Molecular Analysis of Non–Small Cell Lung Cancer Identifies Subsets with Different Sensitivity to Insulin-like Growth Factor I Receptor Inhibition

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Abstract

Purpose: This study aimed to identify molecular determinants of sensitivity of non–small cell lung cancer (NSCLC) to anti–insulin-like growth factor receptor (IGF-IR) therapy.

Experimental Design: A total of 216 tumor samples were investigated, of which 165 consisted of retrospective analyses of banked tissue and an additional 51 were from patients enrolled in a phase II study of figitumumab, a monoclonal antibody against IGF-IR, in stage IIIb/IV NSCLC. Biomarkers assessed included IGF-IR, epidermal growth factor receptor, IGF-II, IGF-IIR, insulin receptor substrate 1 (IRS-1), IRS-2, vimentin, and E-cadherin. Subcellular localization of IRS-1 and phosphorylation levels of mitogen-activated protein kinase and Akt1 were also analyzed.

Results: IGF-IR was differentially expressed across histologic subtypes (P = 0.04), with highest levels observed in squamous cell tumors. Elevated IGF-IR expression was also observed in a small number of squamous cell tumors responding to chemotherapy combined with figitumumab (P = 0.008). Because no other biomarker/response interaction was observed using classical histologic subtyping, a molecular approach was undertaken to segment NSCLC into mechanism-based subpopulations. Principal component analysis and unsupervised Bayesian clustering identified three NSCLC subsets that resembled the steps of the epithelial to mesenchymal transition: E-cadherin high/IRS-1 low (epithelial-like), E-cadherin intermediate/IRS-1 high (transitional), and E-cadherin low/IRS-1 low (mesenchymal-like). Several markers of the IGF-IR pathway were overexpressed in the transitional subset. Furthermore, a higher response rate to the combination of chemotherapy and figitumumab was observed in transitional tumors (71%) compared with those in the mesenchymal-like subset (32%; P = 0.03). Only one epithelial-like tumor was identified in the phase II study, suggesting that advanced NSCLC has undergone significant dedifferentiation at diagnosis.

Conclusion: NSCLC comprises molecular subsets with differential sensitivity to IGF-IR inhibition.
IGF-IR could be a viable approach for the treatment of NSCLC.

Figitumumab is a selective inhibitor of the IGF-IR and has been well tolerated in initial studies (4). Figitumumab increases the tumor growth inhibition of chemotherapy and targeted agents in preclinical models (5). A recently completed phase II study concluded that fitigumumab increases the response rate and progression-free survival (PFS) benefit of paclitaxel and carboplatin as first-line treatment of patients with advanced NSCLC (6, 7). However, pivotal trials of this agent in NSCLC were recently discontinued due to potential futility. These results stress the need to identify patient subpopulations that may preferentially benefit from fitigumumab therapy. This article summarizes a series of preliminary ancillary studies conducted to identify the tumor tissue molecular determinants of sensitivity to fitigumumab that could guide the design of future trials of this agent in NSCLC.

Materials and Methods

Tumor tissue

Formalin-fixed, paraffin-embedded, primary NSCLC tumors were obtained from two sources. As to the first source, 165 tumors came from patients who underwent surgery at Yale University/New Haven Hospital between January 1995 and May 2003 (Yale cohort). Samples were sequentially obtained from the archives of the Pathology Department of Yale University (New Haven, CT) and were prepared in tissue microarray format as previously described (8). Briefly, representative tumor areas obtained from two to four 0.6-mm cores of each tumor block per patient were arrayed in a recipient block. Tissue microarray spots were examined for confirmation of tumor presence by a pathologist. As to the second source, patients enrolled in a phase Ib/II study of fitigumumab provided slides from diagnostic biopsies (n = 51) prior to study entry (phase II cohort; ref. 6). The provision of slides was optional. Formalin-fixed, paraffin-embedded cell line pellets from transfectant NIH 3T3 cells overexpressing the IGF-IR were used as experimental controls. Studies were conducted in accordance with Good Clinical Practice guidelines and were approved by each participating institutional ethics review board. All patients signed written informed consent.

