Cancer-Associated Fibroblasts Derived from EGFR-TKI–Resistant Tumors Reverse EGFR Pathway Inhibition by EGFR-TKIs

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Abstract

Epidermal growth factor receptor (EGFR) plays a critical role in oncogenesis, which makes it an attractive target for pharmacologic inhibition. Yet, EGFR inhibition with tyrosine kinase inhibitors (TKI) does not result in a measurable and sustainable clinical benefit in a vast majority of tumors. This emphasizes the need for further investigations into resistance mechanisms against EGFR-TKIs. We previously reported the generation of an in vivo adenocarcinoma model of EGFR-TKI–acquired resistance that was devoid of the known mechanisms of resistance. Using this same xenograft model, we now show that the tumor stroma plays an important role in limiting responsiveness to EGFR-TKIs. EGFR-TKI–resistant tumors display increased surface expression of CD44hi/CD24lo and markers of epithelial to mesenchymal transition (EMT), SNAI1, and N-cadherin. An in vivo green fluorescent protein–tagging approach reveals that the tumor stroma of the EGFR-TKI–resistant tumors is distinct in that 24% of its cancer-associated fibroblast (CAF) population is composed of EMT-derived tumor cells that represent the in vivo escape from EGFR-TKIs. We further show that EMT subpopulation–harboring CAFs isolated from the EGFR-TKI–resistant tumors are tumorigenic and express the biomarker of gefitinib resistance, epithelial membrane protein-1. Finally, we provide evidence that paracrine factors secreted from the EGFR-TKI–resistant CAFs mitigate the EGFR-TKI–mediated blockade of pEGFR and pMAPK in cocultured tumor cells, regardless of their EGFR mutational status. This is the first demonstration that the tumor stroma is modified with acquisition of EGFR-TKI resistance and that it further contributes in promoting drug resistance. Mol Cancer Res; 8(6); 809–20. ©2010 AACR.

Introduction

Epidermal growth factor receptor (EGFR) kinase signaling pathway plays an integral role in the tumorigenic process of all solid tumors (1, 2). This knowledge was the basis for the development of pharmacologic kinase inhibitors against EGFR. However, the clinical benefit realized with anti-EGFR treatment is both variable and unpredictable among epithelial cancers. This is attributed to inherent differences in these cancers that hamper a uniform response to such targeted therapeutics. Attempts to identify distinguishing features that select tumors for response have revealed activating somatic mutations (3, 4) and gene amplifications (5) within the EGFR gene. These features are associated with dramatic clinical responses to EGFR tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, as they are suggested to enhance the “dependence” of tumor survival on the EGFR pathway. However, such mutations and gene amplifications are primarily limited to a subset of non–small cell lung cancers (NSCLC). Most other solid tumors are modestly dependent on the EGFR pathway and harbor a nonamplified, wild-type EGFR. Such tumors have shown limited and short-lived responses to EGFR-TKIs in very few patients precluding the development of anti-EGFR therapeutics for these cancer types. Well-known examples include clinical experiences in renal cell carcinoma (6, 7), breast cancer (8), prostate cancer (9), glioblastoma, and several others (10). Known features that determine nonresponse to EGFR-TKIs include pathway gene mutations and alternative signaling pathway activations (3, 11-16). However, these mechanisms of EGFR-TKI resistance, which are mostly focused on genetic and posttranslational modifications within tumor cells, are not always present in all epithelial cancers that are nonresponsive to EGFR-TKIs. This emphasizes that a gap exists in our understanding of factors that limit EGFR-TKI responsiveness and highlights the need to extend investigations into mechanisms of resistance beyond the tumor epithelial cells.
Solid tumor initiation, sustenance, and progression are heavily influenced by the supporting stroma or the tumor microenvironment that includes the extracellular matrix, cancer-associated fibroblasts (CAF), and the angiogenic component (17–19). Much of the current knowledge, with respect to the role of tumor microenvironment, has been generated from research in epithelial cancers that are dependent on tumor-stromal interactions for survival as shown in breast, prostate, and renal cancers (20–22). Coincidentally, these are also the cancer types that have not responded well to EGFR-TKI treatments. Tumor microenvironment is known to limit or modulate therapeutic responses (23) such as to gemcitabine and radiation therapy in pancreatic cancer (24), doxorubicin and cis-platinum in breast and prostate cancer (25), and cisplatin and paclitaxel in ovarian cancer (26). However, it is presently unclear if the tumor microenvironment similarly influences responses to kinase inhibitors such as those targeted to the EGFR.

