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Quantifying the sensitivities of EGF receptor (EGFR) tyrosine kinase inhibitors in drug resistant non-small cell lung cancer (NSCLC) cells using hydrogel-based peptide array

Gargi Ghosh[a], Xiaoliang Yan[b], Andrew G. Lee[a], Stephen J. Kron[b], Sean P. Palecek[a,*]

[a] Department of Chemical and Biological Engineering, University of Wisconsin, 1415 Engineering Drive, Madison, WI 53706, United States
[b] Ludwig Center for Metastasis Research, University of Chicago, Chicago, IL 60637, United States

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ABSTRACT

Epidermal growth factor receptor (EGFR) signaling plays an important role in non-small cell lung cancer (NSCLC) and therapeutics targeted against EGFR have been effective in treating a subset of patients bearing somatic EFGR mutations. However, the cancer eventually progresses during treatment with EGFR inhibitors, even in the patients who respond to these drugs initially. A large variety of distinct irreversible inhibitors have been developed, which may combat therapeutic resistance. Nonetheless, major challenges in tailoring patient-specific treatment regimens involve predicting the most effective inhibitors and monitoring for acquisition of resistance. A patient-customized, predictive diagnostic that quantifies the effects of specific anti-EGFR therapies may improve outcomes in cancers where EGFR plays a mechanistic role. In this study we used an EGFR-phosphorylatable peptide, AEEEEYFELVAKKK, immobilized within a polyacrylamide hydrogel as a substrate for profiling the activation status of EGFR in the cellular extracts of erlotinib-resistant cancer cells. The hydrogel array was able to detect therapeutic resistance as well as identify inhibitors capable of combating therapeutic resistance. These findings establish the potential of this protein–acrylamide copolymer hydrogel array to not only evaluate EGFR status in cancer cell lysates but also to screen for the most promising therapeutics for individual patients and monitor effects of treatment on acquisition of resistance to EGFR inhibitors.

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1. Introduction

Lung cancer is the leading cause of death globally. The first line of treatment involving platinum-based chemotherapeutics yields a response rate of only 30% in advanced non-small cell lung cancer (NSCLC) (Schiller et al., 2002). Frequent abnormal amplification or activation of epidermal growth factor receptor (EGFR) in NSCLC has been correlated with hallmarks of cancer including increased cell proliferation, angiogenesis, invasion and metastasis (Laskin and Sandler, 2004; Yarden, 2001). EGFR is a member of the ErbB family of receptor tyrosine kinases (RTKs) which comprised of EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Ligand binding leads to homo or heterodimerization of these receptors which in turn initiates downstream signaling cascades involving the phosphatidylinositol-3-kinase (PI3K), Akt, mitogen activated protein kinase (MAPK), phospholipase cγ and STAT pathways (Laskin and Sandler, 2004; Yarden, 2001).

Identification of the oncogenic potential of EGFR spawned the development of several therapeutics directed against EGFR. Two small molecule EGFR tyrosine kinase inhibitors (EGFR-TKI), gefitinib (Iressa, AstraZeneca International) and erlotinib (Tarveca, OSI Pharmaceuticals) have been evaluated in patients with NSCLC (Inoue et al., 2006; Shepherd et al., 2005). These ATP competitive, reversible EGFR-TKIs have been effective only in a small subset of NSCLC patients (10–15% of Caucasian and 30–40% of Asian) bearing somatic mutations in the kinase domain of EGFR (Sequist et al., 2007). EGFR mutations associated with increased sensitivity to erlotinib and gefitinib are found in exons 18–21, a region that encodes the EGFR tyrosine kinase domain (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Deletions in exon 19 and the L858R mutation constitute around 90% of these mutations. These mutations impart increased affinity for gefitinib or erlotinib as well as decreased affinity for ATP. Nevertheless, patients initially responding to TKI therapy invariably develop resistance to these drugs, thereby limiting the progression-free survival to approximately 9–13 months with a median survival of 2 years (Rossell et al., 2009). Studies have been undertaken to identify the biomolecular properties associated with drug resistance in tumor specimens and also in resistant cell lines. Different molecular techniques involving direct

* Corresponding author. Tel.: +1 60 8 262 8931; fax: +1 60 8 262 5434.
E-mail address: palecek@engr.wisc.edu (S.P. Palecek).

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sequencing, subcloning or cycleave PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), Scorpion amplified refractory mutation system (SARMS) as well as immunohistochemical detection using mutation-specific antibodies have been employed to identify the mechanisms of *de novo* or acquired resistance (Balak et al., 2006; Engleman et al., 2006; Kitamura et al., 2010; Kobayashi et al., 2005; Kosaka et al., 2006; Kuang et al., 2009; Pao et al., 2005). Moreover, mutation scanning based on enzymatic digestion of PCR products by SURVEYOR enzymes combined with HPLC chromatography or real time melting curve analysis has also been used for mutational analysis (Kuang et al., 2009; Li et al., 2007). These studies revealed that 50% of drug resistant tumors are associated with the emergence of a secondary mutation, substitution of methionine for threonine at the position 790 (T790M), in the EGFR kinase domain (Kobayashi et al., 2005; Pao et al., 2005). By increasing ATP affinity, the T790M mutation negates the sensitivity of reversible TKIs and generates a resistance to the achievable clinical doses of the drugs. Studies have also identified the presence of other secondary mutations in the resistant tumors, including D716Y, L747S, E884K, and T854A, although these mutations occur less frequently than T790M (Balak et al., 2006; Choong et al., 2006; Costa et al., 2008). An additional survival mechanism adopted by NSCLC cells in 20% of therapeutic resistance to EGFR-TKIs involves amplification of the MET proto-oncogene (Bean et al., 2007; Engelman et al., 2007a). The molecular mechanism involved in 30–40% of drug resistance cases is yet to be unraveled, illustrating the need to develop assays to directly monitor EGFR activity in cancer cells treated with EGFR-TKIs.

