Mutation and drug-specific intracellular accumulation of EGFR predict clinical responses to tyrosine kinase inhibitors

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ABSTRACT

Background: Clinical responses to EGFR tyrosine kinase inhibitors (TKIs) are restricted to tumors harboring specific activating mutations and even then, not all tyrosine kinase inhibitors provide clinical benefit. All TKIs however, effectively inhibit EGFR phosphorylation regardless of the mutation present.

Methods: High-throughput, high-content imaging analysis, western blot, Reversed phase protein arrays, mass spectrometry and RT-qPCR.

Findings: We show that the addition of TKIs results in a strong and rapid intracellular accumulation of EGFR. This accumulation mimicked clinical efficacy as it was observed only in the context of the combination of a TKI-sensitive mutation with a clinically effective (type I) TKI. Intracellular accumulation of EGFR was able to predict response to gefitinib in a panel of cell-lines with different EGFR mutations. Our assay also predicted clinical benefit to EGFR TKIs on a cohort of pulmonary adenocarcinoma patients (hazard ratio 0.21, \(P=0.0004\) [Cox proportional hazard model]) and could predict the clinical response in patients harboring rare mutations with unknown TKI-sensitivity. All investigated TKIs, regardless of clinical efficacy, inhibited EGFR phosphorylation and downstream pathway activation, irrespective of the mutation present. Intracellular accumulation of EGFR depended on a continued presence of TKI indicating (type I) TKIs remain associated with the protein even after its dephosphorylation. Accumulation therefore is likely caused by two consecutive conformational changes, induced by both activating mutation and TKI, that combined block EGFR-membrane recycling.

Interpretation: We report on an assay that mimics the discrepancy between molecular and clinical activity of EGFR-TKIs, which may allow response prediction in vitro and helps understand the mechanism of effective inhibitors.

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1. Introduction

The epidermal growth factor receptor (EGFR) gene is a key oncogene that is mutated in many different cancer types including gliomas, colorectal cancer and pulmonary adenocarcinoma. Tumors depend on EGFR signaling for their growth and this dependency makes EGFR an attractive target for therapy. Indeed, many pulmonary adenocarcinoma patients harboring EGFR mutations show strong clinical response to EGFR tyrosine kinase inhibitors (TKIs) [1–4]. Unfortunately, other tumor types that depend on EGFR signaling, such as glioblastomas (the most common and aggressive type of primary brain cancer), show no response to EGFR-TKIs [5–7].

Not all EGFR-mutated pulmonary adenocarcinoma patients benefit from EGFR TKIs: responses are predominantly observed in the context of deletions in exon 19 or missense mutations L858R, G719X and S768I. Patients with other, less common activating mutations such as exon 20 insertions show no benefit from EGFR TKIs (see e.g. mycancergenome.org) despite EGFR being effectively dephosphorylated [8–10]. Apart from this mutation-specificity, there is also a drug-specificity of clinical responses: where the type I EGFR-TKIs (erlotinib, gefitinib, afatinib, dacomitinib and osimertinib) that bind to the active conformation have provided clinical benefit to EGFR-mutated pulmonary adenocarcinoma patients, type 1.5 inhibitors that bind to the inactive conformation (e.g. lapatinib) do not show any sign of

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Evidence before this study

Preclinical studies have shown that EGFR-mutated tumors depend on this protein for their growth and several randomized phase III clinical trials demonstrated benefit of EGFR inhibitors in patients. These trials also showed that benefit was not universal for all oncogenic mutations; only specific EGFR-mutations appear to respond. In addition, a phase II clinical trial on laptatinib failed to meet its primary endpoint demonstrating not all inhibitors are effective. The molecular activity of inhibitors therefore does not explain its clinical activity.

Sources investigated: Pubmed and mycancergenome.org. Search terms used: pulmonary adenocarcinoma, glioma, EGFR, EGFR and inhibitor [laptatinib, erlotinib, gefininib, dacomitinib, osimertinib] and clinical trial, EGFR and conformation, EGFR and activating mutation, EGFR and T751-I759delinsATA or L747-E749del or P848L or E746A. Searches were not limited to a specific timeframe. No selection was made on reporting clinical activity of rare mutations.

Added value of this study

We here describe and validate an assay that mimics the discrepancy between molecular and clinical activity of EGFR inhibitors and demonstrate that this in vitro assay allows response prediction of individual patients. We show that EGFR inhibitors remain associated with the protein, but only in the context of inhibitor-sensitive mutations and clinically effective inhibitors, this association results in a block in receptor recycling. These data help understand the mechanism of effective inhibitors.

Implications of all the available evidence

Our data can aid in the clinical decision making in patients harboring novel EGFR mutations. Since we show that sensitivity to EGFR inhibitors is largely independent of the genetic background, all patients with sensitive EGFR mutations should (pending independent validation), regardless of the type of tumor, be considered for treatment with EGFR-TKIs. The block in receptor recycling can aid the development of novel EGFR inhibitors of mutations refractory to the ones currently used in clinical practice.

clinical activity [10–12]. This lack of clinical activity is surprising as both type I and 1.5 inhibitors are highly potent in blocking EGFR phosphorylation. In summary, clinical responses to EGFR TKIs are restricted to a limited set of mutations only, and not all TKIs are clinically effective. The molecular mechanisms for this mutation- and drug-specificity remains unknown.

We here describe a simple in-vitro assay, based on a TKI-induced intracellular accumulation of EGFR, that can predict which mutation is sensitive to which TKI. Similar to the responses observed in the clinic, our assay is both mutation and TKI-specific, and is independent on the inhibition of EGFR-phosphorylation and downstream pathway activation. The observed TKI-induced intracellular accumulation is likely a result of a block in intracellular trafficking due to a continued association of the TKI with EGFR. Because the intracellular accumulation was observed independent of the genetic background of the cell, our results suggest that accumulation and associated clinical responses are almost entirely dictated by the combination of mutation and TKI. When validated in a prospective setting this independence argues that all patients with sensitive EGFR mutations should, regardless of the type of tumor, be considered for treatment with EGFR-TKIs.

