Overcoming $\text{EGFR}^{G724S}$-mediated osimertinib resistance through unique binding characteristics of second-generation EGFR inhibitors

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The emergence of acquired resistance against targeted drugs remains a major clinical challenge in lung adenocarcinoma patients. In a subgroup of these patients we identified an association between selection of $\text{EGFR}^{T790M}$-negative but $\text{EGFR}^{G724S}$-positive subclones and osimertinib resistance. We demonstrate that $\text{EGFR}^{G724S}$ limits the activity of third-generation EGFR inhibitors both in vitro and in vivo. Structural analyses and computational modeling indicate that $\text{EGFR}^{G724S}$ mutations may induce a conformation of the glycine-rich loop, which is incompatible with the binding of third-generation TKIs. Systematic inhibitor screening and in-depth kinetic profiling validate these findings and show that second-generation EGFR inhibitors retain kinase affinity and overcome $\text{EGFR}^{G724S}$-mediated resistance. In the case of afatinib this profile translates into a robust reduction of colony formation and tumor growth of $\text{EGFR}^{G724S}$-driven cells. Our data provide a mechanistic basis for the osimertinib-induced selection of $\text{EGFR}^{G724S}$-mutant clones and a rationale to treat these patients with clinically approved second-generation EGFR inhibitors.
The identification of EGFR mutations and the discovery of their exquisite sensitivity to epidermal growth factor receptor (EGFR) inhibitors dramatically changed the therapeutic routine for lung adenocarcinoma (LADC) patients. Selective inhibition of EGFR with tyrosine kinase inhibitors (TKI), such as erlotinib or gefitinib, significantly prolongs the progression-free survival (PFS) up to 13.6 months in the first-line setting. However, under therapeutic pressure resistant clones emerge in virtually all tumors and ultimately lead to progressive disease and failure of therapy. Third-generation EGFR inhibitors such as osimertinib have been designed to overcome acquired resistance induced by the EGFR T790M gatekeeper mutation. Clinical results show that patients treated with osimertinib respond in up to 71% in the background of an acquired EGFR T790M mutation. More recent data indicate that osimertinib treatment is even superior to single agent first-generation inhibitors such as erlotinib or gefitinib in terms of PFS and overall survival (OS) in the first-line setting.

The recurrent acquisition of EGFR G724S mutations is currently thought to be the most frequent mechanism of resistance to osimertinib. Alternative by-pass mechanisms involving MET amplification or activation of the MAPK pathway may also play a role in the development of resistance to third-generation EGFR inhibitors. Here, we characterized the role of the acquired EGFR G724S mutation that was diagnosed in osimertinib-resistant lesions of four individual EGFR G724S-mutant LADC patients. We performed systematic biochemical, cellular, and structural analyses to determine the functional relevance of this mutation in the context of targeted EGFR inhibition.

Results

Acquisition of EGFR G724S is associated with cancer progression. Within our LADC re-biopsy program we performed targeted sequencing of lesions that progressed under treatment with third-generation EGFR inhibitors. Interestingly, we identified two patients with no detectable EGFR G724S reads (P1, EGFR E746_S752delinsV; P2, EGFR S752_T759delE) and two patients with low levels of EGFR G724S mutation (P3, EGFR E746_S752delinsIP; P4, EGFR E746_T751delinsIP) prior to start of third-generation EGFR inhibitor therapy (Fig. 1a–d; Supplementary Fig. 1A, Supplementary Table 1). Patient P1 (UICC stage IIIA, 59 years old, female) received osimertinib within the AURA trial (NCT01802632) after progression on erlotinib and the detection of an acquired EGFR T790M mutation (T1) (Fig. 1a). Osimertinib treatment resulted in a partial response (54% reduction based on RECIST 1.1) (Supplementary Fig. 1B, 1D). Even though progression occurred after 8.2 months with the growth of target lesions, a new EGFR T790M-negative and EGFR G724S-positive pleural effusion with a molecular fraction (MF, estimate of allelic fraction) of 6.3% (T2) (Supplementary Table 1C).

Patient P2 (UICC stage IV, 47 years old, female) received two consecutive lines of third-generation EGFR inhibitors upon progression on erlotinib and a combination of carboplatin/pemetrexed/bevacizumab. Treatment with the third-generation EGFR inhibitor EGFR816 (CEG816S2101; NCT02108964) was initiated after detection of EGFR T790M (Fig. 1c). EGFR816 treatment resulted in a stable disease according to RECIST 1.1 for almost 6 months. At the time point of progression to EGFR816 two pulmonary lesions were resected (T2). In one (EP1), the initially detected EGFR T790M mutation was lost and an EGFR G724S mutation was acquired with a MF of 71.1%. In the other sample (EP2), the EGFR T790M mutation (AF 39.3%) was still present and no EGFR G724S reads were detected (Fig. 1c, Supplementary Table 1). Osimertinib treatment was initiated and resulted in a metabolic response of the liver metastases and the stabilization of other solid tumor lesions, including the remaining pulmonary lesions, as assessed by 18FDG positron emission tomography (PET)-CT scans (Fig. 1d). However, a progressive malignant pleural effusion that contained EGFR G724S mutant cells and no trace of the previously acquired EGFR T790M mutation was recorded after seven months of treatment (T3). In this example the MF of EGFR G724S exceeds the MF of our reference EGFR816 reads but it is likely that the inherent noise of our PCR-based method overestimates the EGFR G724S reads and we assume similar levels of both mutants in this tumor.