CAFs are the principal component of the tumor microenvironment that regulate tumor cell function by secreting growth factors, chemokines, and extracellular matrix (17, 21, 27). CAFs are typically not considered tumorigenic themselves but rather known to potentiate tumor growth and invasion (28), thereby modulating therapeutic outcome through active signaling cascades with tumor cells (28) and subsequent acquisition of genomic instabilities (29, 30). However, the CAF population can also include epithelial to mesenchymal transition (EMT)– or endothelial to mesenchymal transition–derived tumor cells (31–34) that can influence their functional properties. Markers of EMT have been extensively correlated with tumorigenesis (34) but more importantly with therapeutic resistance including that to EGFR-TKIs (12, 35, 36). Regardless of the tissue of origin, EGFR-TKI–resistant tumors expressing EMT markers lack EGFR-activating somatic mutations. Ectopic reintroduction of E-cadherin, a marker of the epithelial phenotype, can reverse nonresponse to EGFR-TKIs (37). These correlative studies suggest that the tumor microenvironment may have a crucial role in governing EGFR-TKI responsiveness in solid tumors; however, a direct demonstration of such a phenomenon has not been reported.

Our group previously reported the derivation of an EGFR-TKI–acquired resistance adenocarcinoma model (38) from a xenograft line that was modestly responsive to EGFR kinase inhibition. The acquired resistance model was devoid of EGFR kinase domain mutations and other known EGFR-TKI resistance mechanisms but overexpressed several markers of the extracellular matrix. Using this model, epithelial membrane protein-1 (EMP-1) was identified as a biomarker of gefitinib resistance, which was clinically validated in NSCLC samples. In the current study, we present direct evidence that the tumor-associated stroma plays a distinct role in modulating EGFR-TKI responsiveness in this clinically validated model of EGFR-TKI–acquired resistance. Specifically, our data show that a subpopulation of the CAFs isolated from the EGFR-TKI–resistant tumors are a result of EMT and, in collaboration with the host fibroblasts, inhibit the EGFR-TKI–mediated blockade of the EGFR pathway in cocultured tumor cells. Overall, our study uncovers a role for tumor-stromal interactions in governing response to EGFR-TKIs.

**Materials and Methods**

**Cell lines and primary tumor ex vivo cultures**

*Ex vivo* tumor cells were cultured in DMEM/F12 medium with 10% fetal bovine serum. HCC827 NSCLC cells (American Type Culture Collection) were maintained in RPMI containing 10% fetal bovine serum. CAFs were cultured in stromal cell basal medium with supplements, using the SGBM BulletKit™ (Lonza).

**CAF isolation**

Gefitinib parental (GP) or gefitinib-resistant (GR) tumors excised from euthanized mice were rinsed with phenol red-free RPMI (containing 200 U/mL Penicillin and 200 μg/mL of Streptomycin) and enzymatically digested using Collagenase II (100 U/mL) for 5 hours at 37°C. The digested tissue was washed and resuspended in stromal cell basal medium. The epithelial tumor cells were separated from the fibroblasts by a low-speed centrifugation method (400 rpm for 5 min) at room temperature and sorted by fluorescence-activated cell sorting using an epithelial cell adhesion molecule (EpCAM)–phycoerythrin (PE)–conjugated antibody (BD Biosciences). These cells were the purified epithelial tumor cells. The supernatant from the differential centrifugation of the digested tumor tissue that contained the fibroblasts was gently aspirated, plated in cell culture dishes with fresh stromal cell basal medium, and incubated in a humidified chamber at 37°C with 5% CO2. Confluent fibroblast cultures were negatively sorted by fluorescence-activated cell sorting using the epithelial cell adhesion molecule (EpCAM)–PE–conjugated antibody. The data were analyzed using the CellQuest software version 3.1 (BD Biosciences). These negatively sorted fibroblast cells lacking EpCAM expression were the purified GP or GR CAFs. Immunocytochemistry with fibroblast-specific marker, α-smooth muscle actin (α-SMA), and another epithelial-specific marker, E-cadherin, further confirmed the purity of the fibroblasts.

**Coculture and viability assays**

For coculture experiments, HCC827 or the GP tumor cells were plated in the lower chamber of six-well Transwell plates with inserts of pore size 3 μm (Costar), and the various CAFs were plated in the accompanying inserts. The tumor cells were treated with the indicated gefitinib concentrations for 24 hours. At the assay end point, the tumor cells were harvested and lysed for immunoblotting. Cell viability was analyzed using the CellTiter™ 96 AQueous Non-Radioactive Cell Proliferation Assay colorimetric kit (Promega).