2. Methods

2.1. Constructs

EGFR mutation constructs were generated by in-fusion cloning. The backbone of all constructs were essentially as described [13], with eGFP cloned in-frame 3’ to the transmembrane domain. This position was chosen to avoid potential interference with ligand binding or receptor internalization signaling sites. Constructs were cloned into a piggybac vector (System Biosciences, Palo Alto, Ca) allowing for rapid integration using transposase into the host genome. Cell lines were obtained from the ATCC (Manassas, Virginia). Cells were plated in 96 or 384 well plates for further analysis.

2.2. Image analysis

All images were obtained using an Opera Phenix high-throughput high-content confocal microscope (Perkin Elmer, Hamburg, Germany). At least 10 images were obtained per well so that an experiment involving a single construct, 6 conditions (5 inhibitors + control) at 10 different dilutions typically would produce >600 images per time point in which data of ~1000 cells were obtained per condition. Channels were independently excited to minimize potential spectral overlap. Image analysis was performed in bulk using Harmony software (Perkin Elmer) using identical settings within each experiment. Experiments described in current manuscript were performed at least in two independent replicates. Data was further analysed using R.

2.3. Stainings

EGFR antibody (clone H11, DAKO, Amstelveen, the Netherlands) and a phospho-specific EGFR antibody (AB32430, anti phospho Y1068, Abcam, Cambridge, UK) were used at 1:500 dilution for both western blot and immunohistochemistry. Secondary antibodies used were Alexafluor 647 goat anti-mouse (A21240, Invitrogen, Bleiswijk, the Netherlands) and Alexafluor 488 goat anti-rabbit (A11008, Invitrogen, Bleiswijk, the Netherlands). Hoechst and WGA were used as counterstain to visualize nucleus and membranes respectively.

2.4. RT-QPCR

RNA was extracted from cells using the RNeasy mini kit (Qiagen, Venlo, the Netherlands). RT-QPCR was performed using Taqman probes (Applied Biosystems, Bleiswijk the Netherlands) according to the manufacturers’ instructions. Expression levels of cFOS and EGR1 were evaluated relative to POP4 and GAPDH controls.

2.5. Patients

We identified pulmonary adenocarcinoma patients harbouring EGFR mutations from routine diagnostics within the Erasmus MC. For patients screened in 2016, no selection was made other than presence of a mutation in the EGFR gene. The data was further expanded with patients screened in 2017 and 2018 but not including patients with exon 19 deletions or the L858R missense mutation (thus selecting for rare mutations). Patient data were collected in compliance with national and institutional guidelines. We generated constructs for these mutations. If multiple mutations were identified, the prediction of response was made based on the one with highest IC50. Response predictions were performed with the experimenter blinded to the clinical outcome. The separation into responders/non-responders was performed blinded to clinical outcome using a predefined
cutoff of 500 nM. This cutoff was chosen prior to the analysis and was based on maximal concentrations of inhibitor that are achieved in patients, though there is a large inter patient variability [14]. Progression free survival was defined as the time to progression to first line TKI treatment. Patients were censored in case of enduring clinical response or when lost to follow-up.

2.6. RPPA

All samples were prepared according to the guidelines of the MD Anderson functional proteomics RPPA core facility, where all RPPA experiments were subsequently run. Cells were maintained under normal (serum supplemented) culture conditions and inhibitors or DMSO were added two hours prior to cell lysis. RPPA experiments were generated in three experiments, with each experiment performed in a separate week at a different cell-passage number to ensure complete independence.

3. Results

3.1. Clinically effective TKIs induce an intracellular accumulation of EGFR

To examine mutation- and TKI-specificity of clinical responses, we generated eGFP-tagged EGFR mutation constructs, stably expressed them in HeLa cells and monitored response to inhibitors in vitro. When erlotinib was added to cells expressing EGFRL858R, we observed a striking intracellular accumulation of the protein visible as intracellular EGFR-protein ‘spots’ (dozens per cell and up to thousands per imaging field, Fig. 1a). Using an automated quantitative imaging analysis setup, we showed that the response was dose dependent, occurred within 5 min following drug administration and persisted for >3 days (Fig. 1b/c and supplementary Fig. 1 and supplementary movie 1). In contrast, erlotinib did not induce the intracellular accumulation in cells expressing EGFR-wildtype or EGFRvIII (a deletion of exons 2-7, the most common mutation in GBMs, Fig. 1a). We then generated constructs for all EGFR mutations, stably expressed them in HeLa cells and monitored response to inhibitors that do not show clinical efficacy. We then generated constructs for all TKI-sensitive mutations (H596 and H4006) and not in cell lines that do not harbour TKI-sensitive mutations (H596 nor H460). Effective EGFR TKIs therefore lead to the intracellular accumulation of EGFR in cell lines harbouring TKI-sensitive mutations.

3.2. Intracellular accumulation predicts response to gefitinib in cell lines

Because of the correlation of the intracellular accumulation with responses observed in the clinic, we tested whether intracellular accumulation was able to actually predict response to EGFR TKIs. For this, we screened the Genomics of Drug Sensitivity in Cancer (GDSC) database that contains drug-sensitivity data in >1000 genomically characterized cell lines [15–17]). We selected 11 cell lines with a known EGFR mutation (10 different mutations) with documented response to gefitinib. We then generated constructs for all EGFR mutations, stably expressed them in HeLa cells and screened for inhibitor-induced intracellular accumulation. EGFRL858R, EGFR746_A750del, EGFRL747_E749del, EGFRS768I and EGFR719S all responded to gefitinib by rapidly inducing intracellular accumulation of EGFR; none of the other mutation constructs showed such accumulation (supplementary Fig. 3). Dose response analysis indicated that EGFRL858R and EGFR746_A750del were highly sensitive to gefitinib (IC50 <20 nM) whereas EGFR747_E749del, EGFRS768I and EGFR719S showed considerably higher IC50 values (156, 625 and 456 nM respectively, Fig. 3).