Two patients (P3, EGFR E746_S752delinsIP; P4, EGFR E746_T751delinsP) were identified with a low MF of EGFR G724S before initiation of osimertinib treatment and persisting EGFR G724S mutant reads at time of progression (Fig. 1a, Supplementary Table 1). Patient P3 (68 years old, UICC stage IV, female) showed a robust response to gefitinib and at time of progression after 32 months presented with a growing paravertebral EGFR T790M-positive lesion (MF of 6.9%, T1) as well as an EGFR G724S mutation (MF of 5.3%, T1; Supplementary Table 1). Treatment with osimertinib resulted in a good partial response according to RECIST 1.1 for a period of 6.8 months (Supplementary Fig. 1C). However, the lesion in which the EGFR G724S mutation was detected showed no significant decrease. A subsequent re-biopsy of the same paravertebral lesion showed a loss of EGFR T790M and the persistence of EGFR G724S (MF 49.6%, T2) (Fig. 1b).

The last patient (P4) (69 years old, UICC stage IV) with a known mutation was initially treated with erlotinib (Fig. 1a, Supplementary Fig. 1A, Supplementary Table 1). However, progression occurred after 36 months of treatment and a re-biopsy of a growing lesion revealed EGFR T790M, as well as EGFR G724S-positive sequencing reads (T1). Treatment with osimertinib resulted in a PFS of 2.5 months (objective efficacy not determined by RECIST 1.1). Another re-biopsy of the growing lesion in the left lower lobe was collected revealing the loss of EGFR T790M and the outgrowth of a EGFR G724S-mutant subclone (AF 39.3%, T2) (Supplementary Table 1). Thus, although EGFR G724S-positive clones may be partially selected in tumors treated with first-generation EGFR inhibitors, a pronounced increase of EGFR G724S-positive sequencing reads is primarily associated with third-generation EGFR inhibitor treatment.

EGFR G724S mutations have been identified as very rare driver mutations and more recently, case reports have shown their potential role in acquired osimertinib resistance in LADC patients. To assess the overall frequency of the acquired EGFR G724S mutation and other changes in EGFR, we revisited re-biopsy samples obtained from EGFR T790M-positive patients at time of progression under treatment with third-generation EGFR inhibitors. This cohort spans 30 patients; 22 of them received osimertinib (73.3%), four EGFR816 (13.3%) and four rociletinib (13.3%). EGFR G724S was detected in three patients (10%), loss of EGFR T790M without no detectable EGFR acquired mutation was detected in 10 (33.3%) and loss of EGFR T790M and presence of EGFR G724S was seen in four patients (13.3%) (Fig. 1e). As this mutation seems to be less frequent in other cohorts, the actual prevalence across a broader panel of patients with acquired osimertinib resistance remains to be assessed. Although only EGFR G724S mutations have a direct impact on the binding of third-generation EGFR inhibitors within the kinase, the mutual exclusivity between EGFR G724S and EGFR G724S indicates that the EGFR G724S mutations do not represent passenger events (Fig. 1f).

Overall, our data show that EGFR G724S mutations may emerge or persist in osimertinib-resistant clones that may evolve independently of acquired EGFR T790M mutations. The data further suggest a negative relation of the allelic frequencies of...
**EGFR<sup>G724S</sup>** and **EGFR<sup>T990M</sup>** under third-generation EGFR inhibition: increasing **EGFR<sup>G724S</sup>** frequencies were accompanied by decreasing **EGFR<sup>T990M</sup>** frequencies.

**EGFR<sup>G724S</sup>** mediates resistance to third-generation EGFR inhibitors. To test the functional relevance of the identified **EGFR<sup>G724S</sup>** mutation, we overexpressed different combinations of **EGFR<sup>G724S</sup>** and **EGFR<sup>19del</sup>** mutations in NIH-3T3 cells. Erlotinib as well as osimertinib treatment resulted in a major reduction of phospho-EGFR levels in **EGFR<sup>19del</sup>**-mutant cells already at concentrations of 0.3 µM of osimertinib but not in cells that expressed **EGFR<sup>G724S</sup>** either alone or in combination with **EGFR<sup>19del</sup>** (Fig. 2a, b). We observed similar results with the third-generation EGFR inhibitor rociletinib, despite its lower potency against the **EGFR<sup>19del</sup>** mutant (Supplementary Fig. 2A).

To validate our findings in an independent cellular model we generated Ba/F3 cells that overexpress **EGFR<sup>19del</sup>** or **EGFR<sup>G724S</sup>** alone and the combination of these mutations. The survival of these murine cells initially relies on IL-3 but can be switched to an oncogene such as mutant **EGFR<sup>G22,23</sup>**. As expected the introduction of **EGFR<sup>19del</sup>** or **EGFR<sup>G724S</sup>** alone led to a transformation of Ba/F3 cells, but only **EGFR<sup>G724S</sup>** showed high sensitivity to erlotinib and osimertinib (Fig. 2c, d, Supplementary Fig. 7A, B).

