BRIEF REPORT

The Epidermal Growth Factor Receptor-L861Q Mutation Increases Kinase Activity without Leading to Enhanced Sensitivity Toward Epidermal Growth Factor Receptor Kinase Inhibitors

Rama Krishna Kancha, MSc, Christian Peschel, MD, and Justus Duyster, MD

Introduction: Mutations in the epidermal growth factor receptor (EGFR) kinase domain such as EGFR-L858R and EGFR-G719S have been reported to activate the kinase and also sensitize a subset of patients with non-small cell lung cancer to EGFR kinase inhibitor treatment. Nevertheless, for other common point mutations such as EGFR-L861Q, it is unclear whether and to what extent they sensitize toward gefitinib and erlotinib. Thus far, there is no reliable cellular assay to compare in a ligand-independent manner intrinsic kinase activity and drug sensitivity of the unmutated (wild type) and mutated EGFR kinase domain.

Methods: To overcome this obstacle, we introduced L858R, G719S, and L861Q into the backbone of EGFRvIII. EGFRvIII has a wild type-kinase domain but is activated in a ligand-independent manner through a deletion in the extracellular domain.

Results: Using this tool, we show that the L861Q mutation displays enhanced kinase activity and transforming potential compared with L858R, G719S, and also to the wild type-EGFR kinase domain. Interestingly, L861Q does not increase drug sensitivity toward clinically used EGFR kinase inhibitors in contrast to the L858R and G719S mutation. In addition, we demonstrate that EGFR-L861Q could be effectively inhibited with the irreversible second-generation EGFR inhibitor WZ-4002.

Conclusions: Thus, in the common EGFR-L861Q mutation, activation of the kinase domain is uncoupled from a sensitizing effect toward clinically approved kinase inhibitors. Therefore, patients with EGFR-L861Q may not have the same clinical benefit from gefitinib/erlotinib treatment as patients with EGFR-L858R and EGFR-G719S mutations. Treatment with irreversible second-generation kinase inhibitors such as WZ-4002 may be an attractive option in the future for patients with EGFR-L861Q.

Key Words: EGFR, Kinase activity, Gefitinib, Erlotinib, Drug sensitivity.

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A subset of patients with non-small cell lung cancer (NSCLC) with activating mutations in the epidermal growth factor receptor (EGFR) kinase domain was shown to respond to targeted therapy. Nevertheless, not all mutations in the EGFR kinase domain are activating and/or are drug sensitive. Recently, we have established a comprehensive profile of kinase activity, transforming ability, and drug sensitivity of a panel of EGFR kinase domain mutations. We have shown that mutations L858R and G719S are very sensitive to all the drugs tested, whereas another common mutation L861Q is relatively insensitive towards inhibitor treatment. This is in agreement with a previous report.

So far, it was not possible to compare the drug sensitivity of ligand-independent mutant EGFR with that of EGFR-dependent wild-type EGFR in a ligand-independent cellular assay. Thus, we aimed to establish a cell-based system to compare the drug sensitivities of wild type (wt) and mutant receptor kinase domains.

EGFRvIII is an active oncogenic kinase, which is frequently found in glioblastoma patients. EGFRvIII lacks part of the extracellular ligand-binding domain leading to constitutive, ligand-independent activation. In a recent report, we have successfully used EGFRvIII to demonstrate that certain EGFR mutants that were reported in patients with NSCLC in fact are kinase dead. Because EGFRvIII contains a wt-kinase domain and does not require ligand for activation, it can be used as test backbone to study the effect of mutations on kinase activity and inhibitor sensitivity in cells in a ligand-independent manner. Using this system in this study, we compared the kinase activity and transforming ability of common EGFR mutations.

