Epithelial-to-Mesenchymal Transition and Integrin-Linked Kinase Mediate Sensitivity to Epidermal Growth Factor Receptor Inhibition in Human Hepatoma Cells

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Abstract

Hepatocellular carcinoma (HCC) is associated with a poor prognosis due to late diagnoses and a lack of effective treatment options. Epidermal growth factor receptor (EGFR)–targeted therapies have been effective in other cancers. However, erlotinib and cetuximab have shown only modest efficacy in clinical trials of HCC. We examined epithelial-to-mesenchymal transition (EMT) as a determinant of sensitivity of HCC to EGFR inhibitors. A panel of 12 human hepatoma cell lines were classified as epithelial or mesenchymal based on their expression of E-cadherin and vimentin. The resulting classification correlated with a previous microarray analysis of human hepatoma cell lines whereby the mesenchymal cell lines were shown to have increased expression of genes involved in metastasis and invasion. Sensitivity to erlotinib, gefitinib, and cetuximab was assessed and the epithelial cell lines were found to be significantly more susceptible to all three agents. Analysis of the EGFR pathway showed that EMT status was independent of EGFR expression or downstream extracellular signal–regulated kinase activation and only the epithelial cell lines expressed ErbB3. Interestingly, mesenchymal cells resistant to EGFR inhibitors had increased AKT and signal transducer and activator of transcription-3 activation through elevated expression of integrin-linked kinase (ILK). Mesenchymal cell lines were therefore experimentally transformed with kinase-inactive ILK (KI-ILK) with a resulting decrease in ILK activity and activation of AKT. KI-ILK transformants showed increased sensitivity to EGFR inhibitors both in vitro and in an in vivo xenograft model. These data suggest that EMT predicts HCC sensitivity to EGFR-targeted therapies and that ILK is a novel target to overcome HCC resistance to EGFR inhibition.

Note: T. Fujii, J.D. Dorfman, and J.M. Goodwin contributed equally to this research.

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Research Article

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Previous reports have shown that erlotinib, gefitinib, and cetuximab induce apoptosis in human hepatoma cell lines (27–29). In the current study, we analyzed the role of EMT in HCC sensitivity to EGFR-targeted therapies. We observed that mesenchymal cells are less susceptible to EGFR inhibition than epithelial cells. This resistance is at least partly due to ILK overexpression in mesenchymal cells, and ILK inhibition is a novel mechanism to overcome resistance to EGFR inhibitors.

Materials and Methods

Cells and culture conditions. Human hepatoma cell lines (SNU-182, SNU-387, SNU-398, SNU-423, SNU-449, and SNU-475) were obtained from American Type Culture Collection. The hepatoma cell lines SK-Hep, PLC/PgR5/F, HepG2, and Hep3B were kindly provided by Barrie Bode (Saint Louis University, St. Louis, MO), whereas Huh-7 was provided by Jake Liang (National Institutes of Diabetes, Digestive and Kidney Diseases, NIH, Bethesda, MD), and Focus by Jack Wands (Brown University, Providence, RI). All the cell lines were propagated in DMEM (4.5 mg/mL glucose, 2 mmol/L l-glutamine) with 10% fetal bovine serum (both from MediaTech CellGro), supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified incubator with 5% CO2 in air. Stock solutions of erlotinib (Tarceva, Genentech) and gefitinib (Iressa, AstraZeneca) were prepared in DMSO and stored at –20°C. Cetuximab (Erbitux, ImClone Systems) was supplied as a 2 mg/mL stock and PBS was used as a vehicle control.