Immunohistochemical methods

Tumor tissues were deparaffinized then hydrated in water, and antigen retrieval was done using standard techniques. Incubation with primary antibodies was conducted for 1 hour at room temperature. Antibodies used for fluorescence immunohistochemical staining included rabbit anti-IGF-IR (Cell Signaling; 1.5 μg/mL), mouse anti-E-cadherin (DAKO, clone NCH38; 2.05 μg/mL), rabbit anti-IGF-IR (Santa Cruz; 6.7 μg/mL), mouse anti-IGF-II (Millipore, clone S1F2; 10 μg/mL), rabbit anti-IRS1 (Santa Cruz; 2.0 μg/mL), rabbit anti-IRS2 (Novus, clone EP976Y; 1:100 dilution), mouse anti-EGFR (Invitrogen, clone 31G7; 1.3 μg/mL), and mouse anti-vimentin (Thermo Scientific; 0.067 μg/mL). Each primary antibody was included in a cocktail with either mouse anti-pan-cytokeratin (Dako; 1:50) or rabbit anti-pan-cytokeratin (Dako; 1:200) for the identification of epithelial regions and nonnuclear regions. Additional methods were as described previously (8). For immunohistochemical staining of phospho-Akt and phospho–mitogen-activated protein kinase (MAPK), 4-μm tissue sections were deparaffinized, endogenous peroxidase was blocked by 3% H2O2, and endogenous avidin and biotin were blocked with the AB blocking kit (Vector) as described. Slides were incubated with anti-phosphorylated MAPK rabbit antibody in a 1:80 dilution (Cell Signaling Technology; 2-hour incubation) or anti-phosphorylated Akt at 1:80 dilution (Cell Signaling Technology; 2-hour incubation). This was followed by incubation with appropriate secondary antibody (DakoCytomation), labeled streptavidin–horseradish peroxidase (DakoCytomation), 3,3′-diaminobenzidine plus chromogen (DakoCytomation), and 0.2% osmium tetroxide (Sigma Chemicals), followed by counterstaining with light hematoxylin. Immunohistochemistry of IGF-IR was conducted using antibody SC-713 at 1:100 dilution (Santa Cruz Biotechnology).

Automated quantitative analysis of fluorescence immunohistochemical stainings

Image review, validation, and scoring were done with AQUAAnalysis software (HistoRx). Relative protein concentration within subcellular compartments was measured as described in detail previously (9). Briefly, high-resolution, 12 bit (resulting in 4,096 discrete intensity values per pixel...
of an acquired image) digital images of the nuclei along with the cytokeratin or CD68 staining were visualized with 4′, 6-diamidino-2-phenylindole (DAPI) and Alexa555, whereas target staining with Cy5 was captured and saved for every histospot on the array using the PM2000 epifluorescence microscopy system (HistoRx, Inc.). Prior to statistical analysis, images were reviewed for quality (e.g., saturation and focus) and signal intensity of the DAPI and Cy3 signals. For all markers, the pan-cytokeratin signal was used to create an epithelial “mask” to distinguish regions of epithelial tissue from stromal elements within both the normal and the tumor samples. Compartmentalization of expression using DAPI to identify nuclei and pan-cytokeratin to identify cytoplasm/membrane was as described previously (10). Automated quantitative analysis (AQUA) scores were calculated and reported as compartment-specific scores. Phospho-Akt and phospho-MAPK immunohistochemical stainings were quantified using an Aperio Digital Slide Scanner following standard recommendations from the manufacturer.

**Serum markers**

Plasma samples were obtained within 4 hours prior to the first study treatment dose. Levels of IGF-II and IGFBP-3 were determined using the enzyme-linked immunosorbent assay (ELISA) method according to the recommendations of the assay kit manufacturer. Kits were from Beckman-Coulter Diagnostic System Laboratories: IGF-II (DSL 10-2600) and IGFBP-3 (DSL 10-6600).