**Lentivirus-based green fluorescent protein tagging of tumor cells**

pLenti6.3-green fluorescent protein (GFP; Invitrogen) was cotransfected with the ViralPower packaging mix (Invitrogen)
into 293T cells using Fugene-HD (Roche). Seventy-two hours posttransfection, the supernatant was collected and filtered through a 0.45-μM cellulose acetate membrane. The GFP-expressing virus was subsequently used to infect 2 × 10^6 of GP or GR tumor cells at an multiplicity of infection of 50. Ninety-six hours postinfection, lentivirus-infected cells were dislodged from the culture plates and surface stained for the epithelial marker, EpCAM. Tumor cells were double sorted for GFP and EpCAM, and ∼5 × 10^4 of the dual-positive tumor cells were mixed 1:1 with Matrigel (BD Biosciences), injected s.c. into nude mice, and monitored for tumor growth. Upon reaching a tumor volume of ∼1,000 mm^3, the GFP-expressing tumors were used to isolate the CAFs and tumor cells as described above. CAFs isolated from the GP or GR GFP tumors were subsequently used for immunocytochemical and flow cytometric studies.

**Immunohistochemistry**

Flow cytometric staining of GP and GR tumor cells and CAFs was carried out using the following antibodies: PE-conjugated CD44 (BD Pharmingen), FITC-conjugated CD24 (BD Pharmingen), PE-conjugated EpCAM (BD Pharmingen), α-SMA (Sigma), and PE-conjugated CDH2 (R & D Systems). Data were analyzed on the MoFlo Cell Sorter (Beckman Coulter, Inc.).

For immunoblotting, pEGFR (Cell Signaling), total EGFR (Cell Signaling), pMAPK (Cell Signaling), and pAkt (Cell Signaling) antibodies were used with β-actin (Sigma) as the normalization control.

For immunofluorescence, GP or GR CAFs were seeded in chamber slides, fixed with 3% paraformaldehyde in PBS, blocked in 5% goat serum, and incubated with antibodies against α-SMA (Sigma) or N-cadherin (BD Biosciences). After antibody incubation, cells were washed with PBS, were incubated with Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (Invitrogen), and mounted in the Prolong Gold Antifade reagent (Molecular Probes). Cells were stained with the nuclear stain TOPRO-3 or 4',6-diamidino-2-phenylindole (DAPI; Sigma). Confocal microscopy images were acquired at ×20 or ×60 magnification and analyzed using Adobe Photoshop.

For immunohistochemistry, GR CAF– or GR CAF–derived or GFP-tagged tumors were fixed using 3% paraformaldehyde and processed for immunohistochemical staining using standard procedures. The samples were probed with antibodies against Ki67 (DakoCytomation), pan-cytokeratin (Biogenex), E-cadherin (BD Biosciences), α-SMA (Sigma), or GFP (Abcam) antibodies. Visualization of immune complexes was done with a 3,3′-diaminobenzidine peroxidase substrate kit (Vector Laboratories, Inc.) or using Alexa Fluor 488– or Alexa Fluor 568–conjugated secondary antibodies (Invitrogen). The images were acquired using the Leica microscope at ×20 or ×60 magnification and analyzed using Adobe Photoshop.

**Fluorescence in situ hybridization**

Fluorescence in situ hybridization was performed on methanol-fixed GP and GR CAFs. The samples were probed for CEP Y (DYZ1) SpectrumGreen (Abbott Molecular), a probe-specific for human Y-chromosome (37). The specificity of the probe was confirmed using mouse sample as the negative control. Codenaturation and hybridization were done using the HyBrite machine. Slides were washed and counterstained using the manufacturer’s protocol. One hundred interphase nuclei were examined and counted.

**Real-time quantitative reverse transcription-PCR**

RNA was isolated from tumors using the Trizol reagent (Invitrogen) and from cells in culture using the RNeasy Extraction kit (Qiagen). One-Step reverse transcription-PCR (RT-PCR) was done using AmpliTaq gold PCR Master Mix Reagent (Applied Biosystems) on a 7900HT Sequence Detection System (Applied Biosystems). One hundred nanograms of RNA were run in triplicate for each sample in a 384-well plate, normalized to the indicated genes, and analyzed by either the absolute quantification (AQ) or the comparative CT (RQ) method. The primer and probe sets for CD44, CD24, CDH1, SNAI2, TWIST, and ZEB1 were purchased from Applied Biosystems. The sequences for EMP-1, CDH2, and SNAI1 used were as shown in Table 1.