Real-time PCR. Human hepatoma cells were plated at 1 × 105/mL in 10-mL medium in a 100-mm plate and allowed to grow for 48 h to represent log-phase growth. Total RNA was extracted from each cell line with TRIzol (Invitrogen) according to the manufacturer’s instructions and subsequently treated with DNase I (Promega). Total RNA (250 ng) from each sample was used to create cDNA by single strand reverse transcription [SuperScript III First-Strand Synthesis SuperMix (Invitrogen)] for quantitative real-time PCR (RT-PCR). All of the sample cDNAs were pooled together to create a quantitative standard control. Expression of E-cadherin, vimentin, α-fetoprotein (AFP), CD44, and insulin-like growth factor II (IGF-II) mRNA in the human hepatoma cell lines was analyzed by quantitative real-time PCR (LightCycler, Roche Diagnostics Corporation). mRNA expression present was normalized to the expression of β-actin. Primer sequences are as follows: E-cadherin, forward AGTGGGCCACAGATGGTGTGA and reverse TAGGGTGGAGTCCCCAGCTGA; vimentin, forward CTTCACTGTT-GAAATGGAATG and reverse CAACGCAAGATCTCTCCTCA; AFP, forward TGGCACCAGAATCCGCGCAAGAGA and reverse CATTACGAGGCAG-CACAGGAGAC; CD44, forward CAACCTCACCTGGCAACCAA and reverse GTAACCCTCAGTGGCTC; IGF-II, forward CCACTCCGACCTGGTCT and reverse TCAATTTGGAGAACCTTGGCCA; and actin, forward CCTGGACTCTGACAGGATG and reverse GCCGATCCACAGGAGACT. All reactions were done in duplicate and the experiment was performed to ensure reproducibility.

Western blotting. Cell lines were plated as described above and, after 48 h, the cells were washed in ice-cold PBS and harvested in 500 μL of radioimmunoprecipitation assay buffer (Boston BioProducts) containing protease inhibitors (Sigma). Protein concentration of lysates was analyzed with the bicinchoninic acid (BCA) method (Pierce Chemical Co.). Cellular lysates (30 μg) were prepared in Laemmli’s reducing sample buffer (Boston BioProducts) and separated by electrophoresis on 4% to 20% polyacrylamide gradient gels (Cambrex Bio Science), and transferred electrophoretically by the bicinchoninic acid (BCA) method (Pierce Chemical Co.). Cellular lysates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were incubated with primary antibodies overnight at 4°C and with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP; GE Healthcare) for 1 h at room temperature. After each incubation period, membranes were washed thrice with TBS/T. Immunoreactive bands were visualized on X-ray film (Denville Scientific) with a chemiluminescent HRP substrate (Perkin-Elmer). Relative levels of total and phosphorylated proteins were determined by Western blot analysis with the following antibodies: E-cadherin, HER3/ErbB3 (1B2), p44/42 mitogen-activated protein kinase [extracellular signal–regulated kinase (ERK)], phospho-ERK (Thr202/Tyr204), AKT, phospho-AKT (Ser473), signal transducer and activator of transcription-3 (STAT3), phospho-STAT3 (Tyr705), ILK1, IGF-1 receptor (IGF-IR)-β (all from Cell Signaling Technology), EGFR, and vimentin (both from BD Biosciences). An antibody directed against β-actin (Abcam) was used to verify equal loading. Band intensities were quantified by image analysis software (LabWorks 4.5, UVP Inc.). Each Western blot was repeated to ensure reproducible results.

Cell growth assay. Briefly, cells were plated in triplicate at a density of 4 × 103/mL in a 24-well plate. After 24 h, cells were washed once with PBS and medium containing inhibitors at the indicated concentrations or vehicle control was added. After 72 h, the effect of each EGFR inhibitor on the growth of human hepatoma cells was measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide (Sigma) assay. The absorbance at 562 nm was measured with a spectrophotometric plate reader (Emax, Molecular Devices) and the experiment was repeated twice for each cell line. IC50 value is defined as the drug concentration yielding 50% nonsurviving cells compared with vehicle-treated controls, and the percent of maximal inhibition is calculated at the highest concentration for each drug.