**Statistical analysis**

Analyses were conducted using the SPSS statistical program (SPSS Inc.). A log-base-2 transformation was used to stabilize variance and normalize data, except for IGF-IR, which showed a normal distribution of raw AQUA scores. AQUA scores were compared using Kruskal-Wallis tests, and correlations were determined using Pearson’s correlation tests. Principal component analysis was carried out using the built-in functions of SPSS, and variance was captured in two eigenvectors. Unsupervised data segmentation was accomplished using a Bayesian two-step clustering method with log-likelihood distances.

**Results**

**Differential expression of the IGF-IR pathway across NSCLC histologies**

A cohort of 165 tumors was obtained from the Yale University Department of Pathology. Tumors were sequentially obtained from stage I to IIIA patients who underwent surgery at the Yale University/New Haven Hospital between January 1995 and May 2003. Most specimens were stage I disease (55%). A small subset of specimens came from stage IIIB (n = 8) and IV patients (n = 10). Available patient demographics and tumor pathology characteristics are summarized in Table 1. The presence of tumor was confirmed by a pathologist. All patients were treatment naive before tumor resection. Tissue arrays were generated from these samples and analyzed using fluorescence immunohistochemistry and an AQUA system. Protein levels of IGF-IR, epidermal growth factor receptor (EGFR), IGF-II, IGF-IIR, IRS-1, IRS-2, vimentin, and E-cadherin were determined. Each slide was stained for the marker of interest, cytokeratin, to differentiate epithelium from stromal components as well as to identify cytoplasm, and with DAPI to distinguish nuclei. Figure 1 shows the fluorescent (Cy5) staining patterns for each of these markers in two representative samples. Of note, differences in tumor cell subcellular localization were evident for some of the markers. Figure 1 (IRS-1, left) shows a tumor with high nuclear IRS-1 expression relative to cytoplasm (nuclear:cytoplasmic ratio, 2.63), as well as a tumor with low IRS-1 nuclear expression (IRS-1, right) relative to cytoplasm (nuclear:cytoplasmic ratio, 0.65). The IRS-1 nuclear:cytoplasmic ratio was >1 in 77% (115 of 165) of tumors; thus, the majority of samples had a more predominant nuclear expression of IRS-1. Figure 1 (IRS-2, left) also shows a tumor with high nuclear and perinuclear IRS-2 expression relative to cytoplasm (nuclear:cytoplasmic ratio: 1.89), as well as a tumor with low IRS-2 nuclear expression (IRS-2, right) relative to cytoplasm (nuclear:cytoplasmic ratio, 0.48). Thus, IRS-2 showed a predominant cytoplasmic expression with 88% (134 of 165) of tumors having a nucleocytoplasmic ratio of <1. Membrane levels of IGF-IR, IGF-IIR, EGFR, and cytoplasmic E-cadherin, vimentin, and IRS-2 were investigated in subsequent experiments. Cytoplasmic and nuclear IRS-1 were investigated as indicated in the text.

Marker expression was investigated across classical NSCLC histologic subtypes (Fig. 2). IGF-IR expression was differentially expressed (P = 0.04), with higher IGF-IR levels in squamous cell and adenosquamous carcinomas (P = 0.03 and P = 0.02, respectively) compared with