Based on these results and the increasing relevance of osimertinib in the front-line setting we focused on the resistance phenotype against this third-generation EGFR inhibitor<sup>13</sup>. As in the patients we assume the **EGFR<sup>G724S</sup>** resistance mutations to occur in the background of **EGFR<sup>19del</sup>** we compared **EGFR<sup>19del</sup>** and **EGFR<sup>G724S</sup>+<sup>19del</sup>** sensitivity to third-generation EGFR inhibitors in vivo. To this end we employed murine xenograft models in which genetically modified NIH-3T3 cells were injected subcutaneously into nude mice (NCR<sup>nu/nu</sup>). Again, we observed efficient tumor formation for both double-mutant **EGFR<sup>19del</sup>+<sup>G724S</sup>** and single-mutant **EGFR<sup>19del</sup>** NIH-3T3 cells (Fig. 2e). Confirming our in vitro results osimertinib (5 mg/kg daily) treatment significantly slowed down tumor growth of **EGFR<sup>19del</sup>** NIH-3T3 cells compared with vehicle-treated tumors (p = 0.027). Of note, osimertinib has a favorable pharmacokinetic profile and is known to halt tumor growth in EGFR-dependent patient-derived cell line xenografts at doses as low as 1 mg/kg daily<sup>10</sup>. However, we observed virtually no therapeutic effect for osimertinib (5 mg/kg daily) treated in double-mutant **EGFR<sup>19del</sup>+<sup>G724S</sup>** NIH-3T3 cells compared with vehicle-treated tumors (Fig. 2e, f). Since NIH-3T3 cells can partially form tumors in the absence of an oncogenic driver we did not observe any tumor shrinkage in our xenograft model, as one would expect for xenografts implanted with patient-derived cells<sup>24,25</sup>. As expected, we observed a significant induction of cleaved caspase-3-positive cells (p = 0.037) (Fig. 2g, h) and a robust reduction of Ki67 positive cells only in **EGFR<sup>19del</sup>-mutant** but not in
**Fig. 2** EGFR<sup>G724S</sup> mediates resistance to third-generation EGFR inhibitors in vitro and in vivo. 

**a** Immunoblotting results of NIH-3T3 cells (empty vector, EGFR<sup>19del</sup>, EGFR<sup>G724S</sup>, or EGFR<sup>19del+G724S</sup>) monitoring phospho-EGFR and total EGFR under erlotinib treatment (24 h). HSP90 was used as loading control. **b** Immunoblotting of NIH-3T3 cells under osimertinib treatment (24 h) is shown. Dose–response measurement of Ba/F3 cells expressing EGFR<sup>19del</sup>, EGFR<sup>G724S</sup>, or EGFR<sup>19del+G724S</sup> treated for 72 h with **c** erlotinib or **d** osimertinib. Experiments were performed at least three times. **e**, **f** Graphs show tumor volume of mice injected with NIH-3T3 EGFR<sup>19del</sup> and EGFR<sup>19del+G724S</sup> cells treated with osimertinib (5 mg/kg, i.p., once daily). **e** Tumor volumes (EGFR<sup>19del</sup> vehicle, n = 7 mice; EGFR<sup>19del</sup> osimertinib, n = 8 mice; EGFR<sup>19del+G724S</sup> vehicle, n = 7 mice; EGFR<sup>19del+G724S</sup> osimertinib, n = 10 mice) were assessed for 20 days by longitudinal caliper measurements every second day following treatment initiation. **f** Tumor volumes were quantified after 8 days of treatment. Volume changes in the osimertinib treatment cohort (dark gray and green) were compared with the vehicle-treated control group (light gray and green).

Each dot represents a single tumor per mouse. Significance is calculated by two-tailed Student’s t test, n.s.: non-significant. **g** Representative images of Cleaved Caspase-3 stainings. Tumors of mice bearing NIH-3T3 EGFR<sup>19del</sup> or EGFR<sup>19del+G724S</sup> cells were treated with vehicle solution HPMC (0, 5%) or osimertinib. Scale bar 100 μm. **h** Quantification of Cleaved Caspase-3 staining. Number of apoptotic cells in the osimertinib-treated cohort (dark gray and green) was compared with the vehicle-treated control group (light gray and green).
EGFR<sup>19del+G724S</sup>-mutant tumors that received osimertinib (Supplementary Fig. 2B–F).

These results clearly indicate that the EGFR<sup>G724S</sup> point mutation may confer resistance against third-generation EGFR inhibitors.

**Structural impact of an altered glycine-rich loop conformation.** The glycine-rich loop is a crucial structural element for substrate and ligand binding. It is a highly conserved flexible element located in the N-lobe of the kinase domain and contains the canonical GxGxxG motif, where x may be any amino acid<sup>26,27</sup>.
It does not come as a surprise that mutations in the glycine-rich loop can interfere with ligand binding and thus mediate resistance to kinase inhibitors as it was described previously for chronic myelogenous leukemia, where mutations in the glycine-rich loop in BCR-ABL cause resistance to imatinib\textsuperscript{28,29}.

To assess the structural impact of the EGFR\textsuperscript{G724S} mutation on the EGFR kinase we performed structural analysis based on a previously published co-crystal structure of rociletinib bound to EGFR (PDB ID: 5UWD) (Fig. 3a\textsuperscript{30}). As described before the glycine-rich loop is an essential element for ligand binding and the glycine at position 724 is in direct contact with the adjacent ELREA motif and the glycine-rich loop may undergo conformational changes upon ligand binding, the rearrangement might be hindered in the case of third-generation TKIs.

In line with these considerations, molecular dynamics (MD) simulation (based on PDB ID: 4ZAU)\textsuperscript{33} revealed an altered ELREA motif in EGFR\textsuperscript{G724S}, as compared with simulated wild-type protein when bound to osimertinib (Fig. 3c, Supplementary Fig. 3B; raw data are represented by the determined root mean squared fluctuations (RMSF) values (Fig. 3c, Supplementary Fig. 3B; raw data are reported in the B-factor column of PDB structures for osimertinib bound to the EGFR variants studied in Supplementary Data 1–4). These enhanced fluctuations extend toward the methylindole residue of osimertinib. As pointed out in seminal work by
Kuriyan, Shaw and co-workers, substantial conformational impact of the exon 19 deletion on the helix αC can be expected, whereas the structural and dynamical influence of additionally introducing the G724S mutation is unknown. Hence, we additionally simulated the corresponding EGFR19del and EGFR19del+G724S systems (Fig. 3d, Supplementary Fig. 3A right). Remarkably, despite the strain introduced by deleting the ELREA motif, increased flexibility particularly of the G-rich loop within the regulatory network is also evident for EGFR19del+G724S relative to EGFR19del. This finding is robust with respect to varying the starting structures of the simulations (Supplementary Fig. 3B). It appears that the mutant Ser724 side chain renders the regulatory network more flexible and induces altered conformations to the G-rich loop.

