Characterizing Acquired Resistance to EGFR Tyrosine Kinase Inhibitors in an EGFR Mutant Lung Adenocarcinoma Cell Line

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Traditional treatments for lung adenocarcinomas do not produce impressive improvements in patient health. Newly developed biologically-based treatments exploit sensitivity to tyrosine kinase inhibitors (TKIs) conferred by somatic mutations in the epidermal growth factor receptor (EGFR) found in certain lung adenocarcinomas. However, almost every patient acquires resistance to TKI treatment, and resistance mechanisms in about 30% of cases remain unclear. In the past, the Pao Lab has characterized multiple mechanisms of acquired resistance using isogenic pairs of drug-sensitive and −resistant EGFR-mutant cell line models as well as correlative human data. To potentially discover novel resistance mechanisms, in this project a new in vitro cell line model of acquired resistance was developed using human lung adenocarcinoma EGFR-mutant HCC2279 cells. Preliminary characterization of mechanisms of resistance in these cells was done using mutational profiling, growth inhibition assays, immunoblotting, and light microscopy. Compared to parental cells, the HCC2279 resistant (HCC2279R) cells contained the same lung cancer mutations (exon 19 deletion only), appeared to have an EGFR-independent mechanism of resistance, and were not dependent upon signaling through MET, a receptor tyrosine kinase that shares downstream targets of EGFR that can bypass the need for EGFR in some patients with acquired resistance. HCC2279R cells did display histological and molecular features of epithelial to mesenchymal transition (EMT), where cells lose cell-cell junctions and became more aggressive. EMT has been found in some patients with acquired resistance. Thus, HCC2279R cells may be useful in understanding how EGFR-mutant lung cancer becomes resistant to TKIs via EMT.

INTRODUCTION

Lung cancer is one of the deadliest cancers in the world and is the number one cause of all cancer-related deaths. In the United States alone, lung cancer kills almost 160,000 people per year. Even with treatment, the 5-year survival rate for all stages of lung cancer is only 16%. In response to the dire need for more effective methods of fighting lung cancer, new biologically-based treatment options have been developed. Some show great promise for certain subsets of lung cancer.

One subset of lung cancer currently being targeted by these biological treatments is adenocarcinoma, a non-small-cell lung cancer (NSCLC) which accounts for 40% of all lung cancer cases. Certain adenocarcinomas can harbor mutations in the gene encoding the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK) involved in tumor cell proliferation and survival. Two key mutations that have been found in EGFR are L858R, an arginine for leucine substitution in exon 21, and an exon 19 deletion.

Both these mutations increase EGFR kinase activity, but the most interesting feature of these mutations is that they also confer sensitivity to EGFR tyrosine kinase inhibitors (TKIs). Both the L858R and exon 19 deletion mutations decrease EGFR binding affinity to ATP while increasing EGFR binding affinity to TKIs. First generation TKIs such as erlotinib (Tarceva) and gefitinib (Iressa) are compounds that bind reversibly to EGFR and inhibit kinase activity by preventing ATP from binding to EGFR.

These new TKI treatments have dramatically increased overall response rates in patients with EGFR-mutant adenocarcinomas when compared to traditional treatments like chemotherapy. For patients with advanced NSCLC, the response rate to chemotherapy is only 20-30% with a median overall survival of 10 months. However, when similar patients are treated with TKIs, the response rate is around 70% with a median overall survival of 25-30 months. Furthermore, patients treated with TKIs more often report an improvement in quality of life when compared to patients treated with chemotherapy. Notably, chemo-
therapies are usually given intravenously, while TKIs such as gefitinib and erlotinib are oral drugs, which are taken daily\textsuperscript{7}.

Although EGFR-TKI treatment is promising, one major roadblock is acquired resistance. Almost all patients treated with TKIs develop resistance to treatment in 10-16 months\textsuperscript{10}. Up to 50\% of resistance is caused by a secondary mutation in \textit{EGFR} called T790M, a methionine for threonine substitution in exon 20, which decreases the binding affinity for TKIs and restores EGFR affinity for ATP\textsuperscript{11,12}. To overcome this resistance, second-generation TKIs, such as BIBW2992 (afatinib), are being developed\textsuperscript{13}. Such drugs irreversibly bind to EGFR and inhibit its autophosphorylation\textsuperscript{13}.