Hepatoma cell transfection. SK-Hep and SNU-449 cells were transiently transfected with pUSEamp (empty vector) and ILK S343A (kinase-inactive; both from UpState) containing Lipofectamine 2000 in OptiMEM serum-free medium (both from Invitrogen) according to the manufacturer’s suggestions. Cells were seeded at a density of 48 h later into 150-mm tissue culture plates and maintained in selective growth medium supplemented with 1,200 μg/mL geneticin (Invitrogen). Individual colonies were isolated 7 to 10 d later and candidate clones were identified by PCR with primers spanning the multiple cloning site of pUSEamp. The primer sequences used are as follows: pUSEamp forward, TAATACGACTCATATAcGG; reverse, CAACA-GATGGCTGGACAAT.

ILK kinase assay. ILK activity was measured as previously described (30). Briefly, cells were plated at 1 × 105/mL in 10-mL medium and grown to log phase. After rinsing in PBS, cells were harvested in cell lysis buffer (Cell Signaling) containing 1 mmol/L phenylmethylsulfonyl fluoride, briefly sonicated, and centrifuged at 12,000 rpm for 10 min at 4°C. Equivalent amounts (250 μg) of the supernatant were immunoprecipitated overnight with 2 μg of mouse monoclonal anti-ILK antibody (Upstate) at 4°C. Immune complexes were isolated with protein A/G agarose beads (Santa Cruz Biotechnology) and washed twice with cell lysis buffer and kinase buffer (Cell Signaling). The pellet was resuspended in kinase buffer supplemented with 200 μmol/L ATP and 2 μg of glycogen synthase kinase (GSK)-3 fusion protein (Cell Signaling) and incubated for 30 min at 30°C. Phosphorylation of the substrate was detected by Western blot with a phospho-GSK-3α/β (Ser21/9) antibody (Cell Signaling).

Caspase assays. Specific activity of caspase-3 was measured in cellular extracts with a colorimetric kit according to the manufacturer’s instructions (CaspACE, Promega). Cells were plated in 6-cm plates (Corning) at a density of 1 × 106/mL, allowed to grow for 2 d, and subsequently treated with the experimental conditions for the indicated times. After the incubations, cells were lysed and protein extracts were collected. In this assay, the caspase-3 recognition sequence peptide (DEVD) conjugated to the chromophore paranitroaniline (pNA) is cleaved by the activated enzyme, and the liberated pNA is quantified by spectrophotometric absorbance at 405 nm. The pNA released was normalized to total protein content in the lysates by the BCA method, and results are expressed as picomoles of pNA released per microgram of protein per hour.

Animal model. Six- to eight-week-old BALB/c nu/nu mice (Charles River Laboratories) were maintained in accordance with the institutional guidelines of the Massachusetts General Hospital animal care facility. SK-Hep cells transformed with either pUSEamp (empty vector) or ILK S343A (kinase-inactive) were injected s.c. into the flank area of mice (5 × 106/100 μL of PBS). Groups 1 and 2 were injected with SK-Hep pUSEamp cells, whereas groups 3 and 4 were injected with SK-Hep ILK S343A cells. When tumors had reached 50 mm2, mice were randomized into two treatment groups of eight mice each. Groups 1 and 3 received vehicle solution and Western...
groups 2 and 4 received 80 mg/kg gefitinib, both for 5 d/wk by i.p. injection. Tumor volumes were measured 3 d/wk for 1 mo using a caliper and the following formula: \( V = \frac{(smaller \ diameter)^2 \times (larger \ diameter)}{2} \). Mice were euthanized at the end of the study.

**Statistical analysis.** A Student’s *t* test was used to compare the IC\(_{50}\) values and overall response of epithelial cells to those of mesenchymal cells, as well as those of the KI-ILK transformants to those of empty vector controls. Similarly, a Student’s *t* test was used to compare caspase-3 activation in KI-ILK transformants to that in empty vector controls after treatment with EGFR inhibitors as well as tumor volumes after treatment with vehicle or gefitinib.