**Xenograft studies**

Eight- to 10-week-old nude athymic BALB/c female mice were obtained from Charles River Breeding Laboratories and were maintained in pressurized ventilated cages at the Cedars-Sinai Medical Center vivarium. All animal experiments were done as per the institutional guidelines and approved by the Institutional Animal Care and Use Committee at Cedars-Sinai Medical Center. For studying tumor generation from pure CAFs, ∼4 × 10^5 of the respective fibroblasts purified as per

**Table 1. Primer and probe sequences for quantitative reverse transcription-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>EMP-1</td>
<td>CCAGTGAGGATTGCCTCAAA</td>
<td>CCAGGAGGGGCAATGACAC</td>
<td>AGTGCAGGCCTTCATGATT</td>
</tr>
<tr>
<td>SNAI1</td>
<td>ATGGGAGGCCCTACTACAG</td>
<td>TCCAGATGAGCATTGG</td>
<td>CTCTAATCCAGTTCCTCAGG</td>
</tr>
<tr>
<td>CDH2</td>
<td>TGAAGGTTTTGCGACGATGACT</td>
<td>CAGCACAAGGATGAAGCAGGATG</td>
<td>CCGGGACTGACAGAGGTGTAGTTG</td>
</tr>
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the protocol mentioned above, or \(5 \times 10^3\) cells for the GFP-expressing tumor cells, were s.c. injected with Matrigel (1:1), and tumor growth was monitored. Tumor sizes were measured twice weekly with vernier calipers, and tumor volumes were calculated as: \(\pi/6 \times \) larger diameter \(\times (\text{smaller diameter})^2\). Data are represented as a plot of mean tumor volumes versus time in days.

**FIGURE 1.** A, GP and GR epithelial tumor cells are equally responsive to gefitinib. Equal numbers of GP and GR epithelial tumor cells seeded in 96-well plates were treated in quadruplicates with increasing concentrations of gefitinib (0, 0.1, 1, 5, 10, and 20 μmol/L) for 5 d. End point cell viability was measured using the MTS assay, and the data are represented as a percentage of the nontreated sample. B, same as in A, except cells from 60-mm dishes were lysed and analyzed by immunoblot for pEGFR, EGFR, pMAPK, and β-actin. C, hEMP-1 is expressed in the GR CAFs. Quantitative RT-PCR analysis of hEMP-1 mRNA comparing GP to GR in the tumor tissue, epithelial tumor cells, and CAFs. Data are normalized to GAPDH and presented as fold induction of GP tumor tissue. D, FISH analysis of purified GP and GR CAFs with CEP Y (DYZ1) probe (green fluorescent signal) depicting human nuclei (white arrow) and murine nuclei (red arrow). Nuclear counter staining with DAPI (blue).
Results

EMP-1, marker of gefitinib resistance, is expressed in the GR CAFs

We previously reported the in vivo derivation of a GR model (38) from an isogenic GP CWR22R xenograft. GP tumors displayed modest but significant tumor growth inhibition (∼63%) in presence of gefitinib, as also reported by others (39). Gefitinib responsiveness was rapidly lost when the GP model was passaged in the presence of gefitinib. Unlike the differential responsiveness observed in vivo, purified GP and GR epithelial tumor cells had comparable viability in presence of gefitinib (Fig. 1A), indicating a role for the tumor microenvironment in modulating EGFR-TKI response. As expected, inhibition of pEGFR and pMAPK was minimal with gefitinib in line with the limited dependency of this model on the EGFR pathway (Fig. 1B). This prompted us to isolate and characterize the CAFs from GP and GR tumors. Isolated CAFs were confirmed for their purity by the absence of epithelial-specific markers, EpCAM (Supplementary Fig. S1) and E-cadherin, and the presence of the fibroblast-specific marker, α-SMA (Supplementary Fig. S2). The definition and composition of CAFs remains relatively poorly defined with some groups favoring a functional definition, i.e., activated fibroblasts that promote tumorigenicity (27, 40). However, in this study, we define CAFs based on their fibroblastic morphology and cell surface expression in GP (left) and GR (right) epithelial tumor cells. The percentage of positive cells is indicated in the respective quadrants.