Comparing ‘gefitinib induced intracellular accumulation in HeLa cells expressing EGFR-mutation constructs’ with ‘gefitinib sensitivity of cells endogenously expressing EGFR mutations’ showed that the IC50 value for intracellular accumulation was highly similar to the IC50 value for viability (extracted from the GDSC database, supplementary table 1) for each of the mutations tested (Fig. 3b). Cell lines that are highly sensitive to gefitinib also harbored mutations that were highly sensitive to gefitinib induced intracellular accumulation (EGFRL858R or EGFR746_A750del), cell-lines with moderate sensitivity harbored mutations that were moderately sensitive to gefitinib induced intracellular accumulation (EGFRS768I or EGFR719S) and cell-lines that are insensitive to gefitinib harbored mutations that do not show gefitinib induced intracellular accumulation (Fig. 3). Note, virtually identical results were obtained using erlotinib in our assay and lapatinib was unable to induce intracellular accumulation in any EGFR mutation. Our relatively simple and straightforward assay therefore was able to predict sensitivity to EGFR TKIs in cell lines harboring endogenous EGFR mutations.

3.3. Intracellular accumulation predicts response to EGFR TKIs in pulmonary adenocarcinoma patients

To determine whether intracellular accumulation of EGFR can predict response to TKIs in patients, we screened all pulmonary
Clinically effective EGFR TKIs induce a rapid and massive intracellular accumulation of EGFR. (a) Erlotinib treatment of HeLa cells ectopically expressing EGFR\textsuperscript{L858R} results in its intracellular accumulation. This accumulation is not observed in cells expressing EGFR\textsubscript{wt} or EGFR\textsubscript{vIII}. Top panels depict the EGFR signal only (Green); bottom panels is a merge

![Image](image-url)

**Fig. 1.** Clinically effective EGFR TKIs induce a rapid and massive intracellular accumulation of EGFR. (a) Erlotinib treatment of HeLa cells ectopically expressing EGFR\textsuperscript{L858R} results in its intracellular accumulation. This accumulation is not observed in cells expressing EGFR\textsubscript{wt} or EGFR\textsubscript{vIII}. Top panels depict the EGFR signal only (Green); bottom panels is a merge.
adenocarcinoma patients treated in 2016 and 2017 within our clinic for the presence of EGFR mutations (Table 1). For each mutation identified in this patient cohort (of which the only selection criterion was the presence of an EGFR mutation), we generated EGFR-mutation constructs and stably expressed them in HeLa cells. In each EGFR mutation we tested the ability of TKIs to induce intracellular accumulation and, if so, determined the IC50 value thereof. All experiments were performed using automated image analysis software and were blinded to clinical outcome. We then split the dataset into ‘predicted responders’ and ‘predicted non-responders’ using a cutoff of 500 nM for intracellular EGFR accumulation. This cutoff was defined prior to performing the experiments and was based on estimates of the in-tumoral concentration of erlotinib (~200ng/g tumor tissue, though there is a wide inter-patient and intra-tumoral variability [14]). On this dataset, we show that ‘predicted responders’ had a significantly longer time to progression to first line EGFR TKIs than the ‘predicted non-responders’ (median survival 7.0 vs 13 months, HR 0.21, P=0.0004 [Cox proportional hazard], Fig. 4). Explorative analysis of other cutoffs points (ranging from 10−1000 nM) is shown in supplementary Fig. 3b.

It should be noted that some tumors harbored more than one EGFR mutation, in which case we used the mutation with least ability for intracellular accumulation to predict treatment response. We defined this prior to any data analysis. However, data from the double mutant EGFR\textsuperscript{L858R,T790M} could suggest that the accumulation may be

including Red: WGA (membrane) and blue: Hoechst (nucleus). (b) intracellular accumulation is dose dependent and only occurs with clinically effective inhibitors erlotinib and gefitinib but not with lapatinib. The intracellular accumulation is retained up to 60 h (c). (d) Erlotinib no longer induces intracellular accumulation in cells ectopically expressing the resistance mutation EGFR\textsuperscript{L858R,T790M}. They do however remain responsive to osimertinib (bottom panels) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).
Table 1
Intracellular accumulation formation predicts response to EGFR TKIs in pulmonary adenocarcinoma patients.