**Results**

**Characterization of EMT in human hepatoma cell lines.** The expression of E-cadherin and vimentin was assessed for each of the human hepatoma cell lines to determine their extent of EMT. Five of the cell lines (SNU-423, HepG2, Hep3B, HuH-7, and PLC/PRF/5) were classified as epithelial based on their expression of E-cadherin by Western blot analysis (Fig. 1A). Conversely, seven of the cell lines (SNU-182, SNU-387, SNU-475, Focus, SK-Hep, SNU-449, and SNU-398) were considered to be mesenchymal because they lack E-cadherin, although these cell lines express vimentin at varying levels (Fig. 1A). Results of quantitative real-time PCR measurement of E-cadherin and vimentin mRNA were consistent with the Western blot analysis (Fig. 1B).

Microarray analysis of human hepatoma cell lines by Lee and Thorgerisson (31) reveals two distinctive subtypes. Group I is characterized by the activation of oncofetal promoters leading to increased expression of AFP and IGF-II. Group II is characterized by overexpression of genes involved in metastasis and invasion, such as CD44 and ILK. We examined AFP and CD44 expression by quantitative real-time PCR in all 12 human hepatomas as compared with primary cultures of human hepatocytes (Fig. 1C). Notably, AFP expression was up-regulated in four of the epithelial hepatoma cell lines (HepG2, Hep3B, HuH-7, and PLC/PRF/5) and similar to expression in normal hepatocytes in the other epithelial cell line (SNU-423). By comparison, expression of AFP was extremely down-regulated in the mesenchymal cell lines. Conversely, expression of CD44 was elevated in the mesenchymal cells and down-regulated in the epithelial cell lines compared with normal hepatocytes. Relative to normal hepatocytes, AFP expression was highest in the HuH-7 cell line (513-fold greater) and lowest in SK-Hep (>1,000-fold less), whereas CD44 was highest in SNU-449 (16-fold greater) and lowest in HepG2 (720-fold less). Thus, EMT strongly correlates with previously described HCC subtypes that are based on invasive gene expression profiles.

**Response of human hepatoma cell lines to EGFR inhibition.** As previous reports have shown a strong correlation between response to EGFR inhibitors and E-cadherin expression in other cell types, we examined the effects of EGFR-targeted therapies on
Interestingly, EMT status was independent of total EGFR levels signaling in response to EGF stimulation (data not shown). 

downstream of EGFR under normal growth conditions (Fig. 2). blot analysis was used to analyze the signaling pathways on susceptibility to EGFR-targeted therapies in HCC cells, Western 
signaling pathways that may be responsible for the effects of EMT 

To further investigate the 

mesenchymal HCC cells. Thus, IGF-II/IGF-IR signaling does not seem to be consis-
tery to erlotinib, gefitinib, and cetuximab (Table 1). Student's t test was done to compare the responses of the five epithelial cell lines to the seven mesenchymal ones. 

Analysis of IGF-II/IGF-IR signaling in human hepatoma cells. 

The IGF-IR pathway has been shown to mediate resistance to EGFR tyrosine kinase inhibitors in NSCLC (32, 33) as well as breast (34) and glioblastoma (35) cell lines. Further, a previous report suggests that IGF-II signaling through IGF-IR may result in resistance to gefitinib in hepatoma cells (36). We therefore correlated IGF-IR and IGF-II expression with EMT status in the 12 hepatoma cell lines. Expression of IGF-IR was observed in both sensitive epithelial cell lines and resistant mesenchymal ones (Fig. 2A). However, elevated expression of IGF-II was only observed in some of the sensitive epithelial cell lines (HepG2, Hep3B, and HuH-7; Fig. 2B) where this pathway seems to mediate some resistance especially in HuH-7 cells. Thus, IGF-II/IGF-II signaling does not seem to be consis-
tently associated with resistance to EGFR-targeted therapies in mesenchymal HCC cells. 

Mesenchymal cell lines have increased AKT and STAT3 expression and elevated levels of ILK. To further investigate the 

signaling pathways that may be responsible for the effects of EMT on susceptibility to EGFR-targeted therapies in HCC cells, Western blot analysis was used to analyze the signaling pathways downstream of EGFR under normal growth conditions (Fig. 2A). All 12 hepatoma cell lines exhibit increased growth and EGFR signaling in response to EGF stimulation (data not shown). Interestingly, EMT status was independent of total EGFR levels and downstream ERK activation. A minor association between total EGFR expression and ERK activation was observed. With few exceptions, the mesenchymal cell lines were observed to have increased activation of AKT and STAT3. The activation of AKT in mesenchymal cells was not due to signaling from EGFR-ErbB3 heterodimers because ErbB3 expression was observed only in epithelial cell lines, as has previously been reported in NSCLC (17, 18). 