Based on these findings, a second line of argumentation could be valid: rather than steric repulsion, the increased flexibility might result in the loss of important interactions between third-generation inhibitors and the binding site that lead to the observed drug resistance. Taken together, we conclude that the EGFR19del+G724S mutation may provoke a conformation of the glycine-rich loop, which is incompatible with ligand binding and accounts for decreased binding efficiency as determined for third-generation EGFR inhibitors.

**Altered EGFR inhibitor activity pattern through EGFR**

We next addressed the question whether the EGFR19del+G724S mutation might directly interfere with the ability of third-generation EGFR inhibitors to bind to the EGFR kinase, as the mutation site is located in the glycine-rich loop, which is an important regulatory element (Supplementary Fig. 4A). Similar to our observations in NIH-3T3 cells, we detected a marked increase of IC50-values in kinase assays using osimertinib against the double-mutant EGFR19del+G724S as compared with the EGFR19del single-mutant protein (100-fold) (Fig. 4a).

Having established the kinase assay platform, we next sought to test whether the EGFR19del+G724S mutation induces resistance against a specific class of EGFR inhibitors. Herein, we collected a library of more than 120 compounds, of which 90 compounds are proprietary and hence the results of 32 readily published compounds with a known anti-EGFR profile covering clinically relevant first-, second-, and third-generation EGFR inhibitors are discussed in the following (Fig. 4b, Supplementary Fig. 4B, Supplementary Table 2). We screened these inhibitors against both the single and the double-mutant EGFR kinase and observed two interesting patterns of inhibitor activity: (i) the introduction of the EGFR19del+G724S mutation in addition to the EGFR19del mutation induces resistance against virtually all clinically used first- and third-generation inhibitors and (ii) all second-generation inhibitors including afatinib, poziotinib, and dacomitinib remained active against the EGFR19del+G724S double-mutant kinase (Fig. 4b, Supplementary Table 2). Although, first- and second-generation inhibitors exhibit the same quinazoline scaffold, a remarkable difference in biochemical potency is evident. Alkylation of Cys797 is a distinct feature of second-generation TKIs that discriminates them from first-generation inhibitors. This finding indicates that a covalent bond formation to the target kinase is crucial to occupy the binding site efficiently. Interestingly, we also identified an aminoindazole-based inhibitor with low nanomolar activity against the double-mutant kinase that would not be classified as a second-generation EGFR inhibitor but indeed exhibits a different binding mode than osimertinib (Fig. 4b, Supplementary Table 2).

We next tested whether the biochemical activity of second-generation EGFR inhibitors in EGFR19del+G724S mutant cells translates into a therapeutically relevant off-target activity in cellular models. We therefore assessed phospho-EGFR levels following afatinib exposure in NIH-3T3 cells expressing either the empty vector or vectors with EGFR19del, EGFR19del+G724S, or the
combination of both mutations \( \text{EGFR}^{19\text{del}+G724S} \) (Fig. 4c, Supplementary Fig. 7C). As expected, afatinib treatment led to a reduction of phospho-EGFR signaling that was independent of the presence of the \( \text{EGFR}^{G724S} \) mutation in the glycine-rich loop at concentrations between 10–100 nM (Fig. 4c, Supplementary Fig. 4C). We validated these findings in our Ba/F3 cell lines and found that \( \text{EGFR}^{G724S} \)-mutant cells largely retained sensitivity to afatinib at low nanomolar concentrations (Fig. 4d).

These results triggered us to revisit our previously analyzed crystal structure of exon 20-mutant \( \text{EGFR} \) (PDB ID: 4LRM) (Supplementary Fig. 5). In line with our biochemical data this structure shows that the binding of second-generation TKIs based on the 4-anilinoquinazoline scaffold (PD168393) to the altered binding site is well tolerated (Fig. 4e).

Thus, our data indicate that the \( \text{EGFR}^{G724S} \) mutation induces resistance toward third- and first-generation but retains sensitivity toward 4-aminoquinazoline based second-generation EGFR inhibitors.

\( \text{EGFR}^{G724S} \) reduces binding of third-generation EGFR TKI. Further in-depth kinetic evaluation including determination of kinetic parameters \( K_i \) and \( k_{\text{inact}} \) was conducted to more accurately define differences between second- and third-generation inhibitor binding. Binding of covalent inhibitors to a kinase is assumed to succeed in a two-step process: first the inhibitor binds to the kinase in a reversible fashion characterized by \( K_i \) and in a second step the covalent bond is formed which can be specified with the rate of inactivation \( (k_{\text{inact}}) \) (Fig. 5a).

