with IR-A inducing a stronger effect compared with IR-B. Expression of either isoform indicated an inverse effect on the antitumor activity of IGF-IR mAb in patient-derived models of lung adenosarcoma, further supporting the role of the “metabolic” B isoform as an important determinant of cixutumumab efficacy.

Earlier publications reported different ligand specificity for the two IR isoforms. Insulin is known to bind to both IR-A and IR-B. IGF-II binds to IR-A with an affinity similar to insulin and seems to be also capable of binding to IR-B with several fold lower affinity (26, 36). Our in vitro experiments showed that treatment with physiologic concentrations of IGF-II induced phosphorylation of IR in both A549 IR-overexpressing cell lines, with downstream PI3K/Akt and MAPK pathway activation. This effect was more prominent in A549-IR-A cells, but was also quite substantial in A549-IR-B cells. Moreover, neutralization of IGF-II in culture media conditioned by A549-IR-B cells was able to restore tumor sensitivity to cixutumumab, suggesting that IR-B-driven resistance to anti–IGF-IR mAb is indeed mediated by IGF-II. Interestingly, A549-IR-B xenografts exhibit sensitivity to cixutumumab initially but became fully resistant to the antibody 4 weeks after treatment initiation. Given that in mice IGF-II expression levels are negligible after birth (35), it is tempting to speculate that IR-B-mediated resistance to IGF-IR mAb can only be observed if large amounts of IGF-II are produced within IR-B-expressing tumor tissue. Because IGF-IR and IR can form hybrid receptors (1), it is also possible that resistance mediated by both IR isoforms is at least in part caused by IGF-II-mediated signaling via hybrid receptors, as a result of ligand binding to the IR arm.
However, because anti–IGF-IR antibodies, including cixutumumab, have been shown to be very proficient at receptor internalization and degradation (37), hybrid receptors are likely effectively neutralized, and therefore not present in abundance on tumor cells following treatment with anti–IGF-IR mAbs. More than any other component of the IGF-IR signaling pathway, IGF-II is frequently deregulated in cancer. It is important to note that mouse may not be the most appropriate species to model tumor resistance to cixutumumab in vivo. In contrast to humans, mice have very low levels of circulating IGF-II. However, in human tumors, IGF-II is suggested to act in an autocrine/paracrine rather than endocrine manner. Loss of IGF2 gene imprinting and IGF-IR downregulation are two fairly common phenomena that increase the bioavailability of IGF-II in the vicinity of IGF-IR and IR on tumor cells (38). Moreover, IGF2 gene is markedly overexpressed in colorectal tumors, and recent studies reported a substantial prevalence of IGF2 amplification in this tumor type (39).

IGF-II targeting may thus represent an interesting alternative to the anti–IGF-IR blockade, with the advantage of reducing signaling via IR. Of note, some pharmaceutical companies have already initiated the development of therapeutic mAbs against IGFs. BI-836845 and MEDI-573 are fully human mAbs currently in preclinical and clinical development, respectively, which neutralize both IGF-I and IGF-II and are efficacious in experimental tumor models (40, 41). In a phase I trial, MEDI-573 demonstrated acceptable safety profile and tolerability with no significant perturbations of glucose homeostasis. The antitumor activity of MEDI-573 was modest (0% responses, 33% stable disease). These safety and efficacy findings should be interpreted with caution as no changes in circulating growth hormone were observed suggesting that negative feedback regulation of growth hormone by IGFs was not altered during treatment with MEDI-573 (42).

IGF-IR TKIs are known to block both IGF-IR and IR tyrosine kinase activity (43), and IGF-II/IR-mediated tumor growth inhibition of tumor cell viability.

**Figure 7.** Insulin-like growth factor II (IGF-II) mediates resistance to cixutumumab in tumor cells overexpressing IR-B. Clonogenic assay in A549 cells that overexpress IR-B (A) in the presence or absence of cixutumumab and/or anti–IGF-II mAb. Represented are means ± SEM, expressed as percentage of colony formation relative to control treated cells. Statistical significance was determined by one-way ANOVA followed by Tukey post-hoc analysis. Statistically significant difference versus control treated cells (a), cixutumumab-treated cells (b), and anti–IGF-II–treated cells (c). B and C, Western blot analysis of signal transduction in A549-IR-B cells treated with IGF-II (25 nmol/L), in the presence or absence of cixutumumab and linsitinib. Serum-starved cells were pretreated with cixutumumab (100 nmol/L) or linsitinib (0.01, 0.1, or 1 mol/L) for 15 minutes and 2 hours, respectively, followed by incubation with rhIGF-II (25 nmol/L) for 10 minutes. Proteins (15–20 μg) extracted from cultured cells were size-fractionated by SDS-PAGE and immunoblotted with antibodies against total Akt and p42/p44 MAPK. D, MCF-7 and A549 cells with or without IR-A or IRB overexpression were treated with increasing concentrations of linsitinib (0.00015–10 μmol/L) for 72 hours, and tumor cell viability was quantified by CellTiter-Glo assay. The results are expressed as the percentage of inhibition of tumor cell viability.
could be suppressed by this class of agents, as demonstrated by the present study. However, the antitumor efficacy of IGF-IR TKIs may require an optimal exposure and tissue distribution, which might be difficult to achieve in patients due to dose limiting toxicities associated with IR inhibition and development of insulin resistance and hyperglycemia in up to 40% of treated patients (43).

Based on the current findings, we argue that discovery of suitable biomarkers to guide patient selection should become a priority to enable successful IGF-IR-targeted therapy. Previous work based on expression profiling in sarcoma and neuroblastoma identified potential predictors of intrinsic and acquired resistance to IGF-IR inhibition. Higher levels of IGF-I, IGF-II, and IGF-IR were linked to a better response to IGF-IR TKIs, whereas IGFBP-3 and IGFBP-6 overexpression were observed in resistant models (44). Similar studies in breast and colorectal cancer indicate that IGF-IR expression together with adaptor proteins IRS-1 and IRS-2 or IGF-IR expression could be linked to sensitivity to IGF-IR mAb treatment (45, 46). In addition, tumor cells can overcome IGF-IR inhibition via an alternative RTK such as EGFR (44, 47). Our work further extends previously disclosed data by demonstrating that insulin receptor mediates resistance to IGF-IR antibodies used in monotherapy or in combination with cytotoxic agents in a number of various indications including lung adenocarcinoma and breast carcinoma. This study also provides ample evidence that both IR isoforms play an important role in the sensitivity to IGF-IR-targeted therapies, sheds a new light on the role of IR-B in tumor biology, and provides a scientific rationale for a clinical trial with a preselection of patients based on the IR expression levels of the tumor.

Furthermore, although many pharmaceutical companies discontinued their IGF-IR programs in oncology, there is growing evidence that the IGF-IR pathway is implicated in the development of resistance to novel targeted agents (ALK inhibitors, next-generation EGFR TKIs; refs. 48, 49). It is therefore possible that there might be a need to reinvigorate the development of IGF-IR antibodies as more targeted agents will be approved for treatment of human malignancies.

Disclosure of Potential Conflicts of Interest

G.P. Donoho has ownership interest (including patents) in Eli Lilly & Co. No potential conflicts of interest were disclosed by the other authors.

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