FIGURE 2. Increased expression of mesenchymal markers and CD44hi/CD24lo surface expression in GR epithelial tumor cells. A, representative photomicrographs of GP and GR epithelial tumor cells. B, relative mRNA expression of human CDH2 (top), human CDH1 (middle), and human SNAI1 (bottom) in GP and GR epithelial tumor cells from three different tumor isolates. Data are normalized to GAPDH and presented as fold expression. C, flow cytometry comparing CDH2 cell surface expression in GP and GR epithelial tumor cells. D, flow cytometry comparing CD44 and CD24 cell surface expression in GP (left) and GR (right) epithelial tumor cells. The percentage of positive cells is indicated in the respective quadrants.
FIGURE 3. In vivo demonstration of EMT in GFP-tagged tumors. A, immunocytochemistry for α-SMA in GP-purified (left) and GR-purified (right) CAFs. Multiple cells dual positive for GFP and α-SMA are indicated (white arrows, right). B, immunocytochemistry for CAF subpopulation dual positive for GFP and α-SMA (a-d) or N-cadherin (e-h). d and h, DAPI-stained human (h) or murine (m) nuclei. d and h, enlarged views for regions outlined in (c and g), respectively. Images were acquired at ×20 (A) or ×60 (B) and analyzed in Adobe Photoshop. C, H&E and α-SMA immunohistochemistry on GP and GR tumor tissues. D, immunohistochemistry for the GFP-expressing GP and GR tumor tissue. GP and GR tumor tissues were coimmunostained with antibodies against GFP and pan-cytokeratin (a and d), or GFP and α-SMA (b and e). c and f, enlarged images for regions outlined in (b and e), respectively. Images were acquired at ×10 magnification (a and d) or ×60 magnification (b and e). White arrows, the positive α-SMA staining in GP tumor sample (c) and overlap of GFP and α-SMA staining in GR tumor sample (f). E, flow cytometry for α-SMA and GFP to estimate the percentage of dual-positive GR CAFs. Percentage of positive cells is indicated in the respective quadrants.
expression of mesenchymal markers such as α-SMA with a simultaneous absence of epithelial cell markers. This definition of CAFs based on morphology and fibroblast marker expression has been previously described and typically includes cells derived as a result of EMT or endothelial to mesenchymal transition (31-33).

As another indicator of fibroblast purity, a quantitative RT-PCR for EMP-1 was done on the purified GP and GR CAFs using both human- and murine-specific gene primers. We were surprised to note that although murine EMP-1 (mEMP-1) mRNA levels were comparable between GP and GR CAFs (data not shown), human EMP-1 (hEMP-1) was overexpressed in the GR CAFs by ∼45-fold (Fig. 1C) compared with the GP CAFs. Expression of hEMP-1 in the GR CAFs implied the presence of human fibroblasts among the murine fibroblast population. This observation was further confirmed by comparative FISH analysis using CEP Y (DYZ1), a human Y-chromosome-specific probe (41), which showed that the GP CAFs were exclusively murine, whereas ∼15% of the GR CAFs were of human origin [Fig. 1D, compare the nuclei indicated in white (human) or red (murine) arrows]. These data provide clear evidence that human fibroblast population characterized by hEMP-1 expression exists within the murine stroma of the EGFR-TKI–resistant GR tumors, supporting the notion of an epithelial origin for this fibroblast subpopulation.

**EGFR-TKI–resistant GR tumors express mesenchymal markers and CD44hi/CD24lo cell surface expression**

The tumor cell morphology was comparable between the purified GP and GR epithelial tumor cells (Fig. 2A). Because the GR CAFs include a human-specific subpopulation, a plausible explanation was that these cells might have originated from the GR tumor cells through an EMT event. To explore this possibility, we compared the expression of classic EMT markers in the ex vivo derived GP and GR epithelial tumor cells. Quantitative mRNA analyses revealed that the mesenchymal marker, human CDH2 (N-cadherin), was expressed approximately 8- to 10-fold higher in the GR tumor cells (Fig. 2B, top) compared with the GP counterparts. However, mRNA expression of the epithelial marker, human CDH1 (E-cadherin), was comparable between the GP and GR epithelial tumor cells (Fig. 2B, middle). We also analyzed the cell surface CDH1 and CDH2 protein expression in GP and GR epithelial tumor cells. Although cell surface CDH1 protein expression was comparable between GP and GR epithelial tumor cells (data not shown), a higher percentage (25.6%) of GR epithelial tumor cells expressed CDH2 (Fig. 2C) than the GP epithelial tumor cells (0.8%). Furthermore, comparative mRNA analyses of the key transcription factors that regulate EMT, i.e., SNAI1, SNAI2, ZEB1, and Twist, revealed approximately 5- to 10-fold overexpression of SNAI1 in three different isolates of GR epithelial tumor cells (Fig. 2B, bottom). The other transcription factors, SNAI2, ZEB1, and Twist, did not display a consistent expression pattern in three different isolates of GP and GR epithelial tumor cells (Supplementary Fig. S3).