**Table 1. Yale cohort demographics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n (%)</th>
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<tr>
<td>Stage*</td>
<td>n (%)</td>
</tr>
<tr>
<td>I</td>
<td>80 (55)</td>
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<tr>
<td>II</td>
<td>24 (17)</td>
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<tr>
<td>III</td>
<td>31 (21)</td>
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<tr>
<td>IV</td>
<td>10 (7)</td>
</tr>
<tr>
<td>Gender</td>
<td>n (%)</td>
</tr>
<tr>
<td>Male</td>
<td>80 (48)</td>
</tr>
<tr>
<td>Female</td>
<td>85 (52)</td>
</tr>
<tr>
<td>Histologic subtype</td>
<td>n (%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>102 (62)</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>13 (8)</td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td>33 (20)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>17 (10)</td>
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*Disease stage was unknown for 20 patients.*
adenocarcinoma (Fig. 2, IGF-IR). EGFR was also differentially expressed ($P < 0.001$), with higher levels observed in squamous cell carcinoma compared with other subtypes (Fig. 2, EGFR; adenocarcinoma, $P < 0.001$; adenosquamous, $P = 0.03$; large cell, $P = 0.02$). There were no significant differences in IRS-1 levels across histologies (Fig. 2, IRS-1); however, the IRS-1 nuclear:cytoplasmic ratio was higher in squamous cell tumors related to adenocarcinoma ($P < 0.001$), although squamous cell carcinomas had overall low levels of nuclear IRS-1 (data not shown). IRS-2 expression was lowest in squamous cell carcinoma compared with other subtypes (squamous versus adenocarcinoma, $P = 0.002$). No subtype-related differences were observed for IGF-IIR, IGF-II, and E-cadherin. Finally, vimentin, a mesenchymal marker, was underexpressed in squamous cell tumors related to adenocarcinoma ($P = 0.039$) and large cell carcinoma ($P = 0.009$).

**IGF-IR is expressed in squamous NSCLC responding to figitumumab**

The potential value of the markers analyzed as potential predictors of the clinical benefit of patients treated with anti-IGF-IR therapy was investigated in a cohort of tumor samples from patients enrolled in a phase II study of paclitaxel and carboplatin chemotherapy (PC) given in combination with figitumumab as first-line treatment of stage IIIb/IV NSCLC. Only 51 pretreatment tumor samples were obtained from the 151 patients enrolled in
the study. This cohort was representative of the overall patient population: 72% of the patients providing tumor biopsies were male, median age was 63 years old, and 84% had stage IV disease; however, squamous cell carcinoma histology was more frequent in the biomarker cohort than in the overall study population (41% versus 18%). Six tumor samples were not included in the analysis due to quality control issues. Of the remaining 45 tumors, 37 were from patients who received figitumumab treatment at 10 or 20 mg/kg together with standard

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Fig. 2. AQUA scores of marker expression by histologic subtype in the Yale NSCLC cohort (n = 165). Central box, values from the lower to upper quartile (25 to 75 percentile). In the box plots, the middle line represents the median. A line extends from the minimum to the maximum value, excluding “outside” and “far out” values that are displayed as separate points. An outside value is defined as a value that is smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range (inner fences). These values are plotted with a square marker. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (outer fences). These values are plotted with a round or triangular marker. The number inside the box indicates the number of patients in the subset.
doses of paclitaxel and carboplatin; 7 patients received chemotherapy alone and 1 patient discontinued study prior to treatment. Analysis of marker expression by objective response (Response Evaluation Criteria for Solid Tumors) identified an association ($P = 0.037$) of IGF-IR levels with partial response in patients treated with the combination of chemotherapy and figitumumab (PCF; Fig. 3A). No significant association was found for tumors receiving PC alone ($P = 0.25$), but the number of PC samples was small ($n = 7$). Furthermore, analysis by histologic subtype indicated that the association with response in PCF-treated patients was limited to those tumors with squamous cell histology (Fig. 3B, SQ; median AQUA scores, 5,885 versus 2,638; $n = 17$; $P = 0.008$). Both the intensity of the IGF-IR fluorescence signal and its membrane localization seemed important for the clinical benefit in terms of PFS derived from figitumumab treatment (Fig. 4). Tumor areas with intense membrane-localized IGF-IR were observed in biopsies from patients who received the combination of figitumumab and chemotherapy and experienced prolonged clinical benefit (Fig. 4A). In contrast, short PFS time characterized the treatment response of patients with tumors with low IGF-IR expression or diffuse subcellular localization (Fig. 4B) despite figitumumab use. Furthermore, high membrane IGF-IR levels were observed in tumors from patients with rapid disease progression on chemotherapy alone (Fig. 4C). The intense membrane IGF-IR localization in figitumumab responders observed by fluorescence immunohistochemistry with the anti-IGF-IR antibody CS-3027 was reproduced with antibody SC-713 in immunohistochemistry (not shown).