MATERIALS AND METHODS

Constructs, Cell Lines, and Reagents

Cloning of MiGR1-EGFRvIII was described previously. Point mutations were introduced into MiGR1-EGFRvIII using the Quickchange Site-Directed Mutagenesis kit (Fermentas, St. Leon-Rot, Germany) and confirmed by sequencing. HEK293 cells were cultured in Dulbecco’s Modified Eagle Medium (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (PAA). Ba/F3 cells were cultured in Roswell Park Memorial Institute 1640 (Life Technologies) supplemented with interleukin-3 (IL-3; R&D, Wiesbaden, Germany). Stable Ba/F3
FIGURE 1. EGFRvIII-based assay identifies L861Q as hyperactive kinase with strongest oncogenic potential. A, Clinically relevant point mutations in EGFRvIII backbone were transiently overexpressed in HEK293 cells and analyzed for autokinase activity and Stat5 activation. B, Comparative activation of EGFR and Stat5 was quantified and plotted as fold increase in activity with respect to wild type. Analysis was done in triplicates, and measured "p values" were shown. C, Ba/F3 cells stably expressing EGFRvIII mutants were tested for their ability to outgrow untransduced cells on IL-3 withdrawal. Percentage of eGFP-positive cells was measured in triplicates at indicated time points by FACS analysis. D, NIH/3T3 cells stably expressing wild-type or mutant EGFRvIII were plated in soft agar assay at a density of $2.5 \times 10^4$ cells/well in triplicates in a six-well plate. Three weeks after plating, colonies were stained with MTT solution and counted. E, Average number of colonies for each cell line was shown. EGFR, epidermal growth factor receptor; IL, interleukin; eGFP, enhanced green fluorescent protein; FACS, fluorescence activated cell sorting; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.
cell lines were established by retroviral infection with MiGR1-EGFRvIII constructs followed by IL-3 withdrawal. Gefitinib was provided by AstraZeneca, and AEE 788 was a kind gift from Novartis Pharma. Erlotinib was purchased from pharmacy. WZ-4002 was purchased from Axon Medchem BV. Each compound was dissolved in dimethyl sulfoxide to make an initial stock solution of 10 mmol/liter (gefitinib, AEE 788 and WZ-4002) and 2.5 mmol/liter (erlotinib).

FIGURE 2. EGFR-L861Q is not a drug-sensitizing mutation unlike L858R and G719S. Stable Ba/F3 cell lines expressing wild-type or mutant EGFRvIII were tested for their sensitivity toward (A) gefitinib, (B) erlotinib, and (C) AEE788 at indicated concentrations. D, Table representing the effect of individual mutations on intrinsic properties of the kinase. E, Ba/F3 cells expressing wild-type or mutant EGFRvIII were treated with increasing concentrations (50, 100, 250, 500, or 1000 nM) of gefitinib or erlotinib for 30 minutes and analyzed for the inhibition of EGFR autophosphorylation and Stat5 phosphorylation. EGFR, epidermal growth factor receptor.
Proliferation Assay, Soft Agar Assay, and Western Blotting

For proliferation analysis, Ba/F3-EGFRvIII cells (1 × 10⁴) were plated into 96-well plates, and inhibitors were added at indicated concentrations. Cell proliferation was measured at 48 hours using CellTiter96 Proliferation Assay (Promega, Madison, WI).

To test anchorage-independent growth in soft agar, 1 ml of 0.75% agar (Difco, Lawrence, KS) solution in Iscove’s Modified Dulbecco’s Medium (Life Technologies) was first measured at 48 hours using CellTiter96 Proliferation Assay added at indicated concentrations. Cell proliferation was counted.

HEK293 cells were transiently transfected with wild-type or mutant MiGR1/EGFRvIII using Lipofectamine 2000 reagent. Cell lysates were prepared for biochemical analysis. Cell lysates of stable Ba/F3 cell lines expressing wild-type or mutant EGFRvIII were prepared after treatment with inhibitors at indicated concentrations for 30 minutes. Cells lysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blotting were performed as described previously. Following antibodies were used for analysis: phosphorylated EGFR-Tyr1068 (Cell Signaling, Danvers, MA), p-EGFR-Tyr845 (Santa Cruz Biotechnology, Heidelberg, Germany), EGFR (Santa Cruz Biotechnology), pStat5-Tyr692 (Cell Signaling), Stat5 (Santa Cruz Biotechnology), pAkt-Ser473 (Cell Signaling), and Akt1/2 (Santa Cruz Biotechnology). Blots were scanned and subjected to analysis using ImageJ software.

RESULTS AND DISCUSSION

The most common point mutations reported in patients with NSCLC, EGFR-L858R, EGFR-G719S, and EGFR-L861Q, were selected for this study. Our aim was to determine kinase activity and transforming potential of these mutants compared with the wt-EGFR kinase domain. Using the wt EGFR as reference has several limitations: ligand stimulation and the required serum starvation of cells may alter cellular responses. In addition, the wt receptor cannot be used as comparison for the transforming potential of certain EGFR mutants. The use of EGFRvIII as reference abrogates several of these limitations. EGFRvIII contains a wt-kinase domain and, thus, can be used to study the impact of mutations on the kinase activity in an unaltered cellular setting.