Some EGFR the secondary mutations, such as L747S or D761Y, confer substantially less resistance to gefitinib or erlotinib compared with the T790M mutation, and administering alternative EGFR-TKIs can be beneficial (Choong et al., 2006; Costa et al., 2008). One study showed that while switching to erlotinib overcame gefitinib resistance in a NSCLC patient with L858R + L747S mutations, it failed for a gefitinib refractory patient with the T790M mutation (Choong et al., 2006). Similarly another report demonstrated that a switch from erlotinib to gefitinib yielded a positive response in a lung adenocarcinoma patient with L858R + E884K mutations (Costa et al., 2008). However, none of the reversible EGFR-TKIs are effective in patients expressing EGFR with the T790M mutation. Thus it appears that the precise nature of the secondary mutations determines the success of these TKIs. However, the realization that cancer cells with T790M EGFR mutation still depend on EGFR for survival spawned the development of a gamut of irreversible EGFR-TKIs. These second-generation irreversible EGFR-TKIs, including CL-387,789, HKI-272, and PF00299804, inhibit EGFR phosphorylation by affecting a Michael addition reaction with the cysteine residue in the ATP binding pocket of the EGFR kinase domain. The covalent attachments ensure a higher occupancy of ATP binding sites and thus enable these TKIs to inhibit the activation of T790M EGFR (Engelman et al., 2007b; Zhou et al., 2009). Other second-generation irreversible inhibitors which have shown promise at different stages of clinical development include BIBW-2992 (EGFR/HER2 dual inhibitor), CI-1033 (pan-EGFR inhibitor) and EKB-569 (pan-EGFR inhibitor).

However, there are some serious issues which prevent a smooth transition of these TKIs from preclinical studies to clinical therapies. Due to the involvement of different resistance mechanisms, a major challenge involves identifying the mechanism of resistance in individual patients. This is because a general therapeutic strategy to overcome EGFR-TKI resistance will not be effective in treating all resistant patients. For example, patients with amplified MET expression will not respond to EGFR-TKI therapy. Similarly, treating patients bearing secondary EGFR mutations or having some other activated kinase pathway with MET inhibitor will be unsuccessful. Hence, there is a need of a diagnostic tool which can differentiate the patients who may benefit from switching to different EGFR-TKIs from patients who will need different therapeutic strategies to overcome the drug resistance.

Here we report the development of a hydrogel-based peptide array capable of quantitatively assessing EGFR tyrosine kinase activity and the sensitivity of different EGFR inhibitors in extracts of cells that have acquired drug resistance. In this study, we have used the EGFR-phosphorylatable peptide sequence AEEEEYFELVAKKK as the substrate for profiling EGFR kinase activity. As opposed to the conventional method of genotyping cancer specimens and cancer cell lines, the peptide array will not identify mutations but instead will detect alterations in the kinase activity due to acquired drug resistance. Sequencing is regarded as the gold standard for mutational analysis. However, allelic dilution due to the presence of a small fraction of mutated alleles in the background of large excess of normal alleles often renders the sequencing/mutation screening technique inadequate (Engleman et al., 2006; Li et al., 2007). Recent effort directed towards immunohistochemical detection of EGFR mutations with mutation-specific antibodies as an alternative of the mutational analysis revealed limited clinical utility of these antibodies (Kitamura et al., 2010). Since, EGFR-TKI resistant cancer cells with secondary mutations not only express active EGFR but also depend on EGFR for survival, an assay measuring activity of EGFR carrying mutations may provide a more accurate report of the disease state than these conventional techniques. AEEEEYFELVAKKK has been reported to be the optimal peptide for EGFR kinase (Songyang et al., 1995). However, to the best of our knowledge, this peptide has never been used to quantitatively assess the activity of EGFR carrying primary or secondary mutations. Here, we report the ability of the hydrogel-based peptide array to profile EGFR kinase activity and assess the sensitivity of different inhibitors in the extracts of cells with wild type EGFR (NCI-H23), cells with activating mutations (NCI-H1650, PC9) and also in cells having acquired resistance to erlotinib and gefitinib (H1650-ER and PC9-GR).

2. Materials and methods

2.1. Preparation and purification of peptide

Amino acids, CLEAR-Amide resin and 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were obtained from Peptides International (Louisville, KY, USA). Piperidine, N-methylmorpholine (NMM), trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), Triisopropylsilane (TIS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All reagents were used as received without any purification. The peptide, AEEEEYFELVAKKK, was synthesized on an automated synthesizer, Prelude™ (Protein Technologies, Inc.), using solid-phase method based on Fmoc-chemistry. Cleavage of the crude peptide was performed with the mixture of TFA/ddH_2O/EDT/TIS (94:2.5:2.5:1, v/v) at room temperature. The crude peptide was then precipitated and washed with cold diethyl ether three times. The crude peptides were tested using ABI 4700 MALDI TOF/TOF mass spectrometry (Applied Biosystems) to confirm the correct molecular masses and Agilent 1200 Series LC/MS system for purity. Purification was done through a preparative C_{18} column in the Agilent 1200 LC/MS system if necessary.

2.2. Cell culture and lysate production

Human lung cancer cell lines NCI-H23 and NCI-H1650 (henceforth referred to as H23 and H1650) were obtained from ATCC (Manassas, VA). PC9 and PC9-GR cells were kind gift from Dr. Pasi A. Janne (Dana–Faeber Cancer Institue, Boston, Massachusetts).

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