Another known mechanism of acquired resistance is \textit{MET} gene amplification. Normally, EGFR phosphorylates ERBB3, which activates phosphatidylinositol 3-kinase (PI3K)/AKT, which in turn inhibits apoptosis and sustains cancer cells\textsuperscript{14} (Fig. 1). Since TKIs inhibit the kinase activity of EGFR, ERBB3 is prevented from activating downstream signals in the presence of TKIs. MET, another RTK, can bypass the need for EGFR by phosphorylating ERBB3 and sustaining the downstream survival signal\textsuperscript{14}. SGX-523 is an ATP-competitive inhibitor that is “exquisitely” selective for MET\textsuperscript{15}. Because EGFR-TKI sensitivity can be restored when MET is inhibited, a combination therapy using both SGX-523 and erlotinib can overcome this resistance in cell line models\textsuperscript{10}.

A third established acquired resistance mechanism that is not clearly understood is epithelial to mesenchymal transition (EMT). In EMT, epithelial cells lose their cell-cell contacts and become more mesenchymal, leading to an increased ability to invade and metastasize\textsuperscript{16}. Although many studies have established a link between EMT and EGFR-TKI resistance, the exact mechanism by which cells undergo EMT remains unclear.

Despite recent advancements in elucidating resistance mechanisms against TKI treatment, the cause of resistance in one-third of all cases still remains unknown\textsuperscript{14}, and this lack of knowledge makes it difficult to advance patient care. In order to identify models of acquired resistance, the Pao lab has developed and characterized multiple isogenic pairs of drug-sensitive and -resistant EGFR-mutant human lung cancer cell lines that exhibited mechanisms of resistance found in patients\textsuperscript{17,18}. These models show what is done in lab can provide valuable insight for what is found in the clinic. In this project, a new \textit{in vitro} cell line model was developed using human lung adenocarcinoma EGFR-mutant HCC2279 cells. These cells were preliminarily characterized by mutational profiling, cell growth assays, immunoblotting, and light microscopy in order to unearth a new mechanism of acquired resistance or to continue developing the knowledge of a current mechanism.

**Materials and Methods**

**Cell Culture**

The isogenic HCC2279 resistant line (HCC2279R) was created from the HCC2279 parental line (HCC2279P) by exposing parental cells to increasing doses of erlotinib as previously described\textsuperscript{18} until resistant cells obtained an erlotinib IC\textsubscript{50} at least 100 times greater than that of parental cells. All cells were maintained in RPMI-1640 complete growth medium supplemented with 10\% fetal bovine serum (FBS) and 1\% penicillin/streptomycin. All cells were incubated at 37°C and 5\% CO\textsubscript{2}.

**Mutation Screening**

The HCC2279R line was screened for recurrent point mutations in 9 genes known to be mutated in lung adenocarcinomas using an established multiplex PCR assay as previously described\textsuperscript{19}. A separate sizing assay was used to screen for common insertion/deletion mutations\textsuperscript{19}.

**Growth Inhibition Assays**

Cell drug sensitivity was analyzed using the Promega CellTiter Blue Cell Viability Assay as previously...
Cell stocks were diluted to accommodate 3000 cells/well, and cells were treated with 5, 25, 50, 100, 1000, or 5000 μM of drug. Cells were prepared for analysis after 120 hours of incubation as previously described. Absorbance was measured at a wavelength of 595 nm and was analyzed using the SPECTRAMAX M5 in the VICB High Throughput Screening Facility core at Vanderbilt. Two independent experiments were run for each cell line, with each experiment containing six replicates of each respective treatment. Cell growth curves were calculated on Excel based on an untreated control.

**Cell Lysis/Protein Preparation**

Before lysis, all cells were exposed to either 1 uM DMSO or 1 uM erlotinib for 6 hours. Cells were lysed using a standard RIPA buffer solution supplemented with protease inhibitor, NaVO₃, NaF, and okadaic acid (Supplementary Table 1). Protein levels were quantified by a Bradford protein assay as previously described. Absorbance was measured at a wavelength of 595 nm. Samples were diluted with supplemented RIPA buffer and 6x SDS with beta-mercaptoethanol to contain 10 mg of protein per 30 ul volume. Samples were boiled at 98°C for 10 minutes before being used or stored in -80°C.