| Patient | drug | mutation 1 | mutation 2 | IC50 mut 1 (nM) | IC50 mut 2 (nM) | PFS (m) | event | response prediction |
|---------|------|------------|------------|-----------------|-----------------|---------|-------|---------------------|
| 034     | gef  | ΔE746_A750 |           | 156             |                 | 11      | 1     | Sens                |
| 041     | erl  | L858R      |           | 30              |                 | 15      | 1     | sens                |
| 060     | erl  | ΔE746_A750 |           | 7               |                 | 10      | 1     | sens                |
| 086     | gef  | ΔE746_A750 |           | 2.4             |                 | 70      | 1     | sens                |
| 088     | erl  | ΔE746_A750 |           | 7               |                 | 23      | 1     | sens                |
| 158     | erl  | ΔE746_A750 |           | 39              |                 | 18      | 1     | sens                |
| 158     | erl  | ΔK745_A750 |           | 7               |                 | 13      | 1     | sens                |
| 175     | erl  | ΔK745_A750 |           | 7               |                 | 15      | 1     | sens                |
| 183     | com  | ΔE746_A750 |           | 7               |                 | 7       | 1     | sens                |
| 196     | erl  | ΔE746_P753 |           | 7               |                 | 16      | 1     | sens                |
| 208     | com  | G719S      | S768I      | 156             | 1250            | 9       | 1     | insens              |
| 228     | erl  | ΔE746_A750 |           | 7               |                 | 28      | 1     | sens                |
| 294     | erl  | G719A      |           | 156             |                 | 3       | 0     | sens                |
| 323     | erl  | L858R      |           | 30              |                 | 12      | 1     | sens                |
| 345     | erl  | L858R      |           | 30              |                 | 13      | 1     | sens                |
| 450     | erl  | ΔE746_A750 |           | 7               |                 | 10      | 1     | sens                |
| 467     | erl  | ΔE746_A750 |           | 7               |                 | 12      | 1     | sens                |
| 475     | erl  | G719S      | E709A      | 156             | 2500            | 19      | 1     | insens              |
| 554     | erl  | ΔE746_A750 |           | 7               |                 | 33      | 1     | sens                |
| 586     | erl  | G719A      |           | 156             |                 | 4       | 1     | sens                |
| 580     | erl  | S768I      | G724S      | 1250            |                 | 2       | 1     | insens              |
| 650     | erl  | L730R      |           | 10000           |                 | 2       | 1     | insens              |
| 655     | erl  | S752I*11   |           | 10000           |                 | 2       | 1     | insens              |
| 700     | erl  | S768I      | L861Q      | 1250            | 625             | 2       | 1     | insens              |
| 715     | erl  | G719S      | E709A      | 156             | 2500            | 11      | 1     | insens              |
| 831     | gef  | ΔE746_A750 |           | 2               |                 | 3       | 1     | sens                |
| 845     | erl  | ΔE746_A750 | S768I      | 39              | 1250            | 10      | 1     | insens              |
| 854     | gef  | ΔE746_A750 |           | 2               |                 | 13      | 1     | sens                |
| 932     | erl  | L861Q      |           | 625             |                 | 6       | 1     | insens              |
| 949     | erl  | L858R      |           | 30              |                 | 12      | 1     | insens              |
| 555     | erl  | P848L      |           | 10000           |                 | 1       | 1     | insens              |
| 225     | crizo| G719C      |           | 1250            |                 | 12      | 1     | insens              |
| 924     | erl  | S768I      |           | 1250            |                 | 12      | 1     | insens              |
| 475     | erl  | G719S      | E709A      | 156             | 2500            | 18      | 1     | insens              |
| 608     | erl  | L861Q      |           | 625             |                 | 1       | 1     | insens              |
| 743     | erl  | L861Q      |           | 625             |                 | 8       | 1     | insens              |
| 924     | erl  | L858R      |           | 30              |                 | 12      | 1     | insens              |
| 890     | erl  | L858R      | L730R      | 30              | 10000           | 2       | 1     | insens              |
| 228     | erl  | ΔE746_A750 |           | 7               |                 | 25      | 1     | sens                |
| 747     | erl  | G719A      |           | 156             |                 | 27      | 1     | sens                |
| 502     | erl  | G719S      | E709K      | 156             | 10000           | 10      | 1     | insens              |

Er: Erlotinib; gef: gefitinib; com: combination of erlotinib/gefitinib. Only one TKI was administered at one timepoint but toxicity of the first TKI led to change in regimen to the second TKI; PFS: progression free survival; sens: sensitive; insens: insensitive.
dictated by the most sensitive mutation, unless of course this concerns a secondary resistance mutation. We therefore also performed a similar analysis but used the mutation with highest ability for intracellular accumulation to predict treatment response. Also in this analysis ‘predicted responders’ had a significantly longer time to progression to first line EGFR TKIs than the ‘predicted non-responders’ (median survival 2.0 vs 12 months, HR 0.14, P<0.0001). These data demonstrate that intracellular accumulation of EGFR’s predictive for clinical response to first line EGFR TKI.

3.4. Predicting response to rare mutations

We further evaluated the intracellular accumulation in mutations where clinical responses to EGFR TKIs is unknown. Because of the rarity of such mutations, we included DIRECT database queries and public domain literature to assess clinical responses (Table 2). The EGFRT751-I759delinsATA mutation showed strong intracellular accumulation (IC50 for gefitinib and erlotinib of 40 and 10 nM respectively) and was classified as ‘predicted responder’. A patient with similar mutation indeed showed a partial response to EGFR TKIs and a progression free survival of 8 months [18]. The EGFRL747-E749del showed sufficient strong intracellular accumulation (IC50 for gefitinib and erlotinib of 156 and 432 nM respectively) to be classified as ‘predicted responder’. The DIRECT database identified two patients harboring such mutations and both showed partial responses to EGFR TKIs (PFS 6 months in one patient, PFS not reported for the other) [19]. The EGFRL746X missense mutation did not show any sign intracellular accumulation and was classified as ‘predicted non-responder’. Two patients have been described harboring a similar mutation and neither patient responded to EGFR TKI treatment (both had stable disease, no PFS reported) [20,21]. Finally, the EGFRL848L was found in one of our patients and, as predicted by a lack of intracellular accumulation, this patient did not respond to EGFR TKI treatment. A patient with identical mutation also did not respond to erlotinib [22]. Therefore, also in these rare mutations with previously unknown sensitivity to EGFR-TKIs, intracellular EGFR accumulation highly correlated to the clinical responses in all seven patients. These results therefore further demonstrate that intracellular accumulation predicts response to EGFR TKIs.