Levels of E-cadherin, vimentin, EGFR, IRS-1 (nuclear and cytoplasmic), phospho-MAPK, and phospho-AKT1 were also investigated. Vimentin levels were significantly elevated in squamous cell and adenocarcinoma patients responding to the figitumumab and chemotherapy combination as compared with nonresponders (3,162 versus 2,206 median vimentin AQUA scores; $n = 37$; $P = 0.037$). Because those responding tumors had also intermediate to elevated E-cadherin levels (3,695 median E-cadherin AQUA score), this finding suggested that tumors in figitumumab responders could be undergoing epithelial to mesenchymal transition (EMT). Tumors of less well differentiated histology, i.e., large cell and NSCLC not otherwise specified, had elevated vimentin levels that were unrelated to response. No vimentin/efficacy interaction was observed in patients who received chemotherapy alone. Elevated levels of nuclear IRS-1 or high nuclear:cytoplasmic IRS-1 ratios were observed in figitumumab-responding tumors, but this association was not significant. No other biomarker associations with objective response were identified.

**NSCLC subsets defined by E-cadherin and IRS-1 expression**

Classical histologic subtyping facilitated the identification of IGF-IR levels as a correlate of figitumumab response in squamous cell NSCLC, but IGF-IR expression was not informative of objective response in other histologies. *IGF1R* gene sequence was not investigated. We had previously screened the *IGF1R* in 92 solid tumors using a mismatch repair detection technology, including 46 NSCLC samples. Two mutations were identified in NSCLC specimens: Gly1199Arg and Ala1206Ser. Both mutations were then confirmed by sequencing. *IGF1R* subcloning, generation of stable cell lines, and functional assays revealed that the *IGF-IR* Gly1199Arg and Ala1206Ser variants do not encode receptors with ligand-independent kinase activity, nor did they respond differentially to figitumumab in vitro as compared with wild-type *IGF-IR* (11). These experiments will be described elsewhere in detail. Because no other single biomarker/efficacy interactions were identified, we segmented the NSCLC population using the biomarker information generated in the tissue arrays to identify molecular subgroups with potential differential sensitivity to anti-IGF-IR treatment. The Yale and phase II cohorts were used, respectively, as training...
and validation groups. The principal component analysis of biomarker expression in the Yale cohort identified uncorrelated markers that could be used as segmentation criteria. Both E-cadherin and IRS-1 appeared distinct in principal component analysis biplots from the other markers investigated, and their AQUA scores were entered into an unsupervised Bayesian clustering analysis to segment the tumor population (not shown). Cytoplasmic IRS-1 levels were used for cluster identification, but the results with nuclear IRS-1 were similar (not shown). Three clusters were observed that represented unique subpopulations based on mean marker expression: (a) E-cadherin
high/IRS-1 low (n = 35); (b) E-cadherin intermediate/IRS-1 high (n = 28); and E-cadherin low/IRS-1 low (n = 74). Twenty-eight of the 165 samples were not included in the analysis due to fluorescence immunohistochemistry quality control issues (both E-cadherin and IRS-1 stainings had to be considered optimal for analysis). Based on the step-wise expression of E-cadherin in the clusters and the previously described roles of E-cadherin and IRS-1 in EMT (12, 13), we named these subsets, respectively, (a) epithelial-like, (b) transitional, and (c) mesenchymal-like.

Differences in marker expression were also investigated (Fig. 5A). Higher IGF-IR expression was observed in epithelial-like tumors compared with mesenchymal-like tumors (P = 0.06), whereas median IGF-IR levels in transitional tumors were intermediate between those in the epithelial-like and mesenchymal-like subsets. Importantly, transitional tumors had the highest levels of IGF-II, IGF-IR, and IRS-2, suggesting that the IGF-IR pathway could be of particular significance in this subset (Fig. 5A). Biomarker correlations were also investigated. IGF-IR expression was correlated with that of E-cadherin (Spearman ρ = 0.429, P = 0.01) in the epithelial-like subset. In contrast, in the mesenchymal-like subset, IGF-IR showed a highly significant moderate correlation to EGFR (Spearman ρ = 0.416, P < 0.001).