**Immunoblotting**

Target proteins were detected using enhanced chemiluminescence (Perkin Elmer) and various monoclonal antibodies (Supplementary Table 2). All primary antibodies were diluted 1:1000 in 5% milk for total protein and 5% BSA for phosphorylated protein. All secondary antibodies were diluted 1:3000 in the same solvent that was used for the primary antibody. Three independent experiments were performed for each of the specific proteins detected. 30 ul (10 mg) of each sample was loaded into a 4-20% precast Tris-Glycine

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**Table 1. SNaPShot of HCC2279R Cells**

<table>
<thead>
<tr>
<th>Locus</th>
<th>WT Nucleotide</th>
<th>Mutant Nucleotide</th>
<th>Amino Acid Substitution</th>
<th>Phenotype</th>
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<td>G</td>
<td>A</td>
<td>G719S</td>
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<td>G</td>
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<td>G719C</td>
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<td>C</td>
<td>T</td>
<td>T790M</td>
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<td>T</td>
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<td>T</td>
<td>A</td>
<td>L861Q</td>
<td>Wild-Type</td>
</tr>
</tbody>
</table>

An excerpt from the mutation screen that shows the absence of the EGFR T790M mutation in resistant cells.

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**Figure 2.** Growth inhibition curves of HCC2279 parental and resistant cell lines treated with increasing concentrations of various TKIs. Percent growth was calculated based on DMSO controls. E + S is a combination therapy containing equal parts of erlotinib and SGX-523 (MET inhibitor). The error bars shown represent standard deviation. All treatment was administered for 120 hours.
gradient gel (Invitrogen) for separation via gel electrophoresis. Total protein levels were used as loading controls because total protein levels (barring gene amplification) are dependent on cell density rather than treatment. The samples were then transferred onto PVDF membranes using the iBlot transfer system as previously described\(^2\).

**RESULTS**

**HCC2279R Cells Lack Known Secondary Mutations that Confer Resistance to Erlotinib**

The HCC2279R line was screened for 40-plus recurrent point mutations as well as for insertions and deletions in 9 genes known to be mutated in adenocarcinomas using a multiplex PCR assay\(^1\). The only mutation that was found was an exon 19 deletion, the original drug-sensitizing mutation (not shown). No other mutations were found; T790M was not present in the resistant cells (Table 1).

**Growth Inhibition Assays Show HCC2279R Cells Are Resistant to TKIs and Combination Therapy Using TKIs and MET Inhibitors**

EGFR mutant cells with acquired resistance to erlotinib can become independent of EGFR signaling\(^7\). In these cases, cells can bypass EGFR signaling through activation of MET via gene amplification\(^1\). To determine EGFR/MET status in HCC2279R cells, these cells were cultured in the presence of EGFR and MET inhibitors and analyzed with standard growth inhibition assays. Parental cells confirmed to be EGFR dependent, because growth was inhibited by EGFR TKIs, and MET inhibitor alone had no effect (Fig. 2A). The resistant cells, on the other hand, seemed to have lost their dependency on EGFR, because there was no significant inhibition of growth in the presence EGFR TKIs, including in the more potent BIBW2992, except at very high drug concentrations (Fig. 2B). The resistant cells also did not appear to be dependent upon MET signaling, as neither single nor combination therapy (erlotinib + SGX-523) inhibited growth (Fig. 2B).

**HCC2279R Cells Appear to be EGFR Independent and Exhibit Signs of EMT**

Immunoblotting was used to confirm the results of the growth inhibition assays as well as to examine the status of proteins of interest. Total EGFR levels were approximately the same for both parental and resistant cells. Upon treatment with erlotinib, EGFR phosphorylation was completely inhibited in both lines (Fig. 3A). These data suggested that resistant cells no longer depend upon EGFR for growth.

Parental cells depended on EGFR to phosphorylate the downstream targets, AKT and ERK, which suppress apoptosis and increase cell proliferation, respectively (Fig. 1). Total levels of AKT and ERK were the same for both parental and resistant cells (Fig. 3 D,F). In parental cells, there was no phosphorylation of AKT or ERK upon treatment with erlotinib (Fig. 3 C, E). However, the resistant cells still had phosphorylated AKT and ERK, despite the presence of erlotinib (Fig. 3 C, E). Collectively, these results suggested that resistant cells were no longer dependent on EGFR for downstream signaling.