In these experiments, we identified marked differences in the binding characteristics of second- and third-generation EGFR inhibitors to \( \text{EGFR} \)-mutants (Fig. 5b, c, Supplementary Table 3). For instance, the \( k_{\text{inact}} \) of osimertinib and rociletinib appears to be similar among the \( \text{EGFR}^{19\text{del}} \)-mutants and the \( \text{EGFR}^{G724S} \) mutation does not negatively affect the covalent bond formation with Cys797. Our data further indicate that the \( \text{EGFR}^{G724S} \) mutation has a strong impact on the formation of the reversible protein–ligand complex in the context of these drugs which prove to bind in a less-affine manner indicated by increased \( K_i \)-values (osimertinib \( \text{EGFR}^{19\text{del}} < 1 \text{nM} \) and \( \text{EGFR}^{G724S} \) 80 nM) (Fig. 5b, c). The second-generation EGFR inhibitor afatinib and the structurally related inhibitor poziotinib exhibit constant affinities and binding kinetics for \( \text{EGFR}^{\text{WT}} \), \( \text{EGFR}^{19\text{del}} \), and \( \text{EGFR}^{19\text{del}+G724S} \) kinases (Fig. 5b, c). In addition, our data reveal that the loss of \( \text{EGFR}^{\text{T790M}} \), as observed in all of the relapsed tumors that were enriched for \( \text{EGFR}^{G724S} \)-positive clones, further enhanced the loss of affinity of third-generation EGFR inhibitors. Third-generation inhibitors are designed to target a methionine gatekeeper residue in position 790, whereas second-generation inhibitors afatinib and poziotinib exhibit a more pronounced affinity toward threonine-carrying EGFR variants. Based on these findings we hypothesized that such a marked difference between afatinib and osimertinib in the engagement of the mutant kinase should be also detectable in cellular assays. We therefore tested...
the ability of afatinib and osimertinib to inhibit phospho-EGFR over time (Fig. 5d, Supplementary Fig. 7D, E). In line with our biochemical data even at 1 μM concentrations osimertinib was not able to reduce phospho-EGFR levels in NIH-3T3 cells expressing EGFR19del+G724S. In contrast to that, afatinib depleted phospho-EGFR levels in these cells as efficiently as in EGFR19del cells (Fig. 5d, Supplementary Fig. 7D, E).

Thus, our data suggest that in EGFR19del+G724S-mutant kinase the reversible binding of second-generation EGFR inhibitors is superior to third-generation EGFR inhibitors and might therefore overcome EGFR19del-driven resistance.

**EGFR**<sup>G724S</sup> is sensitive to second-generation EGFR inhibitors.

A previous case report of a patient with acquired EGFR<sup>G724S</sup> mutation showed a remarkable response to the combination of osimertinib and afatinib<sup>31</sup>. To test whether the observed affinity to second-generation EGFR inhibitors translates into cellular activity we tested the ability of afatinib as single agent to outperform osimertinib activity in EGFR19<sup>19del+G724S</sup> driven cells. To this end, we plated NIH-3T3 cells that express either EGFR<sup>19del</sup> or EGFR<sup>19del+G724S</sup> in soft agar and treated the cells with increasing concentrations of both drugs over the time of 2 weeks. In line with our in vivo results osimertinib was only effective against the formation of EGFR<sup>19del</sup>-driven colonies at sub-micromolar concentrations but not against EGFR<sup>19del+G724S</sup> mutant cells (Fig. 6a, b). However, afatinib largely prevented outgrowth of both EGFR<sup>19del</sup> and EGFR<sup>19del+G724S</sup>-driven colonies at submicromolar concentrations. Thus, the growth inhibition effect of afatinib compared with osimertinib was significantly higher ($p = 0.01$) in EGFR<sup>19del+G724S</sup> mutant cells (Fig. 6a, b).

To further evaluate the sensitivity of EGFR<sup>G724S</sup> either alone or in combination with EGFR<sup>19del</sup> to third-generation EGFR inhibitors in vivo we employed our murine xenograft models. Again, both single-mutant EGFR<sup>19del</sup> and double-mutant EGFR<sup>19del+G724S</sup> NIH-3T3 cells formed fast growing tumors in nude mice (Fig. 6c). As expected, afatinib (20 mg/kg daily) treatment significantly slowed down tumor growth of both single-mutant EGFR<sup>19del</sup> ($p = 0.0192$) and double-mutant EGFR<sup>19del+G724S</sup> ($p < 0.0001$) xenograft when compared with vehicle-treated tumors (Fig. 6d, Supplementary Fig. 6).

Overall, our results indicate that acquired EGFR<sup>G724S</sup> mutations robustly limit the activity of third-generation inhibitors, whereas sparing the second-generation EGFR inhibitors such as afatinib. Existing Food and Drug Administration (FDA)-approvals for some of these drugs may facilitate the translation of our results into clinical practice for genetically defined osimertinib-resistant lung cancer patients.

**Discussion**

EGFR inhibitors represent a showcase for the therapeutic power of precision cancer medicine in genetically selected patients. Building on structural and functional insights, several lines of drug development efforts provided a rich source of clinically available drugs including second-generation EGFR inhibitors<sup>35–40</sup>. So far, these drugs played only a minor role for the targeted treatment of EGFR-mutant tumors as they largely failed to induce pronounced effects in patients that acquired a gatekeeper EGFR<sup>T790M</sup> mutation<sup>22,41</sup>. Although second-generation EGFR inhibitors bind irreversibly to EGFR these drugs lack the flexibility to circumvent a steric clash with the gatekeeper mutation<sup>22</sup>. Second-generation EGFR inhibitors are also very potent inhibitors of EGFR<sup>WT</sup> and therefore these drugs are likely to induce diarrhea or skin rash<sup>41</sup>. At the same time, afatinib is an FDA-approved drug for first-line treatment of EGFR-mutant LADC with a known on-target resistance profile derived from preclinical models<sup>43,44</sup>.