Response to figitumumab treatment in the NSCLC molecular subsets

The significance of the defined molecular subsets to the treatment of advanced NSCLC with figitumumab was investigated. Bayesian clustering of phase II samples defined epithelial-like, transitional, and mesenchymal-like subpopulations. Of the original 51 samples, 8 tumors were unclassified due to limited sample availability (n = 2) or quality control issues (n = 6). Only one epithelial-like tumor was identified, corresponding to one patient with squamous cell carcinoma enrolled in the chemotherapy arm. All patients enrolled in the chemotheraphy plus figitumumab arm had the characteristics of transitional or mesenchymal-like tumors, suggesting that, in general, stage IIIb/IV NSCLC tumors have undergone de-differentiation by the time of diagnosis. Twenty transitional and 22 mesenchymal-like tumors were identified. There was no apparent relationship between molecular clustering and histologic subtyping, although most squamous cell tumors (65%) were transitional rather than mesenchymal-like (35%). Median levels of E-cadherin and cytoplasmic IRS-1 in transitional and mesenchymal-like tumors were respectively 6,155 and 5,789, and 3,017 and 3,819 AQUA scores. Expression of E-cadherin, IRS-1 (nuclear and cytoplasmic), IGF-IR, and vimentin is shown in Fig. 5B. E-cadherin, IGF-IR, IRS-1, IRS-2, and vimentin were more highly expressed in transitional tumors, recapitulating the findings in the Yale cohort.

Objective tumor response was then analyzed according to molecular subset. Ten of 14 patients (71%) with transitional tumors responded to the combination of figitumumab and chemotherapy whereas only 7 of 22 (31.8%; P = 0.03) with mesenchymal tumors did so. Two of six patients with transitional tumors responded to chemotherapy alone. Median PFS times for patients with transitional and mesenchymal-like tumors receiving the chemotherapy and figitumumab combination treatment were, respectively, 166 and 96 days, but no significant difference in PFS was observed.

Biomarker correlations in the NSCLC molecular subsets

A strong correlation between IGF-IR and E-cadherin expression was observed (P = 0.562, P < 0.0001) in the overall cohort, whereas cytoplasmic IRS-1 expression was significantly correlated to that of vimentin in mesenchymal tumors (P = 0.695, P = 0.004). No significant association between EGFR and IGF-IR expression was identified. To investigate potential correlations with markers of downstream receptor signaling activation, phosphorylation levels of MAPK and Akt1 were quantified. In the overall population, P-Akt correlated directly with EGFFR expression (ρ = 0.467, P = 0.01), cytoplasmic (ρ = 0.521, P = 0.01) and nuclear IRS-1 (ρ = 0.478, P = 0.02), and inversely with P-MAPK (ρ = -0.367, P = 0.05). A trend for a negative correlation between P-Akt and IGF-IR was also observed in mesenchymal-like tumors (ρ = -0.476, P = 0.08). Upstream markers of IGF-IR pathway activation were investigated by measuring patient pretreatment circulating blood levels of IGF-II and IGFBP-3 using standard ELISA techniques. No significant differences in plasma levels of these growth factors were observed between the molecular subsets, but a direct correlation between circulating IGF-II levels and vimentin (ρ = 0.558, P = 0.03) and an inverse correlation between circulating IGF-II levels and E-cadherin (ρ = -0.575, P = 0.01) were identified in mesenchymal-like tumors.