AKT and ERK are shared downstream targets for EGFR and MET. Although the total levels of MET were much higher in resistant cells than in parental cells (Fig. 3H), levels of phosphorylated MET were not higher and were completely inhibited in the presence of erlotinib (Fig. 3G). This suggested that MET signaling in these cells was mediated by EGFR but did not play a major role in acquired resistance.

 Normally, PTEN, a phosphatase, suppresses AKT signaling and helps regulate apoptosis. Loss of PTEN can lead to resistance to erlotinib\(^2\). For HCC2279 cells, both parental and resistant cells had similar levels of PTEN regardless of treatment (Fig. 3I). Another known route of acquired resistance is EMT, in which epithelial cells transition to a mesenchymal cell phenotype. Hallmarks of this transition include loss of expression of E-cadherin, a protein involved in adheren junctions that is highly expressed in epithelial cells, and increased expression of vimentin, an intermediate filament that is abundantly expressed in mesenchymal cells but rarely expressed in epithelial cells\(^16\). In comparison to parental cells, the resistant cells displayed a dramatic decrease in E-cadherin levels and a slight increase in vimentin levels. These data suggested that the resistant cells may have undergone EMT (Fig. 3 J,K).

**Light Microscopy Further Suggests that HCC279R Cells Have Undergone EMT**

One prominent sign of EMT is a loss of cell polarity, as cells transition to a mesenchymal state. When examined by light microscopy, in comparison to parental cells, resistant cells had no visible cell polarity and very few cell-cell contacts (Fig. 4B), suggesting that resistant cells had acquired features of EMT.
**DISCUSSION**

Certain somatic mutations in *EGFR* can predict for highly successful treatment of lung cancers with EGFR-TKIs\(^7,9\). However, acquired resistance to TKIs develops in nearly all patients\(^24,25\). Currently, known mechanisms of acquired resistance include the secondary *EGFR* mutation T790M, *MET* amplification, and EMT\(^11,12,14,16\). All of these have been previously modeled in the lab through the development of isogenic pairs of drug-sensitive and -resistant cell lines\(^9\). To establish another potentially clinically relevant model of resistance, in this project, a new *in vitro* model using HCC2279 cells, which harbor a drug-sensitive *EGFR* mutation, was developed. HCC2279R cells were characterized using a variety of techniques including mutational profiling, growth inhibition assays, immunoblotting, and light microscopy.

Collectively, the characterization of HCC2279R cells suggested that they lacked the common *EGFR* T790M mutation and had become independent of both EGFR and MET signaling for survival. By immunoblotting and light microscopy, resistant cells appeared to have acquired features suggestive of EMT. In lung cancer, EMT has been correlated with a more aggressive tumor and metastasis, because cells lose adherens junctions and become more mobile and capable of invading\(^26\). EMT has also been shown to confer primary EGFR-TKI resistance\(^16,26\). Although EMT could be invoked to explain resistance, it should be noted that is difficult to say if EMT directly causes resistance or if newly activated pathways actually cause resistance.

Although EMT has been established to exist in cancer patients, it is unclear what fraction of patients acquires resistance through this mechanism\(^16\). However, previous studies have shown that only specific factors in EMT affect resistance to TKI treatments\(^27\); thus, it would be helpful to identify specific factors that can trigger EMT and affect sensitivity to erlotinib. Knowing which markers of EMT to screen for could help more accurately tailor a patient’s treatment. Furthermore, if there are specific EMT factors that could be inhibited by a small molecule, new combination therapies could be used to resensitize EMT cells to erlotinib while simultaneously preventing the tumor from becoming more aggressive. To search for such factors in an unbiased manner, a comparison between the mRNA expression profiles of HCC2279 resistant cells and parental cells can be used to identify any differentially expressed genes. The status of overexpressed or underexpressed genes could then be corroborated in an orthogonal fashion via immunoblotting. Then, by overexpression or knockdown studies, it could be shown if these targets mediate erlotinib resistance and/or EMT.
in the cells.

Despite recent advances in understanding the progression of cancer, there is still much to uncover and elucidate. Having a clear understanding of how cancer cells acquire resistance to treatment is crucial for developing the next generation of therapy. By using HCC2279R cells, it is possible to further examine how EMT affects acquired resistance to EGFR-TKIs. Understanding this mechanism could be a stepping stone to uncovering new or more efficient methods of treatment for patients with EGFR-mutant lung cancer.

REFERENCES


Invitrogen. iBlot Dry Blotting System. Grand Islands: Life Technologies; 2011.