Our clinical and functional characterization of the acquired EGFR<sup>G724S</sup> resistance mutation suggests a revision of the properties and clinical liabilities that are associated with second-generation EGFR inhibitors. This is becoming an even more relevant issue in the light of the encouraging results achieved with osimertinib in the front-line setting that might challenge the standard use of first-generation EGFR inhibitors in EGFR mutant patients<sup>11</sup>. First of all, the emergence of an EGFR<sup>G724S</sup> resistance mutation is associated with loss of EGFR<sup>T790M</sup>, positive subclones, and thus providing the right genetic context for the use of second-generation EGFR inhibitors. These observations are in line with previous reports on acquired osimertinib resistance that frequently report a loss of EGFR<sup>T790M</sup> -positive clones<sup>15,16</sup>. Although we cannot exclude the fact that additional signaling layers including FAK or SFK or co-occurring mutations that were not part of our panel may be involved in the observed resistance phenotype<sup>45–47</sup>, our genomic and functional data strongly support a dominant role of the acquired EGFR<sup>G724S</sup> mutation in the context of third-generation EGFR inhibitors. Thus, our findings might be relevant for a large group of patients receiving third-generation inhibitors. Interestingly, previous studies that characterized resistance patterns to third-generation EGFR inhibitors did not capture recurrent EGFR<sup>G724S</sup> mutations<sup>14,48</sup>. Although our limited sample size precludes broad conclusion on the real prevalence of this mutation it is conceivable that the size of the previous studies or a potential compound selection bias (osimertinib vs. rociletinib) may have underestimated the relevance of EGFR<sup>G724S</sup> mutations as a potential resistance mechanism. Our data indicate that EGFR<sup>G724S</sup> mutations seem to primarily affect the reversible first step of third-generation inhibitor binding within the ATP-binding pocket before irreversible attachment to Cys797 can occur. We conclude that the observed fluctuation of the glycine-rich loop plays a role in this phenotype, similar to other systems where anti-correlations between flexibility and ligand-binding affinity have been observed<sup>49,50</sup>. However, although the kinetics of third-generation EGFR inhibitor binding are perturbed dramatically, second-generation EGFR inhibitors are potent enough to establish such a reversible binding despite the EGFR<sup>G724S</sup> mutation. Thus, their liability in terms of an efficient engagement of EGFR<sup>WT</sup> in the context of EGFR<sup>G724S</sup> resistance mutations turns into an asset.

Given the increasing number of patients receiving osimertinib our data are of high clinical relevance. Importantly, our study provides a molecular basis for the ability of EGFR<sup>G724S</sup> to induce resistance and suggest that second-generation EGFR inhibitors might overcome osimertinib resistance in these patients.

**Methods**

**Experimental design.** The aim of this study was to examine the effects of the acquired EGFR<sup>G724S</sup> mutation that was observed in lung tumors that become resistant to osimertinib treatment. We conducted systematic cellular, in vivo, biochemical, and structural analyses to determine the functional relevance of this mutation in the context of first-, second-, and third-generation EGFR inhibition. To investigate the EGFR<sup>G724S</sup> mutation in combination with the different EGFR inhibitors we used the mouse fibroblast cell line NIH-3T3 and female nude mice. All experiments including immunoblotting, tumor volume measurement, soft agar assays, and biochemical assays were performed at least three times. For the biochemical analysis, we used activity-based assay for IC<sub>50</sub>-determination and kinetic characterization. Each reaction was performed in duplicate, and at least three independent determinations of each IC<sub>50</sub> were made. To characterize the acquired EGFR<sup>G724S</sup> resistance mutation on a molecular level we used structural modeling of EGFR kinase and validated our observations using computational modeling of publically available co-crystal structures. For detailed information please see "Methods". The local animal protection committee and the local authorities approved all animal procedures. All patients consented into the analyses according to the local practice.
Patients, efficacy assessments, and sample collection. The four patients included in this analysis were treated with the third-generation EGFR inhibitor osimertinib in the AURA trial (NCT020108964). All patients consented to treatment according to the local clinical practice guideline and were treated according to the trial protocol and/or local practice. Patients received osimertinib at a dose of 80 mg qd and were treated until progression. Treatment doses were adapted if necessary in case of toxicity and adverse events. Tumor assessment was performed by computed tomography (CT) or 18F-FDG PET and magnetic resonance imaging according to the specifications given in the trial protocols and/or according to local standards. Efficacy was assessed using the response evaluation criteria for solid tumors, version 1.1 in patients P1 and P3 (RECIST 1.1)24. In patients P2 and P4 RECIST evaluation was not feasible. Response to osimertinib treatment was performed by 18F-FDG PET-CT in P2. In P4 baseline CT scan was older than 4 weeks, not fulfilling the requirements set up by RECIST 1.1. In patients where RECIST evaluation was not possible, progression was defined by the treating physician as growth of clinically significant lesions. Biopsy collection was performed through core needle biopsy, excisional biopsy, or cytology according to local standard procedures at time points T0 to T3. Samples at time points T1, T2, and T3 were collected in preserving tumor lesions. At tumor samples were fixed in formalin (4%) and embedded in paraffin (FFPE). To assess the frequency of EGFR T790M in the setting of acquired resistance to third-generation EGFR inhibitors, we analyzed FFPE tissue of patients from the Network Genomic Medicine and collaborating institutions. EGFR-mutant NSCLC patients who fulfilled the following criteria were included into the analysis: (1) sufficient tumor tissue for characterization, (2) progressive disease while on treatment with a third-generation EGFR inhibitor. All patients consented into the analyses according to the local practice.