ILK is up-regulated in mesenchymal hepatoma cell lines (31) and is known to phosphorylate and activate AKT (37) and promote cell survival. Inhibition of ILK activity in human pancreatic cancer xenografts results in decreased activation of AKT and STAT3 but not ERK (38), similar to the differences observed between epithelial and mesenchymal cells. We therefore examined ILK expression in the 12 hepatoma cell lines. Notably, increased ILK expression was restricted to mesenchymal cells, with the exception of one epithelial cell line (SNU-423; Fig. 2A). Compared with the expression in the five epithelial cell lines, ILK protein level in the seven mesenchymal cell lines was significantly (P < 0.001) 2.2-fold higher by densitometric analysis (Fig. 2C). 

Inhibition of ILK decreases AKT activation and induces mesenchymal-to-epithelial transition. To further investigate the role of ILK in the observed resistance of mesenchymal cells to EGFR-targeted therapy, we transduced the mesenchymal cell lines SK-Hep and SNU-449 with a plasmid containing a KI-ILK (ILK S343A). Transformed clones expressing either the KI-ILK or the empty vector controls were isolated (Fig. 3A). KI-ILK–transformed clones were observed to have less ILK activity compared with empty vector controls as measured by a kinase activity assay (Fig. 3B). Corresponding with this reduction in ILK activity, KI-ILK–transformed SK-Hep and SNU-449 had a 47% and 41% reduction in AKT activation, respectively (Fig. 3C). Importantly, the KI-ILK–transformed SK-Hep and SNU-449 cells also underwent a partial mesenchymal-to-epithelial transition. Compared with the empty vector controls, E-cadherin mRNA expression was increased 397- and 360-fold in the SK-Hep and SNU-449 KI-ILK transformants, respectively (Fig. 3D). Correspondingly, a 14- and

### Table 1. EMT determines sensitivity of human HCC cell lines to EGFR-targeted therapies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EMT status</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Cetuximab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50} (µmol/L)</td>
<td>%Max inhibition</td>
<td>IC_{50} (µmol/L)</td>
<td>%Max inhibition</td>
</tr>
<tr>
<td>SNU-423</td>
<td>Epithelial</td>
<td>2.2</td>
<td>82</td>
<td>4.9</td>
</tr>
<tr>
<td>HepG2</td>
<td>Epithelial</td>
<td>4.4</td>
<td>80</td>
<td>8.8</td>
</tr>
<tr>
<td>Hep3B</td>
<td>Epithelial</td>
<td>8.3</td>
<td>66</td>
<td>8.1</td>
</tr>
<tr>
<td>HuH-7</td>
<td>Epithelial</td>
<td>13.8</td>
<td>58</td>
<td>10.3</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>Epithelial</td>
<td>9.7</td>
<td>69</td>
<td>8.3</td>
</tr>
<tr>
<td>SNU-182</td>
<td>Mesenchymal</td>
<td>11.3</td>
<td>64</td>
<td>7.6</td>
</tr>
<tr>
<td>SNU-387</td>
<td>Mesenchymal</td>
<td>16.4</td>
<td>54</td>
<td>18.3</td>
</tr>
<tr>
<td>SNU-475</td>
<td>Mesenchymal</td>
<td>&gt;20</td>
<td>37</td>
<td>15.1</td>
</tr>
<tr>
<td>Focus</td>
<td>Mesenchymal</td>
<td>&gt;20</td>
<td>38</td>
<td>14.2</td>
</tr>
<tr>
<td>SK-Hep</td>
<td>Mesenchymal</td>
<td>12.5</td>
<td>62</td>
<td>19.6</td>
</tr>
<tr>
<td>SNU-449</td>
<td>Mesenchymal</td>
<td>20.0</td>
<td>50</td>
<td>17.4</td>
</tr>
<tr>
<td>SNU-398</td>
<td>Mesenchymal</td>
<td>19.1</td>
<td>52</td>
<td>19.0</td>
</tr>
<tr>
<td>T tests</td>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td>0.003</td>
</tr>
</tbody>
</table>