3.5. All EGFR TKIs effectively inhibit EGFR and its pathway

Because of the strong phenotype induced by effective EGFR TKIs, but only on TKI-sensitive mutations, we explored whether these TKIs and/or mutations differ with respect to pathway activation and inhibition. Western blot analysis showed that all inhibitors effectively blocked EGFR phosphorylation in HCC827 cells (that contains an endogenous EGFRL747-A750del mutation, Fig. 5a). In a cell line containing the T790M resistance mutation (H1975), only osimertinib reduced EGFR phosphorylation (supplementary Fig. 4a). Two other lung cancer cell-lines (H460 and H596, EGFR wt and amplified respectively), showed no EGFR phosphorylation under normal serum culture conditions (supplementary Fig. 4a, see also [23,24]). Quantitative image analysis, using pan- and phospho-specific EGFR antibody stainings, confirmed the efficacy of EGFR-TKIs: In cell lines containing activating EGFR mutations (HCC827 and HCC4006), EGFR is phosphorylated and the addition of all tested TKI effectively inhibited this phosphorylation (Fig. 5b/c). In cell lines without activating EGFR mutations (NCI-H460 and H596), EGFR is not phosphorylated and EGFR stimulation resulted in a rapid increase in EGFR phosphorylation levels. Addition of EGFR TKIs prior to EGFR stimulation prevented EGFR-phosphorylation and the addition of TKIs after EGFR stimulation resulted in a rapid dephosphorylation of EGFR (Fig. 5d/e, supplementary Fig. 4b). Also in stably transfected HeLa cells, all intracellular accumulation consisted of dephosphorylated EGFR (supplementary Fig. 5). All examined TKIs therefore effectively block EGFR phosphorylation and therefore cannot explain the differences in the observed intracellular accumulation.

We performed reversed phase phosphoprotein arrays (RPPA) to study whether different TKIs and/or mutations differentially affect pathway activation. We find that erlotinib and lapatinib are equally effective in blocking downstream EGFR signaling (Fig. 6a–c, supplementary Table 2) irrespective of the type of EGFR mutation present and irrespective of the inhibitor used: in all three cell lines tested phosphorylation of AKT (serine 473), mTOR (serine 2448) and P90 (threonine 573) was inhibited by the addition of erlotinib or lapatinib. We also did not identify differences in other molecular pathways interrogated by the RPPA arrays between the two inhibitors. RT-qPCR further demonstrated that EGFRTKIs effectively blocked the expression the immediate early genes EGR1 and cFOS, also irrespective of EGFR mutation type or inhibitor used [13,25,26] (Fig. 6d).

| Mutation | Response prediction | Clinical response | PFS | ref |
|----------|---------------------|-------------------|-----|-----|
| L747_E749del | sensitive | PR | 6 | Yeh et al., 2013 |
| L747_E749del | sensitive | PR | 6 | Yeh et al., 2013 |
| E746X | insensitive | SD | | Kalikaki et al., 2010 |
| E746X | insensitive | SD | 1 | Pallis et al., 2007 |
| P848L | insensitive | 1 | 4.6 | Faehling et al., 2017 |
| P848L | 1 | | 8 | Schrock et al., 2016 |

PR: partial response; SD: stable disease.
Fig. 5. All investigated TKIs effectively inhibit EGFR phosphorylation. (a) all EGFR TKIs effectively block EGFR phosphorylation on western blot in HCC827 and H4006 cell-lines. (b) Imaging analysis showing effects of EGF stimulation on EGFR and EGFR phosphorylation in H460 (left panels) and H596 cells (middle and right panels). In H460 cells, EGF stimulation results in internalization of the receptor. Co-staining for phospho-EGFR shows a rapid increase in EGFR-phosphorylation, which overlaps with the pan-EGFR signal. Right panels are an inset of the yellow square in EGF-stimulated H596 cells, depicting phospho-EGFR staining (top) and pan-EGFR staining (red). (c) Quantification of the phospho-EGFR signal in areas staining for pan-EGFR. As can be seen, EGF stimulation of H596 cells (top panel) results in a very pronounced increase in phospho-EGFR staining per cell (each dot represents an individual area that stained positive for EGFR). In cells HCC827 cells (lower panel) that have constitutive active EGFR phosphorylation, gefitinib significantly decreases the phospho-EGFR signal. (d) HCC827 cells stained for EGFR (red, left panels) and phospho-EGFR (green, right panels). As can be seen, all inhibitors effectively reduce EGFR phosphorylation. (e) Quantification of images presented in (d) as presented in (c) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).
We also performed pull-down assays to examine whether different TKIs differentially affect EGFR protein-protein interactions. Although some inhibitor-specific protein-protein interactions were identified across the various cell lines examined ([HCC827, HCC4006 and HeLa cells expressing EGFRL858R, supplementary Table 3]), no difference that was common between erlotinib/gefitinib with lapatinib was observed. The various TKIs therefore have similar inhibition of EGFR, its pathways and its interactome and therefore do not provide an explanation for the TKI- and mutation-specific intracellular accumulation in EGFR.

3.6. A two-step conformational change model may explain the intracellular accumulation

EGFR is phosphorylated and internalized after its activation by ligand (see e.g. Fig. 5b and [27]). Once trafficked into early endosomes, the protein is eventually dephosphorylated and either recycled back to the plasma membrane or transported to the lysosome for degradation. As activated EGFR remaining in the cytoplasm will be recycled back to the membrane, it follows that the inhibition of EGFR activity will result in a relative increase in the membrane fraction of the protein. Indeed, quantification of the membrane/cytoplasm ratio of EGFR shows that EGFR-TKIs result in an increased membrane association in cells expressing EGFRwt (Fig. 7). Interestingly, only lapatinib resulted in this increased membrane association in cells expressing EGFRL858R; other TKIs resulted in an increased intracellular accumulation.