Targeted next-generation sequencing. Tumor tissue of patients was genotypically characterized by massively parallel sequencing (MPS) and fluorescence in situ hybridization (FISH), if feasible. Until March 2015, MPS was carried out with an Ion AmpliSeq Custom Panel (Thermo Fisher Scientific, Waltham, MA, USA) (Lun3 panel) and a MiSeq benchtop sequencer (Illumina, San Diego, USA). As from March 2015 MPS was carried out with a GeneRead DNASeq Custom Panel V2 (Qagen, Hilden, Germany) consisting of 205 amplicons (Lun4 panel). Library preparation was performed according to the GeneRead DNAseq Gene Panel Handbook (Qagen) as described earlier25.

Cell viability. In all, 5000 Ba/F3 cells/well were seeded in triplicates in a white-bottom 96-well plate at 90 µl media/well. Compounds were prepared by serial dilution. Dimethyl sulfoxide was added to control wells in the highest dilution used in the assay. The cells were treated for 72 h with the compounds following determination of ATP content as surrogate for viability by CellTiter-Glo® assay (CTG, Promega) incubated for at least 10 min on the cell at 37 °C. Luminiscence was assessed on an Infinite 200 Pro microplate reader (Tecan). Data were analyzed and plotted in PRISM.

Cell culture. NIH-3T3 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and the following medium was used for at least 8 weeks and for 8 weeks before, yielding 5075 water molecules, six sodium ions, 5075 protein- and 72 ligand atoms. The G724S system was composed of 25,651 TIP3P molecules, 7 sodium cations, 5162 protein atoms, and 72 ligand atoms. For both, the structure 7425 for MD simulations, the proteins and ligand complexes by deleting residues 746 to 750 followed by system preparation steps as described earlier. The structure 4ZAU deposited in the PDB was used as basic template for modeling the noncovalently bound EGFR-osimertinib complex. Mutated residues were obtained from PDB entry 7Z3S for the RESPAKE (LREATSPA/LREATSPK), 863–865 (GAE/GAE), 873–874 (GG/GG), 985–991 (ERHM/HLM), 1003–1007 (DEEDM/DEDEM) and from PDBP24 for residues 748–755 (LREATSPA/LREATSPK), 863–865 (GAE/GAE), 873–874 (GG/GG), 985–991 (SPTDSNYFRL/PLDFVYRSL), Terminal regions 693–697 and 1017–1020 were truncated, the structure 19del and NIH-3T3 were introduced by Mode 9.155. For MD simulations, the proteins and ligand bodies were purchased from Cell Signaling (Cleaved Caspase-3, #Asp175, dilution 1:100) and Cell Marque (K67, #SP6, dilution 1:100). Corresponding secondary antibody detection kits were used from Histone® Simple stain and stained on an automated stainer (LabVision AutoStainer 4805, Thermo Fisher).

Immunohistochemical staining. Tissue samples were incubated in 4% formalin overnight and subsequently embedded in paraffin. For tissue analysis, 3–5 µm sections were cut, de-paraffinized, and antigen retrieval was performed using either citrate at pH 6.0, or ethylenediaminetetraacetic acid (EDTA) at pH 9.0 for 20 min. Wash steps were performed with phosphate-buffered saline. Primary antibodies were purchased from Cell Signaling (Cleaved Caspase-3, #Asp175, dilution 1:100) and Cell Marque (K67, #SP6, dilution 1:100). Corresponding secondary antibody detection kits were used from Histone® Simple stain and stained on an automated stainer (LabVision AutoStainer 4805, Thermo Fisher).

Computational modeling. The structure 4ZAU deposited in the PDB was used as basic template for modeling the noncovalently bound EGFR-osimertinib complex. Mutated residues were obtained from PDB entry 7Z3S for the RESPAKE (LREATSPA/LREATSPK), 863–865 (GAE/GAE), 873–874 (GG/GG), 985–991 (ERHM/HLM), 1003–1007 (DEEDM/DEDEM) and from PDBP24 for residues 748–755 (LREATSPA/LREATSPK), 863–865 (GAE/GAE), 873–874 (GG/GG), 985–991 (SPTDSNYFRL/PLDFVYRSL), Terminal regions 693–697 and 1017–1020 were truncated, the structure 19del and NIH-3T3 were introduced by Modeler 9.155. For MD simulations, the proteins and ligand bodies were treated by the st4Sb force field49 and the GAFF model52, respectively, within AMBER 1456. The resulting simulation system for the wild type consisted of 24,358 TIP3P water molecules, seven sodium cations 57, 5138 protein atoms, and 72 ligand atoms. The G724S system was composed of 25,651 TIP3P molecules, 7 sodium cations, 5162 protein atoms, and 72 ligand atoms. For both, EGFR WT and EGFR G724S mutation, the same simulation protocol was used, starting with a geometry optimization down to a final RMS gradient of 0.0001 kcal mol−1 Å−1 followed by 4 ns heating to 298.15 K in the canonical ensemble (Langvin thermostat) while applying harmonic restraints on protein C¢ atoms from the respective initial snapshots of the protein system was then simulated over 4 ns in the isothermal-isobaric ensemble (Berendsen barostat) at 1 bar pressure, also under der action of restraints. Finally, restraints except for fixed hydrogen bond distances were removed and the systems were run over 200 ns with a 2 fs time step using AMBER 1456. The stability of the simulations systems was checked by computing the structural root mean square deviations (RMSD) of Ca atoms from the respective initial snapshots of the production runs over time. (Supplementary Fig. 3C). The final 100 ns were used for clustering structures taken every 10 ps using the DBSCAN algorithm53 in AMBER 16 with distance cutoffs 1.18 Å (ERGRT2) and 1.205 Å (EGFR7245) and a minimum number of points to form a cluster set to 5. Final structures were obtained from centroids of the maximally populated clusters by geometry optimization in an implicit water environment (ALPB)61. RMSD fluctuations were computed over the final 100 ns and mapped onto the resulting structures (Supplementary Data 1). For further analysis. These structures were then modified by Modeller 9.155 to generate starting structures of EGFR G19del and NIH-3T3 complexes by deleting residues 746 to 750 followed by system preparation steps as before, yielding 24271 water molecules, six sodium ions, 5075 protein- and 72
lignand atoms for EGFRL858R and 26,866 water molecules, six sodium ions, 5079 protein- and 72 lignand atoms EGFRL858R. 75 trajectories were generated and analyzed. Before using the last 75 ns (see RMSD plot Supplemental Fig. 3A) for clustering (DBSCAN cutoffs of 1.16 Å for EGFRL858R and EGFRL790M [G724S] and RMSF calculations. The simulations of the pure deletion and the double-mutant complexes were performed in order to check the independence of the system stability and fluctuation analysis of the initial conditions.