Discussion

The data presented in this article describe several biomarker interactions with potential implications for the development of anti-IGF-IR therapy in NSCLC. We found that IGF-IR was differentially expressed across histologic subtypes, with higher levels observed in squamous cell tumors compared with other histologies. These data are consistent with recent reports (14, 15). The pattern of expression and prognostic value of IGF-IR expression in NSCLC remains controversial. Prior studies have shown that IGF-IR expression is associated with longer survival in patients treated with gefitinib (16), poorer survival and higher expression in surgically treated lung adenocarcinomas versus squamous cell carcinomas (17), and shorter disease-free survival when coexpressed with EGFR in resected NSCLC (18). These apparent differences may result in part from the use of different reagents and methodologies. The molecular mechanisms responsible for IGF-IR overexpression in squamous cell NSCLC are also unknown. Recent data indicate that increases in IGFIR copy number may result from polysomy but this...
phenomenon does not seem to follow a specific histologic pattern (15). Importantly, we observed that high levels of IGF-IR expression in patients with squamous cell tumors were associated with objective response to the combination of the anti-IGF-IR inhibitor figitumumab with chemotherapy. These results should be considered preliminary due to the small cohort of patients investigated. It is uncertain whether higher IGF-IR expression could

Fig. 5. AQUA scores of marker expression by molecular subtypes in the Yale (A; n = 137) and phase II (B; n = 43) NSCLC cohorts. Box plots were constructed as in Fig. 2. M, mesenchymal-like; T, transitional; E-cad, E-cadherin.
have been predictive of response to chemotherapy alone if sufficient samples would have been available in the PC cohort. Thus, the predictive value of IGF-IR expression for figitumumab containing therapy requires further investigation in larger trials, and ancillary studies investigating that question are currently under way. On the other hand, our data are consistent with prior observations in cell lines indicating that IGF-IR expression may be a predictor of sensitivity to IGF-IR inhibition in NSCLC (19, 20). Furthermore, recent studies have also linked IGF-IR levels to response to anti-IGF-IR antibodies and small molecule tyrosine kinase inhibitors in other cancer types (21–23). We observed, however, that IGF-IR expression by itself was not informative of response to IGF-IR therapy in patients with nonsquamous histologies. Furthermore, although IGF-IR overexpression in squamous cell tumors was observed in the Yale cohort, no significant differences in IGF-IR levels across histologies were observed in a pool of stage IIb/IV samples from the Yale and phase II cohorts (n = 63). Thus, the use of IGF-IR levels for the selection of advanced NSCLC patients to be treated with anti-IGF-IR therapy should be approached with caution. Of interest, vimentin levels also seemed to be associated with response to the combination of chemotherapy and figitumumab in squamous and adenocarcinoma patients, suggesting that factors related to tissue differentiation could be as important as IGF-IR expression for predicting sensitivity to anti-IGF-IR therapy.

Molecular analysis identified a subset of advanced NSCLC characterized by overexpression of IRS-1, IRS-2, and intermediate levels of E-cadherin and IGF-IR that was highly sensitive to the combination of figitumumab and chemotherapy. These initial results may contribute to our understanding of the mechanisms of activation of the IGF-IR pathway in NSCLC and will require confirmation in larger tumor series. These data are consistent with prior observations indicating that IRSs, particularly IRS-1, play a crucial role in tumor progression, and that coexpression of IRS-1 and IGF-IR may be important for sensitivity to IGF-IR inhibition (24, 25). A role for IGF-IR in tumor remodeling has been also well documented. IGFs can induce in vitro neo-expression of mesenchymal markers such as vimentin, perinuclear E-cadherin localization, and E-cadherin downregulation (reviewed in ref. 26). We have previously seen that IGF-IR–induced EMT is in part mediated by the transcriptional repressor Snail (13). IGFs have also been shown to enhance the phosphorylation of β-catenin, causing its dissociation from E-cadherin and translocation to the cytoplasm/nucleus (27, 28). These interactions are thought to facilitate the coupling of IGF-IR activation with migration, invasiveness, and metastasis. It is unclear whether high levels of IGF-IR expression are associated with high IGF-IR kinase activity. The techniques for the measurement of phospho-IGF-IR epitopes produced variable results in our hands (not shown) and are complicated by the high homology between the kinase domains of the IGF-IR and insulin receptor (IR) isoforms. We observed that IGF-IR expression was highest in epithelial-like tumors and that it was correlated with that of E-cadherin, except in mesenchymal-like tumors. Previous reports have shown that the IGF-IR and E-cadherin are able to form a
complex at cell-cell contact sites (29), and that E-cadherin expression correlates with tumor cell differentiation in squamous cell carcinomas (30). Together, these data suggest that IGF-IR is overexpressed in differentiated NSCLC tumors, predominantly in early stages of squamous cell carcinoma.