We hypothesized that the difference between lapatinib and other TKIs on EGFRL858R may lie in the differential conformational preference of TKIs: erlotinib (a type I inhibitor) associates with the active conformation while the type 1.5 inhibitor lapatinib traps the protein in an inactive conformation [28-30]. In EGFRwt such conformational
preference is TKI-independent: once EGFRwt is dephosphorylated, the protein will adopt an inactive conformation and the protein is recycled to the membrane. However, specific activating mutations such as EGFR_L858R destabilize (or even are incompatible with-) the inactive conformation and promote the protein to adopt its active conformation [28,29,31]. Since erlotinib associates with the active conformation it is possible that, in the context of EGFRL858R, the TKI remains associated with the protein and this association blocks recycling to the plasma membrane.

To demonstrate clinically effective TKIs remain associated with EGFRL858R, we washed out the various inhibitors and monitored intracellular accumulation. The intracellular accumulation indeed depended on the continued presence of the inhibitor (despite EGFR being de-phosphorylated) as removal of competitive inhibitor erlotinib or osimertinib, but not the non-competitive inhibitor dacomitinib, resulted in a reversal the intracellular accumulation in HeLa cells expressing EGFR_L858R after >30 min of erlotinib/osimertinib withdrawal (Fig. 8, supplementary Fig. 6). In lung cancer cell lines harbouring endogenous EGFR mutations, EGFR cannot be re-phosphorylated even after four hours after washout of the inhibitors further confirming that TKIs remain associated with EGFR (supplementary Fig. 7).

These results are compatible with the hypothesis that the mutation and TKI-specificity of the intracellular accumulation is due to two sequential effects: activating mutations firstly lock the protein in an active conformation, TKIs that associate with the active conformation then further affect the conformation of EGFR. Structural studies confirm that TKIs actively affect the conformation of EGFR [28,29,31]. This altered conformation then prohibits recycling to the plasma membrane resulting in an intracellular accumulation of the protein.

4. Discussion

In this study, we have performed functional analysis on EGFR-mutation constructs to understand why only specific tumor-types respond to EGFR inhibitors, and why only specific inhibitors are clinically effective. We show that the addition of TKIs to cells expressing EGFR-mutation constructs results in a rapid intracellular accumulation of EGFR, but only on mutations that show clinical response to EGFR TKIs and only to EGFR-TKIs that are clinically effective. The accumulation is highly correlated to sensitivity to gefitinib in EGFR-mutated cell lines, and we show that it predicts response to EGFR-TKIs in patients.

Our data has two important clinical implications. First of all, our relatively simple assay can be used to predict the response EGFR TKIs in tumors harboring mutations where this is not yet known. The assay can be performed in vitro, and is independent of availability of patient material: it only requires knowledge on the mutation present. A large database containing the TKI-induced intracellular accumulation of all possible EGFR-mutations (alone or in combination with resistance mutations), stably expressed in HeLa cells, would suffice predicting clinical responses, and to which TKI the mutation is likely to be most sensitive. Second, since the intracellular accumulation is seen in cell lines that do not depend on EGFR, our data imply that response to EGFR-TKIs is almost entirely dictated by the type of mutation present, and thus is independent of the cell or tumor type. The tumor type independence of TKI efficacy is supported by several reports where clinical responses to EGFR TKIs have been observed in various (non-pulmonary adenocarcinoma) tumor-types harboring TKI-responsive mutations. In fact, of eight reports found, only one recurrent thymoma patient harboring an exon 19 deletion (E746-A705 del) failed to respond to gefitinib; all other patients responded [32–39]. However, the use of ectopic expression however does not allow screening for intrinsic resistance of cells. Nevertheless, mutation-specificity indicates that all patients with EGFR mutated tumors (regardless of tumor type), that are sensitive to EGFR-TKIs in lung cancer, should be considered for treatment with EGFR-TKIs.

It should be noted that we did not observe overt differences between different TKIs (see e.g. supplementary Fig. 1b) that could be related to the varying clinical responses (e.g. response duration). It is
Therefore possible that clinical efficacy is dictated by the properties of the inhibitor itself (reversible vs irreversible, IC50, bioavailability) or by the probability of acquisition of secondary resistance mutations and/or initiation of other resistance pathways.

Our data also provides some mechanistic insight into how clinically effective EGFR-TKIs may function: they require two sequential effects on the conformation of the protein. Firstly activating mutations lock the protein in an active conformation. Secondly, TKIs that associate with the active conformation further affect the conformation of EGFR which ultimately prohibits the protein recycling to the plasma membrane. It remains to be determined why the intracellular accumulation results in effective clinical responses. It is possible that intracellular accumulation results in an inactivation of all functions of EGFR, perhaps including those that may not depend on phosphorylation. Such a ‘TKI-induced sequestering of EGFR’ would explain why many (non-pulmonary adenocarcinoma) tumors remain dependent on EGFR for growth, but that inhibition of EGFR-phosphorylation alone is ineffective [40,41]. If so, targeting EGFR would remain a valid option for tumors that depend on its signalling for growth.

In summary, we provide an assay that can predict whether a tumor harboring an unknown mutation will respond to EGFR-TKIs, and if so, which TKI is most effective. We show that response to EGFR-TKIs is dictated by the mutation, and not the cell or tumor type. If our observations are validated, preferably in a prospective setting, it indicates that all patients with sensitive EGFR mutations should, regardless of the type of tumor, be considered for treatment with EGFR-TKIs.

Declaration of Competing Interest

JA has served in advisory boards for Astra-Zeneca and Roche-Genentech. PJF received grant support from AbbVie.

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Author contributions

Conceptualization, PJF; Methodology, P.J.F, M.v.R., J.A. and P.S.S.; Investigation, Y.G., M.d.W., I.d.H. and B.V.; Writing – Original Draft, P. J.F; Writing – Review & Editing Y.G., M.d.W., D.M, I.d.H., B.V., M.v.R. J. A and P.S.S.; Funding Acquisition, P.J.F. and P.S.S.; Resources, M.v.R and D.M.; Supervision, P.J.F, J.A. and P.S.S.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102796.