Activity-based assay and kinetic characterization. For biochemical assays EGFRL858R, EGFRL790M, and EGFRL858R/L790M were purchased from Carna Biosciences (lot13CRS-0053B for EGFRL858R, and Carna, lot12CRS-0765B for EGFRL858R/G724S). However, EGFRL856R and EGFRL858R/L790M were expressed and purified as follows. First DNA-encoding residues comprising the juxtamembrane segment, the kinase domain and the C-terminal tail of human EGFR (UniProt entry P00533, residues 695–1210) were synthesized (GeneArt, Life Technologies). The construct was cloned into pIEX-Bac3 expression vector (MercerMillipore), using BamHI and Bsu36I restriction sites. Mutations were introduced by side-directed mutagenesis (QuikChange, Stratagene/Agilent Technologies). Transfection, virus generation, and amplification were carried out in Spodoptera frugiperda cell line Sf9 following the Bacmid protocol. After three days of expression (27 °C, 110 rpm) the cells were harvested (3000 g, 20 min), resuspended in buffer A (50 mM Tris, 500 mM NaCl, 10% glycerol, 1 mM dithiothreitol, pH 8) and homogenized by frozen press. The lysate was cleared by centrifugation at 40,000 × g for 1 h at 4 °C and loaded on a prepacked Q-Sepharose HP column (Pharmacia Biotech). The elution was performed with a gradient of buffer B (buffer A + 10 mM glutathione). For the final purification step the fractions containing the target protein were combined, concentrated and applied to a HiLoad 16/60 superdex 75 pg column (GE Healthcare) in buffer C (25 mM TRIS, 250 mM NaCl, 10% glycerol, pH 8). The purified protein was concentrated by adding buffer containing EDTA as well as an antiphosphatase cocktail and only labeled with europium cryptate and streptavidin labeled with the fluorophore XL665. FRET between europium cryptate and XL665 was measured after an additional hour of incubation to quantify the phosphorylation of the substrate peptide. An EnVision multimode plate reader (PerkinElmer) was used to measure the fluorescence of the samples at 620 nm (Eu-labeled antibody) and 665 nm (XL665 labeled streptavidin) 50 µs after excitation at 320 nm. The quotient of both intensities for reactions made with eight different inhibitor concentrations was then calculated using the EnVision Software Suite for IC_{50} determination. Each reaction was performed in duplicate, and at least three independent determinations of each IC_{50} value were performed. The inhibitors were incubated with EGFR mutants over different periods of time (2–90 min), whereas durations of enzymatic and stop reactions were kept constant as stated above. A sixfold dilution series (eight data points per IC_{50} curve) starting at 20 µM final compound concentrations was applied. Calculated IC_{50} values were plotted versus incubation time, and data were fit as described in the literature to determine k_{act} and K_{CYP}. MAB PT66-Eu cryptate (6T166KLB) stock solution was prepared according to manufacturer instructions and diluted 1:1 with detection buffer for activity-based assay.

MET and HER2 FISH analyses. FISH was performed for determination of MET gene copy number using ZytoLight SPEC MET/CEN7 Dual Color Probe (ZytoVision). High-level amplification was defined in tumors with (a) MET/CEN7 ratio ≥ 5 (b) an average MET gene copy number (CGH) ≥ 6.0 or (c) ≥ 40% of tumor cells containing ≥ 5 MET signals. Intermediate level of gene copy number gain being defined as (a) ≥ 50% of cells containing ≥ 5 MET signals and (b) criteria for high-level amplification are not fulfilled. Low level of gene copy number gain was defined as (a) ≤ 40% of tumor cells showing ≥ 4 MET signals and (b) criteria for high-level amplification are not fulfilled. Low level of gene copy number gain are not fulfilled. All other tumors were classified as negative. For determination of HER2 (ERBB2) status FISH was performed using a ZytoLight SPEC ERBB2/CEN17 Dual Color Probe (ZytoVision). Amplification status was classified in analogy to the recommendations of the American Society of Oncology for HER2 testing in breast cancer. Amplification of HER2 was positive if (a) ERBB2/CEP17 ratio ≥ 2.0 or (b) HER2 CEN ≥ 6.0.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc). Data obtained from mice tumor analysis and in vitro assays were subjected to unpaired Student’s t-test. Data are plotted as means ± standard error of the mean. Quantification of high-power-field analysis was calculated by Mann-Whitney U test.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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