EMT induction is linked with the acquisition of CD44hi/CD24lo cell surface expression (42). We observed a higher (84.64%) CD44hi/CD24lo population in the GR epithelial tumor cells, whereas only 1.56% was detected in the GP epithelial tumor cells (Fig. 2D). Further, we compared CD44 and CD24 mRNA expression in three separate isolates of GP and GR epithelial tumor cells. Alongside, we also examined tumor cells derived from an isogenic paclitaxel-resistant model, which was also derived from the parental CWR22R xenograft.3 As shown in Supplementary Fig. S4, CD44 mRNA was expressed at two orders of magnitude higher in GR epithelial tumor cells compared with the GP counterparts, whereas CD24 expression remained unchanged. The paclitaxel-resistant cells did not overexpress CD44. These cells also failed to overexpress the EMT markers observed in the GR model (data not shown).

Taken together, these analyses indicate the possible occurrence of EMT in the GR model. Furthermore, presence of an explicit CD44hi/CD24lo cell surface signature, specifically in the GR tumor cells that are presumably undergoing EMT, is suggestive of a link between the two phenomena in this EGFR-TKI nonresponsive model.

**EGFR-TKI–resistant GR tumors undergo EMT**

Our observations thus far indicate the occurrence of EMT in the GR tumors; however, a direct demonstration is lacking. Therefore, we tagged both ex vivo GP and GR epithelial tumor cells with GFP, sorted them with EpCAM-positive cells to ascertain epithelial cell purity, and injected them s.c. into nude mice with the idea that if EMT is occurring, then the CAFs in the resulting tumors would be GFP-positive (procedure outlined in Supplementary Fig. S5). Consistent with our hypothesis, GFP expression was observed in a subset of purified GR CAFs after the GR tumors grew out (Fig. 3A). Coexpression of two different mesenchymal markers, α-SMA and N-cadherin, with GFP confirmed the mesenchymal lineage of the GFP-positive GR CAFs, which thus showed that these cells were indeed the result of an in vivo EMT event (Fig. 3B). Immunohistochemical staining of paraffin-embedded sections of the tumor also revealed a colocalization of GFP and the mesenchymal marker, α-SMA, specifically in the GR tumors (Fig. 3D, compare c and f). A closer inspection of the GFP-expressing mesenchymal cells derived from GR tumors revealed that their morphology was very different from the host fibroblasts in that their phenotype appeared at an intermediate stage between an epithelial and a mesenchymal cell morphology (Fig. 3B). Their nuclei had a diffused DAPI staining pattern (characteristic of human nuclei) as opposed to the punctate staining of the host murine nuclei (Fig. 3B, d and h; ref. 43). Although this analysis revealed the presence of EMT-derived CAFs within the

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3 Jain et al., unpublished data.
GR tumors, an overall comparative morphologic and immunohistochemical evaluation suggested similar epithelial to stromal ratios in the GP and GR tumors (Fig. 3C).

Next, we determined the percentage of the EMT-undergoing GFP cells within the GR CAFs. Flow cytometric analysis of the GP and GR CAFs with coexpression of $\alpha$-SMA and GFP indicated that unlike the GP CAFs, 23.9% of the GR CAFs were dual positive for GFP and high $\alpha$-SMA expression (Fig. 3E). This cell percentage is a little bit higher than what was estimated by FISH analysis (Fig. 1D), which could be due to the difference in sensitivity of the two techniques. Overall, these studies show that the GR tumors, but not the GP tumors, undergo EMT, and that a subpopulation of the GR CAFs comprises the EMT-undergoing GR tumor cells.

**GR CAFs are tumorigenic**

We next asked if the EMT-derived GR CAFs maintain their tumorigenicity while they are resident within the host fibroblast population. To test this, equivalent numbers of purified GP and GR CAFs were s.c. injected into immunodeficient mice and monitored for tumor growth. Two independent isolates of the CAFs were used. Equivalent numbers of GP and GR tumor tissues, GR CAFs, and two different GR CAF–derived tumor tissue isolates. Data are presented as fold induction relative to GP tumor tissue.

![FIGURE 4. Tumorigenicity of GR CAFs. A, tumor growth curves of tumors derived from purified GP and GR CAFs. Purified GR epithelial tumor cells and naïve murine fibroblasts are used as positive and negative controls, respectively. Mean tumor volumes are plotted against time in days. Note the delay of $\sim$14 d before the appearance of the GR CAF–derived tumors. B, GR tumor tissue (top) and GR CAF–derived (bottom) tumor tissue were stained with H&E (a and e) or antibodies against Ki67 (b and f), E-cadherin (c and g), or human-specific pan-cytokeratin (d and h). C, quantitative RT-PCR analysis of hEMP-1 mRNA from GP and GR tumor tissues, GR CAFs, and two different GR CAF–derived tumor tissue isolates. Data are presented as fold induction relative to GP tumor tissue.](image-url)
(E-cadherin positive) and genotypically human (positive for human-specific pan-cytokeratin), thus showing that the GR CAFs are tumorigenic (Fig. 4B, g and h, respectively). GR CAF–derived tumors were histologically similar to the GR tumors (Fig. 4B, compare a and e) and were actively proliferating as shown by positive Ki-67 expression (Fig. 4B, f).