References

[1] Maemondo M, Inoue A, Kobayashi K, Sagawa S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362(25):2380–8.
[2] Mok TS, Cheng Y, Zhou X, Lee KH, Nakagawa K, Nishi S, et al. Improvement in overall survival in a randomized study that compared Dacomitinib with Gefitinib in patients with advanced non-small-cell lung cancer and EGFR-activating mutations. J Clin Oncol 2018;JCO2018787994.
[3] Popat S, Osimertinib as first-line treatment in EGFR-mutated non-small-cell lung cancer. N Engl J Med 2018;378(2):192–3.
[4] Takano T, Ohe Y, Sakamoto H, Tsuta K, Matsuno Y, Tateishi U, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23(28):6829–37.
[5] Uhm JH, Ballman KV, Wu W, Giannini C, Krauss JC, Buckner JC, et al. Phase II evaluation of gefitinib in patients with newly diagnosed Grade 4 astrocytoma: Mayo/north central cancer treatment group study N0704. Int J Radiat Oncol Biol Phys 2011;80(2):347–53.
[6] van den Bent MJ, Brandes AA, Ramlng R, Kouwenhoven MC, Kros JM, Carpentier AF, et al. Randomized phase II trial of erlotinib versus temozolomide or carbustine in recurrent glioblastoma: EORTC brain tumor group study 26034. J Clin Oncol 2009;27(8):1268–74.
[7] Hegi ME, Diserens AC, Bady P, Kamoshima Y, Kouwenhoven MC, Delorenzi M, et al. Pathway analysis of glioblastoma tissue after preoperatory treatment with the EGFR tyrosine kinase inhibitor Gefitinib—a phase II trial. Molecular Cancer Therapeutics 2011;10(6):1102–12.
[8] Ruan Z, Kannan N. Altered conformational landscape and dimerization dependency underpins the activation of EGFR by alphachetaita loop insertion mutations. Proc Natl Acad Sci U S A 2018;115(35):E8162–E71.
[9] Hasako S, Terasaka M, Abe N, Uno T, Ohnawa H, Hashimoto A, et al. TSG6117, A Novel EGFR Inhibitor Targeting Exon 20 Insertion Mutations. Molecular Cancer Therapeutics 2018;17(8):1648–58.
[10] Gao Y, Valletengo WR, French PJ. Finding the Right Way to Target EGFR in Glioblastomas: Lessons from Lung Adenocarcinomas. Cancers (Basel) 2018;10(12).
[11] Ross RL, Askham JM, Knowles MA. PIK3CA mutation spectrum in urothelial carcinoma. Cancer Res 2013;73(10):2846–56.
[12] Smylie M, G. R. Blumschein J, Dowlati A, Garst J, Shcaper FA, Rigs J, et al. A phase II multicenter trial comparing two schedules of lapatinib (LAP) as first or second line monotherapy in subjects with advanced or metastatic non-small cell lung cancer (NSCLC) with either bronchioloalveolar carcinoma (BAC) or no smoking history. J Clinical Oncol 2007;25(18_suppl):7611.
[13] Erdem-Eraslan L, Gao Y, Kloosterhorst NK, Atlisi Y, Demmers J, Sacchetti A, et al. Mutation specific functions of EGFR result in a mutation-specific downstream pathway activation. Eur J Cancer 2015;51(7):983–903.
[14] Lankheet NA, Schaeke EE, Burgers SA, van Pel R, Beijnen JH, Huitema AD, et al. Concentrations of Erlotinib in Tumor Tissue and Plasma in Non-Small-Cell Lung Cancer Patients After Neoadjuvant Therapy. Clin Lung Cancer 2015;16(4):320–4.
[15] Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, et al. Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. Nucleic Acids Res 2013;41(Database issue):D955–61.

[16] Barretina J, Caponigro G, Stranges N, Venkatesan K, Margolin AA, Kim S, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 2012;483(7391):603–7.

[17] Iorio F, Knujiengbaum TA, Vis DJ, Bignell GR, Menden MP, Schubert M, et al. A landscape of pharmacogenic interactions in cancer. Cell 2016;166(3):740–54.

[18] Schroek AB, Frampton GM, Herndon D, Greenbowe JR, Wang K, Lipson D, et al. Comprehensive genomic profiling identifies frequent drug-sensitive EGFR Exon 19 Deletions in NSCLC not Identified by Prior Molecular Testing. Clin Cancer Res 2016;22(13):3281–5.

[19] Yeh P, Chen H, Andrews J, Naser R, Pao W, Horn L. DNA-Mutation Inventory to Refine and Enhance Cancer Treatment (DIRECT): a catalog of clinically relevant cancer mutations to enable genome-directed anticancer therapy. Clin Cancer Res 2013;19(7):1894–901.

[20] Pallas AG, Voutsina A, Kalikaki A, Souglakos J, Pallis AG, Voutsina A, Kalikaki A, Souglakos J, Briasoulis E, Murray S, et al. Clinical outcome of patients with non-small cell lung cancer receiving front-line chemotherapy according to EGFR and K-RAS mutation status. Lung Cancer 2010;69(1):110–5.

[21] Faehling M, Schwenk B, Kramberg S, Eckert R, Volckmar AL, Stenzinger A, et al. ‘Classical’ but not ‘other’ mutations of EGFR kinase domain are associated with clinical outcome in gefitinib-treated patients with non-small cell lung cancer. Br J Cancer 2007;97(11):1560–6.

[22] Kalikaki A, Koutsopoulos A, Hatzidaki D, Trypaki M, Kontopodis E, Stathopoulos E, et al. Clinical outcome of patients with non-small cell lung cancer receiving front-line chemotherapy according to EGFR and K-RAS mutation status. Lung Cancer 2010;69(1):110–5.