Therefore, we first cloned all three point mutations into the EGFRvIII backbone. For analysis of kinase activity and signaling, we chose HEK293 cells, which lack endogenous EGFR. Both EGFRvIII-G719S and EGFRvIII-L858R showed a two- to four-fold increased autophosphorylation compared with EGFRvIII containing a wt-kinase domain (Figures 1A, B). EGFRvIII-L861Q showed the strongest autophosphorylation, which was more than 10-fold higher than EGFRvIII (Figures 1A, B). This data are in agreement with studies using the wt-EGFR receptor with ligand stimulation. EGFRvIII-L861Q was also the strongest activator of Stat5 indicating that not only autophosphorylation but also substrate phosphorylation is enhanced by this mutation.

TABLE 1. IC₅₀ Values (nM) of Wild-Type and Mutant EGFRvIII Kinases against Gefitinib, Erlotinib, AEE788, and WZ-4002 Calculated from Figures 2 and 3

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Gefitinib</th>
<th>Erlotinib</th>
<th>AEE788</th>
<th>WZ-4002</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFRvIII</td>
<td>498</td>
<td>209</td>
<td>198</td>
<td>138</td>
</tr>
<tr>
<td>EGFRvIII + G719S</td>
<td>155</td>
<td>57</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td>EGFRvIII + L858R</td>
<td>55</td>
<td>16</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>EGFRvIII + L861Q</td>
<td>458</td>
<td>366</td>
<td>160</td>
<td>74</td>
</tr>
</tbody>
</table>

EGFR, epidermal growth factor receptor.
eration advantage and highest increase in kinase activity among all mutations tested, it did not lead to enhanced kinase inhibitor sensitivity (Figure 2D). Western blot analysis after treatment of Ba/F3 cells expressing EGFRvIII constructs with gefitinib or erlotinib showed that EGFRvIII-G719S and EGFRvIII-L858R but not EGFRvIII-L861Q displayed enhanced inhibition of downstream Stat5 signaling compared with the wild-type EGFRvIII (Figure 2E). Thus, in this common EGFR mutation, activation of the kinase domain seems to be uncoupled from sensitizing effects toward kinase inhibitors. In a recent report, L858R and G719S mutants were shown to have a reduced binding affinity toward adenosine triphosphate compared with wild-type EGFR, making them more accessible for ATP competitive EGFR inhibitors. In contrast, EGFR-L861Q was demonstrated to retain high binding affinity for ATP. This may explain the lack of enhanced sensitivity toward reversible inhibitors of this mutant. Thus, these results suggest that NSCLC patients...
with the EGFR-L861Q mutation may not benefit as much from EGFR inhibitor treatment with gefitinib or erlotinib as patients with the EGFR-L858R, EGFR-G719S, or EGFR exon 19 deletions.

Variation in drug response toward different activating mutations in oncogenic tyrosine kinases has been reported in various cancers, and accumulating evidence indicates that this may have impact on the clinical outcome on inhibitor treatment. Therefore, it may be beneficial to test alternative EGFR inhibitors toward less sensitive activating EGFR mutations such as EGFR-L861Q. WZ-4002 is a novel irreversible inhibitor of EGFR kinase that was recently shown to have significant activity both in vitro and in vivo. Both EGFRvIII-L858R and EGFRvIII-G719S showed lower IC$_{50}$ values toward WZ-4002 treatment compared with wild-type EGFRvIII (Figure 3, Table 1). Interestingly, EGFRvIII-L861Q also showed significantly more sensitivity against WZ-4002 compared with wild-type EGFRvIII (Figure 3, Table 1). WZ-4002 is an irreversible inhibitor and binds to the active conformation of the EGFR kinase. Because all mutations tested in this study are activating, they may be more sensitive to WZ-4002. Therefore, irreversible second-generation kinase inhibitors such as WZ-4002 may offer a more potent alternative treatment for patients with the EGFR-L861Q mutation in the future. The Ba/F3-EGFRvIII-based system described in this report will be a valuable tool to test novel compounds and strategies.

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REFERENCES