Based on the hEMP-1 expression in the GR CAFs (Fig. 1C), we expected the GR CAF–derived tumors to also express hEMP-1. Indeed the GR CAF–derived tumors expressed hEMP-1 mRNA at levels comparable with the GR CAFs (Fig. 4C). Our data therefore strongly suggest that EMP-1, besides being a biomarker of gefitinib resistance, also identifies the EMT-undergoing tumorigenic cell population within the GR tumors.

**pEGFR and pMAPK are inefficiently blocked with EGFR-TKIs in presence of GR CAFs**

Because GR CAFs were derived from tumors that had acquired resistance to EGFR-TKIs, we wanted to examine if the GR CAFs had any influence on EGFR-TKI activity. We addressed this by directly evaluating the effect of gefitinib on the molecular correlates of the EGFR activity (i.e., pEGFR) and its downstream effectors (i.e., pMAPK and pAkt) in gefitinib-sensitive cells when they were cocultured in the presence of GR CAFs.

GP epithelial tumor cells (Fig. 5A) were cocultured with GP or GR CAFs in the presence of gefitinib for 24 hours before EGFR pathway analysis. In the presence of the GP CAFs, gefitinib treatment resulted in a similar inhibition of the pEGFR signal as in the presence of media alone (compare lanes 1 and 2 to 3 and 4). In contrast, the presence of the GR CAFs caused a resistance to gefitinib-mediated pEGFR inhibition (40% inhibition in presence of GR CAFs compared with 73% inhibition in presence of GP CAFs, compare lanes 3 and 4 to lanes 5 and 6). Because the GP tumor cells are modestly dependent on the EGFR pathway (Fig. 1A and B) and harbor a wild-type EGFR that is not gene amplified, EGFR kinase inhibition at the pMAPK and pAkt levels was minor, if any. This was also the reason why we needed to use high gefitinib concentrations in this assay. We next wanted to examine if the differences in the effects of GP and GR CAFs would also be shown in other gefitinib-sensitive systems that are highly dependent on the EGFR pathway. Therefore, we investigated the influence of GR CAF coculture on a NSCLC cell line, HCC827. HCC827 cells carry a deletion mutation within the EGFR gene, E746-A750, which makes them ultrasensitive to EGFR-TKIs. The differences between the GP and GR CAFs were even more pronounced in the HCC827 cells as their effects were observed on the downstream effectors of the EGFR pathway. As shown in Fig. 5B, 100 nmol/L of gefitinib caused >50% inhibition of the pMAPK signal in presence of the GP CAFs, whereas the presence of the GR CAFs resulted in less than a 15% inhibition. Differences at the level of pEGFR were minor due to the presence of EGFR mutations that increase the affinity of EGFR-TKIs (4). No changes were observed in Akt phosphorylation.

Our data clearly show that the presence of the CAFs, derived from the EGFR-TKI–resistant tumors, significantly compromises EGFR-TKI activity. These data highlight the fact that in addition to the tumor cells, the stroma in the EGFR-TKI–resistant tumors is differentially programmed such that it can limit EGFR-TKI activity in the neighboring tumor cells.

**Discussion**

We have used a unique isogenic pair of a prostate adenocarcinoma model with a limited dependency on the EGFR signaling pathway and its EGFR-TKI–resistant counterpart to uncover novel features of EGFR-TKI–acquired resistance. Using this model pair, we had previously reported the identification of EMP-1 as a biomarker of EGFR-TKI resistance that was validated in EGFR-TKI–resistant lung cancer clinical samples (38). Data presented in this study shows that in addition to the tumor cells, the stroma in the EGFR-TKI–resistant tumors is differentially programmed such that it can limit EGFR-TKI activity in the neighboring tumor cells.
population of the EGFR-TKI–resistant tumors. The CAF population derived from the GR tumors can mitigate EGFR-TKI–mediated EGFR pathway kinase inhibition in tumor cells. The EMT-enriched tumor population in the GR tumors, which represent the in vivo escape from EGFR-TKI inhibition, is marked by EMP-1. Notably, the findings described in our study are specific to EGFR-TKI resistance and were not observed in an isogenic tumor model, which is resistant to the taxane-based chemotherapeutic agent, paclitaxel.