[23] Faehling M, Schwenk B, Kraberg S, Eckert R, Volckmar AL, Stenzinger A, et al. Oncogenic driver mutations, treatment, and EGFR-TKI resistance in a Caucasian population with non-small cell lung cancer: survival in clinical practice. Oncotarget 2017;8(44):77897–914.

[24] Amann J, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res 2005;65(1):226–31.

[25] Li T, Ling YH, Perez-Soler R. Tumor dependence on the EGFR signaling pathway expressed by the p-EGFR:p-AKT ratio predicts erlotinib sensitivity in human non-small cell lung cancer (NSCLC) cells expressing wild-type EGFR gene. J Thoracic Oncol Offic Publ Int Assoc Study Lung Cancer 2008;3(6):643–7.

[26] Mukula M, Krzywicki M, Goryca K, Paczkowska K, Ledwon JK, Statkiewicz M, et al. Genome-wide co-localization of active EGFR and downstream ERK pathway kinases mirrors mitogen-inducible RNA polymerase 2 genomic occupancy. Nucleic Acids Res 2016;44(21):10150–64.

[27] Jimeno A, Kulesza P, Kincaid E, Bousaroud N, Chan A, Forastiere A, et al. C-fos assessment as a marker of anti-epidermal growth factor receptor effect. Cancer Res 2006;66(4):2385–90.

[28] Madhus BH, Stang E. Internalization and intracellular sorting of the EGFR receptor: a model for understanding the mechanisms of receptor trafficking. J Cell Sci 2009;122(Pt 19):3433–9.

[29] Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, et al. Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. Nucleic Acids Res 2013;41(Database issue):D955–61.

[30] Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, et al. A unique structure for epidermal growth factor receptor complexed with the 4-anilinoquinazoline inhibitor gefitinib. Cancer Res 2007;67(6):2384–90.

[31] Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, Meyerson M, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell 2007;11(3):217–27.

[32] Iyevleva AG, Novik AV, Mosseynen VM, Imyanitov EN. EGFR mutation in kidney carcinoma confers sensitivity to gefitinib treatment: a case report. Urologic Oncol 2009;27(5):548–50.

[33] Masago K, Asato R, Fujita S, Hirano S, Tamura Y, Kanda T, et al. Epidermal growth factor receptor gene mutations in papillary thyroid carcinoma. Int J Cancer 2009;124(11):2744–9.

[34] Ali SM, Alpaugh RK, Buell JK, Stephens PJ, Yu JQ, Wu H, et al. Antitumor response of an ERBB2 amplified inflammatory breast carcinoma with EGFR mutation to the EGFR-TKI erlotinib. Clin Breast Cancer 2014;14(1):e14–6.

[35] Voss JS, Holtegaard LM, Kerr SE, Fritcher EG, Roberts LR, Gores GJ, et al. Molecular profiling of cholangiocarcinoma shows potential for targeted therapy treatment decisions. Hum Pathol 2013;44(7):1216–22.

[36] Agatsuma N, Yasuda Y, Ozasa H. Malignant pleural mesothelioma harboring both G719C and S768I Mutations of EGFR Successfully Treated with Afatinib. J Thoracic Oncol Offic Publ Int Assoc Study Lung Cancer 2017;12(9):e141–3.

[37] Lote H, Bhosle J, Thway K, Newbold K, O’Brien M. Epidermal growth factor mutation as a diagnostic and therapeutic target in metastatic poorly differentiated thyroid carcinoma: a case report and review of the literature. Case Rep Oncol 2014;7(2):393–400.

[38] Nakagiri T, Funaki S, Kadota Y, Takeuchi Y, Shinoh H, Akashi A, et al. Does gefitinib have effects on EGFR mutation-positive thymoma? -Case report of thymoma recurrence. Ann Thorac Cardiovasc Surg 2014;20(Suppl):674–6.

[39] Masago K, Muru M, Toyama Y, Togashi Y, Mishima M. Good clinical response to erlotinib. J Thorac Oncol Offic Publ Int Assoc Study Lung Cancer 2008;3(6):643–7.

[40] Klingler S, Guo B, Yao J, Yan H, Zhang L, Vaseva AV, et al. Development of an allosteric mechanism for activation of the kinase domain of epithelial growth factor receptor. Cell 2006;125(6):1137–49.

[41] Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, et al. A unique structure for epidermal growth factor receptor complexed with the 4-anilinoquinazoline inhibitor gefitinib. Cancer Res 2007;67(6):2384–90.

[42] Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, Meyerson M, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell 2007;11(3):217–27.

[43] Iyevleva AG, Novik AV, Mosseynen VM, Imyanitov EN. EGFR mutation in kidney carcinoma confers sensitivity to gefitinib treatment: a case report. Urologic Oncol 2009;27(5):548–50.

[44] Masago K, Asato R, Fujita S, Hirano S, Tamura Y, Kanda T, et al. Epidermal growth factor receptor gene mutations in papillary thyroid carcinoma. Int J Cancer 2009;124(11):2744–9.

[45] Ali SM, Alpaugh RK, Buell JK, Stephens PJ, Yu JQ, Wu H, et al. Antitumor response of an ERBB2 amplified inflammatory breast carcinoma with EGFR mutation to the EGFR-TKI erlotinib. Clin Breast Cancer 2014;14(1):e14–6.

[46] Voss JS, Holtegaard LM, Kerr SE, Fritcher EG, Roberts LR, Gores GJ, et al. Molecular profiling of cholangiocarcinoma shows potential for targeted therapy treatment decisions. Hum Pathol 2013;44(7):1216–22.

[47] Agatsuma N, Yasuda Y, Ozasa H. Malignant pleural mesothelioma harboring both G719C and S768I Mutations of EGFR Successfully Treated with Afatinib. J Thoracic Oncol Offic Publ Int Assoc Study Lung Cancer 2017;12(9):e141–3.