NOTE: Percent of maximal inhibition was calculated for the highest dose for each drug (20 µmol/L for erlotinib and gefitinib and 1,000 µg/mL for cetuximab). 

The growth of human hepatoma cell lines. Similar to the previous 

results, E-cadherin expression strongly correlates with susceptibility 
to erlotinib, gefitinib, and cetuximab in hepatoma cells (Table 1). For example, SNU-423, the cell line with the highest expression of E-cadherin, was far more susceptible to EGFR inhibition by all three agents than SNU-398, a mesenchymal cell line with the highest expression of vimentin. Compared with the result from all seven mesenchymal hepatoma cell lines, the IC_{50} values of the five epithelial cell lines were significantly lower whereas the overall responses were significantly greater for the three EGFR inhibitors. It should be noted, however, that one mesenchymal cell line, SNU-182, was slightly sensitive whereas the epithelial cell line HuH-7 showed some resistance.
3-fold decrease in vimentin mRNA expression was observed in the SK-Hep and SNU-449 KI-ILK transformants as compared with the empty vector clones.

**Inhibition of ILK restores sensitivity to EGFR inhibition.** Of significant interest, KI-ILK–transformed cells were also more susceptible to EGFR inhibition (Table 2). The IC_{50} values for SK-Hep KI-ILK were 6.4 and 6.5 μmol/L for erlotinib and gefitinib, respectively, and these were significantly \((P < 0.01)\) less than those for the empty vector controls (11.5 and 19.4 μmol/L for erlotinib and gefitinib, respectively). SK-Hep KI-ILK cells still remained largely resistant to cetuximab but the overall response increased from 6% to 25%. Similarly, SNU-449 KI-ILK cells were also only marginally responsive to cetuximab, with an overall response of 21% compared with 3% for the empty vector control cells. The IC_{50} values for SNU-449 KI-ILK cells were 9.1 and 11.5 μmol/L for erlotinib and gefitinib, respectively, and these were significantly \((P < 0.01)\) less than the corresponding controls, which had IC_{50} >20 μmol/L.

Previous reports have shown that EGFR inhibitors induce apoptosis in hepatoma cell lines (27–29). We next examined the apoptotic response of the KI-ILK transformants as compared with the empty vector controls after treatment with erlotinib and gefitinib. Apoptosis was assessed by activation of caspase-3. Consistent with the IC_{50} data, caspase-3 activity in general was observed to increase at lower doses of both inhibitors in the KI-ILK transformants, and the KI-ILK transformants had greater overall caspase-3 activity at each individual concentration of erlotinib and gefitinib tested as compared with the empty vector controls (Fig. 4A and B).

To determine if the *in vitro* data were consistent with response *in vivo*, we established mouse xenografts with the SK-Hep KI-ILK or empty vector transformants, and then treated these tumors with either gefitinib or vehicle. No significant change in growth was observed when SK-Hep empty vector tumors were treated with gefitinib at 80 mg/kg (Fig. 4C). However, when the same dose of gefitinib was used to treat SK-Hep KI-ILK tumors, a significant reduction in tumor growth was observed (Fig. 4D). After 28 days, the volume of the SK-Hep KI-ILK tumors treated with gefitinib were significantly \((P = 0.004)\) 30% less than the controls.

Taken together, these results suggest that ILK is responsible for the observed increased activation of AKT in mesenchymal hepatoma cell lines. In addition, whereas other mechanisms of AKT activation may be operant, inhibition of ILK activity increases mesenchymal HCC sensitivity to EGFR-targeted therapies.