Several reports have previously correlated the EMT markers with nonresponsiveness to EGFR-TKIs (12, 35). We show that EGFR kinase inhibition accelerates the selection of EMT-undergoing cells in tumors with a limited dependency on the EGFR pathway that ultimately leads to acquired resistance to EGFR TKIs. Tumor cells undergoing EMT potentially camouflage themselves within the tumor microenvironment, i.e., the GR CAFs to escape EGFR-TKI exposure. Interestingly, according to our data, the EMT-derived tumor cells within the GR CAFs maintain their tumorigenicity and are able to grow out as epithelial tumors most likely by using the reverse EMT phenomenon of mesenchymal to epithelial transition. We did observe a significant delay in subcutaneous tumor growth from the GR CAFs compared with the GR tumor cells. This time lag may have been due to the reverse transition of the mesenchymal cells to an epithelial phenotype, as the resulting tumors were epithelial in nature. Existence of an EMT-mesenchymal-to-epithelial transition program specifically in the GR tumors implies that the enrichment of EMT-undergoing cells likely plays an active role in the maintenance and progression of EGFR-TKI resistance instead of being a bystander effect of therapeutic nonresponse. There is precedence for the role of EMT in maintaining tumorigenicity (44, 45). In addition to the EMT markers, we also observe EMP-1 expression on the EMT-undergoing GR mesenchymal cells. Upregulation of EMP-1 family members have been shown in erlotinib-insensitive lung cancer cell lines expressing EMT markers by our group and others (12). Whether EMP-1 has a direct involvement in the EMT process as a result of EGFR-TKI resistance is still unknown. However, it is known that EMP-1 is a c-myc target gene (46) and c-myc-driven mouse mammary tumors can elicit EMT (47).

Induction of EMT in the GR model is likely due to the upregulation of the SNAI1 transcription factor. An important question is the identity of specific upstream signals that upregulate SNAI1 to initiate EMT within the GR tumors. EMT can be induced by a multitude of signals (48). Our attempts at inducing transforming growth factor β–mediated EMT in ex vivo GP tumor cells have been unsuccessful. It is plausible that chronic gefitinib treatment initiated the EMT phenomenon in the GR tumors by activating compensatory growth signaling pathways. Activation of such pathways (14, 15) is emerging as a generalized escape mechanism that the tumors use to evade treatment with kinase-specific small-molecule inhibitors.

GR tumor cells undergoing EMT display a CD44hi/CD24lo cell surface expression. CD44hi/CD24lo subpopulations are suggested to mark putative cancer stem cell populations in breast (49) and prostate cancers (50) that are implicated with treatment resistance (51). Therefore, it is possible to speculate that GR tumors harbor a preexisting CD44hi/CD24lo cancer stem cell population that escapes EGFR-TKI response and is enriched for in the GR tumors. However, tumor cells from an isogenic paclitaxel-resistant model did not select for CD44hi/CD24lo expression or EMT markers, thus arguing against the idea of a simple enrichment process of cancer stem cells in the GR model. Recent reports (42, 52) show that EMT stimulates cancer cells to adopt stem cell–like characteristics with a CD44hi/CD24lo surface phenotype. We believe that a similar scenario may exist in the GR model with the acquisition of gefitinib resistance and EMT. Although our evidence for a stem cell–like phenotype in the GR model is preliminary, it is provocative to suggest that the acquisition of stem cell–like phenotype might cooperate with the EMT program in the maintenance of EGFR-TKI resistance while amplifying the resistant tumor population.

Besides the occurrence of EMT and an enriched CD44hi/CD24lo phenotype, the CAF population in the GR tumors is also unique. Paracrine factors secreted from the GR CAFs diminish the EGFR-TKI–dependent inhibition of the EGFR pathway in the neighboring tumor cells independent of the presence of EGFR-activating mutations. The GR CAFs include an EMT-derived subpopulation, and the GFP-tagging strategy clearly indicates that this subpopulation maintains a distinct morphology within the mass of host fibroblasts. It remains to be investigated if it is only the EMT-derived GR CAFs or the entire GR CAF population that plays a role in influencing EGFR-TKI nonresponsiveness in the neighboring tumor cells. Genomic instabilities within CAFs are documented to modulate their function (29, 30). Future studies delineating the identity of the genetic modifications and the paracrine factors secreted by the GR CAFs will help decipher their role in EGFR-TKI nonresponsiveness.

In summary, our study is the first comprehensive examination of the tumor-associated stroma in tumors that have acquired resistance to EGFR-TKIs. It suggests that acquired resistance mechanisms to EGFR-TKIs extend beyond the cancer cell and involve reprogramming of the tumor microenvironment, in addition to the tumor cells. Furthermore, our findings highlight the need for investigations for potential targets within CAFs (53) to counter EGFR-TKI resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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