Of interest, high IGF-II levels were observed in transitional tumors. Because no ligand-independent IGF-IR mutants have been identified so far, we can assume that IGFs are required for receptor activity. The presence of high levels of IGF-II, a specific inhibitor of IGF-II but not of IGF-I (1), in transitional tumors suggests that serum circulating IGF-I, including that of liver origin, could be important for IGF-IR activation in patients with transitional NSCLC tumors. This hypothesis is consistent with recent observations (31). We also report here that IGF-II correlated with EMT in mesenchymal-like tumors, further supporting the notion that although a certain level of IGF-IR expression could be a requisite for receptor activity, levels of IGF-IR ligands are likely to be key drivers of such activity. Consequently, the potential use of circulating bioactive IGF-I as a biomarker for the selection of patients who could benefit from anti-IGF-IR therapy deserves further evaluation. The use of IGF-II levels, circulating or locally produced at the tumor site, as a biomarker of anti-IGF-IR therapy may also be helpful but their analysis is complicated by the expression of IGF-IR in NSCLC and by the ability of IGF-II to activate not only the IGF-IR, but also the fetal variant of the IR (IR-A) and IGF-IR/IR hybrid receptors (4, 32). This is particularly relevant to anti-IGF-IR antibodies such as figitumumab, which are active against the IGF-IR and IGF-IR/IR hybrids but not against IR-A homodimers (5). Thus, development of novel methodologies capable of assessing \textit{in vivo} the rate IGF-IR/IR hybrids versus receptor homodimers may be necessary to better tailor anti-IGF-IR therapy and to define its resistance mechanisms.

Finally, IGF-II showed a highly significant correlation with EGFR in the mesenchymal-like subset. EGFR, IRS-1, P-Akt, and vimentin were also directly correlated with each other, whereas a trend for a negative correlation between P-Akt and IGF-IR was observed. A potential explanation for these results is provided by \textit{in vitro} data showing that IRS-1 can be targeted by the EGFR (33). IGF-IR phosphorylates IRS-1 on Y612, a phosphoinositide 3-kinase recruitment site, whereas EGFR preferentially phosphorylates IRS-1 on Y896, a Grb2 binding site. Phosphorylation at the Y612 and Y896 sites seems to be mutually exclusive, with EGFR acting as the dominant recruiter of IRS-1 (34). A corollary of these findings is that EGFR inhibition may sensitize tumor cells to anti-IGF-IR treatment, particularly in tumors that are strongly driven by EGFR. Prior evidence has indicated that acquired resistance to EGFR inhibition in NSCLC may be associated with enhanced dependency on IGF-IR signaling (19, 34). Thus, co-targeting EGFR and IGF-IR could be expected to have additive or synergistic antitumor effects. This hypothesis is currently being investigated in several clinical trials (4).

In summary, the IGF-IR may play diverse functions in NSCLC. In early-stage disease, this protein may be important for the maintenance of the differentiated phenotype. In later stages of disease, this protein may drive EMT, tumor invasiveness, and resistance to chemotherapy and targeted agents. The expression of adaptor proteins, such as IRS family members, may be important for this phenotype switch. Additional research is needed to validate our initial observations and to increase our understanding of the role that the interplay between the IGF-IR and EGFR pathways as well as patient related factors, such as IGFs bioactivity, may play in tumor remodeling and sensitivity to anti-IGF-IR therapy.

**Disclosure of Potential Conflicts of Interest**

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