**Discussion**

To our knowledge, this is the first report characterizing hepatoma cell lines as epithelial or mesenchymal based on their expression of E-cadherin and vimentin. Interestingly, HCC EMT strongly correlates with susceptibility to EGFR-targeted therapies including erlotinib, gefitinib, and cetuximab. Previously, a strong correlation between EMT and erlotinib sensitivity (17–19) as well as

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**Figure 2.** Analysis of the IGF-II/IGF-IR and EGFR signaling pathways in human hepatoma cells. A, Western blot analysis was used to analyze total levels of IGF-IR, EGFR, ErbB3, and ILK, as well as downstream activation of ERK, STAT3, and AKT. B, IGF-II expression was determined by quantitative real-time PCR. C, ILK Western blots were quantified by densitometry.
gefitinib sensitivity (20, 21) had been reported, and this is the first study to show a similar correlation for cetuximab. Overall, the hepatoma cell lines were more responsive to erlotinib and gefitinib and only marginally responsive to cetuximab. These results are consistent with data from clinical trials of advanced HCC patients where 32% (8) and 28% (9) of patients were progression-free at 6 months after treatment with erlotinib compared with 3% after treatment with cetuximab (10). The IC50 values reported in this study for hepatoma cell lines are slightly higher than the serum concentrations observed in patients under treatment and for what has previously been reported for NSCLC cells. Some of these differences may be explained by the fact that the growth curves reported here were done under normal growth conditions rather than in low serum with direct activation of EGFR because the majority of hepatoma cell lines did not grow well under the latter conditions.

A previous report had suggested that IGF-II/IGF-IR signaling could lead to gefitinib resistance in the hepatoma cell lines HepG2 and Hep3B (36). These epithelial cell lines, which express IGF-II/IGF-IR, are responsive to IGF-IR inhibition (39), and combination

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**Table 2. Inhibition of ILK renders mesenchymal cell lines more susceptible to EGFR-targeted therapies**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Cetuximab</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IC50 (μmol/L)</td>
<td>%Max inhibition</td>
<td>IC50 (μmol/L)</td>
</tr>
<tr>
<td>SK-Hep empty</td>
<td>11.5</td>
<td>72</td>
<td>19.4</td>
</tr>
<tr>
<td>SK-Hep KI-ILK</td>
<td>6.4</td>
<td>85</td>
<td>6.5</td>
</tr>
<tr>
<td>SNU-449 empty</td>
<td>&gt;20</td>
<td>47</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SNU-449 KI-ILK</td>
<td>9.1</td>
<td>67</td>
<td>11.5</td>
</tr>
</tbody>
</table>

NOTE: Percent of maximal inhibition was calculated for the highest dose for each drug (20 μmol/L for gefitinib and erlotinib and 1,000 μg/mL for cetuximab).
therapy has been shown to increase susceptibility to EGFR inhibition in these cells (36). Our results suggest that EMT is the primary determinant of response to EGFR inhibition in HCC cells. However, IGF-II/IGF-IR signaling may act as a secondary mechanism of resistance in a subset of epithelial cells and may explain why SNU-423 is the most responsive hepatoma cell line to EGFR-targeted therapies. SNU-423 is epithelial and has low levels of IGF-II and IGF-IR.

EMT status was independent of both total EGFR levels and downstream ERK signaling, suggesting that other mechanisms are responsible for the predictive role of EMT in sensitivity to EGFR inhibitors. ErbB3 expression was observed only in the epithelial cells, which is consistent with the findings in NSCLC cells (17, 18). In a panel of 12 pancreatic and 13 colorectal tumor cell lines, coexpression of EGFR and ErbB3 was only observed in erlotinib-sensitive cells where it mediates AKT signaling (40). Heterodimerization of EGFR-ErbB3 also mediates signaling to AKT in NSCLC and susceptibility to gefitinib (41). Further, gefitinib was shown to reduce phospho-AKT levels only in those NSCLC cells where it inhibits growth. These data suggest that only cells that are dependent on the EGFR pathway for activation of AKT are sensitive to EGFR-targeted therapies. Consistent with this, forced expression of ErbB3 or mutant EGFR does not render resistant cells susceptible to gefitinib (41) because they have developed alternative methods for activating AKT.

Therefore, it is likely that the epithelial cells are more susceptible to EGFR-targeted therapies due to their activation of AKT primarily through EGFR-ErbB3. Mesenchymal cells activate AKT through alternative pathways like ILK (42) and are largely resistant to EGFR inhibitors. ILK is a serine/threonine protein kinase that is localized at the focal adhesion and interacts with the cytoplasmic domains of β1 and β3 integrins. The kinase activity of ILK is stimulated by growth factor stimulation of receptor tyrosine kinases or by engagement of integrins to the extracellular matrix (ECM; ref. 43). Interestingly, exposure of hepatoma cells to the ECM protein laminin 5 increases resistance to gefitinib (44).

Recent reports suggest that overexpression of ILK induces EMT by repressing E-cadherin expression (26), and conversely, inhibition

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**Figure 4.** Apoptosis analysis and in vivo xenograft mouse model. Caspase-3 activity was measured in SK-Hep and SNU-449 KI-ILK and empty vector transformants after treatment with increasing concentrations (2, 5, and 10 μmol/L) of erlotinib (A) or gefitinib (B). The results are expressed as the percent increase as compared with untreated cells. The percent increase of caspase-3 activity elicited by each individual concentration of EGFR inhibitor in the empty vector controls was compared with the percent increase elicited by that same concentration in the KI-ILK transformants by Student’s t test (*, P < 0.05). Tumor growth curves of SK-Hep empty vector (C) or KI-ILK (D) transformants treated with gefitinib (80 mg/kg) or vehicle solution. Differences in tumor volumes were compared by Student’s t test (*, P < 0.01).
of ILK has been shown to induce E-cadherin expression (45). The key mechanism by which ILK regulates E-cadherin levels is through the inhibition of E-cadherin transcription (43). Therefore, we used quantitative real-time PCR to examine the expression of E-cadherin mRNA in the SK-Hep and SNU-449 Ki-ILK transfectants and observed a dramatic up-regulation as compared with the empty vector controls. Interestingly, the Ki-ILK transfectants also had decreased mRNA levels of vimentin, suggesting that they had undergone a mesenchymal-to-epithelial transition. Further, our data suggest that inhibition of ILK leads to decreased AKT activation and increased sensitivity to EGFR-targeted therapies both in vitro and in an in vivo xenograft model. However, it should be noted that some AKT activation remains even after transforming cells with Ki-ILK, suggesting that other mechanisms of AKT activation may be operant in mesenchymal cells as well.

Microarray analysis of human hepatoma cell lines reveals two distinctive subtypes (31). Lee et al. (46) have proposed that human HCC cell lines retain the genomic alterations of the primary tumors, and therefore it is not surprising that multiple microarray studies on primary HCCs have also divided them into two subgroups (16, 47, 48) with gene expression patterns similar to what has been reported for the HCC cell lines (31, 49). We found a strong correlation between EMT and these previously described subtypes. The epithelial cell lines mostly belonged to group I, whereas mesenchymal cells belonged to group II characterized by elevated expression of genes involved in metastasis. These data are consistent with the recent observation that vimentin overexpression is associated with HCC metastasis (50).

It has been suggested that a limited set of quantitative real-time PCR reactions may be sufficient to predict prognosis of patients at the time of HCC diagnosis (47), and we propose that the same strategy may work to determine sensitivity to chemotherapeutic regimens. In particular, the data presented here suggest that HCC cells contain multiple mechanisms of resistance to EGFR-targeted therapies including EMT mediated through ILK and, to a lesser extent, IGF-II/IGF-IR signaling. Therefore, patients with epithelial tumors identified by elevated expression of E-cadherin and ErbB3 may be prime candidates for EGFR-targeted therapies, whereas combination therapies targeting EGFR and ILK may be required for more invasive, mesenchymal tumors. Our data support these hypotheses and merit further examination in clinical studies.

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