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Defining clinically relevant molecular subsets of lung cancer

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Abstract The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, induce dramatic responses in certain patients with non-small cell lung cancer (NSCLC). As such, the drugs provide an unexpected tool to dissect clinically relevant molecular subsets of NSCLC. For example, using mutational profiling of tumor DNA from patients with sensitivity, primary resistance, and secondary resistance to these agents, we and others have demonstrated that somatic mutations in the tyrosine kinase domain of *EGFR* are associated with sensitivity to gefitinib and erlotinib, while mutations in *KRAS*, which encodes a GTPase downstream of *EGFR*, are associated with primary resistance. Furthermore, second site mutations in the *EGFR* kinase domain are commonly found in patients with acquired resistance. We are now using a variety of molecular and biological approaches to help further define molecular subsets of lung cancer that have relevance in the clinic.

Keywords Non-small cell lung cancer · Epidermal growth factor receptor · Tyrosine kinase inhibitors · mutations · Drug sensitivity · Drug resistance

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Introduction

Gefitinib and erlotinib are related quinazoline small molecule inhibitors which compete with ATP for binding at the ATP-binding site in the tyrosine kinase domain of epidermal growth factor receptor (*EGFR*) [23, 33]. During the course of their clinical development, these tyrosine kinase inhibitors (TKIs) induced dramatic clinical and radiographic responses in patients with non-small cell lung cancer (NSCLC), even after progression on multiple lines of systemic chemotherapy [8, 17]. However, only a subset of patients experienced such dramatic responses. Because of the nature of these heretofore unseen responses to a "targeted" therapy, an important question was: who responds best to these compounds?

Retrospective subgroup analyses of the phase II trials of gefitinib revealed several clinical characteristics statistically associated with responses: female gender, East Asian ethnicity, a history of never smoking (i.e. having smoked less than 100 cigarettes in one's lifetime), and adenocarcinoma histology, especially those with features of bronchioloalveolar carcinoma (BAC) [8, 21]. Furthermore, early preclinical

and correlative clinical studies did not reveal any obvious association between *EGFR* expression and response. Thus, multiple groups, including ours at Memorial Sloan-Kettering Cancer Center, became interested in determining if molecular predictors of response could be identified.

Our studies were guided by a tetracycline-inducible mouse model of lung cancer established in the Varmus Laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, in which a different oncogene, activated *Kras*, had been shown to induce Kras-dependent lung tumors in mice [6]. Specifically, when activated *Kras* was "turned on" in the lung epithelial cells of these animals, they developed lung adenocarcinomas. When the activated *Kras* was subsequently "turned off", the lung tumors remarkably disappeared. This phenomenon was even observed in mice lacking the tumor suppressor genes *p53* or *Ink4A-Arf*. These data had three major implications: (1) normal lung epithelial cells can be transformed by an activated oncogene; (2) lung tumor cells somehow become dependent upon or "addicted to" the continued presence of the activated oncogene for survival; and (3) there may be an "Achilles heel" in the RAS pathway that could be exploited for clinical benefit, even in genetically complex tumors.

With this in mind, and aware that mutational profiling of the tyrosine kinome was possible in human tumors [1], we initiated a "Lung Cancer Kinome Project" with the following hypotheses: (1) some NSCLCs are addicted to signaling through one or more kinases; (2) TKIs such as gefitinib or erlotinib block this signaling, inducing tumor responses; and (3) mutations in kinases may be the cause of abnormal signaling. Thus we set out to perform mutational profiling of the lung cancer tyrosine kinome, analyzing drug-sensitive versus drug-refractory tumors as well as untreated tumors.

Our initial pilot project involved analyzing DNA for mutations from three paraffin-embedded tumors derived from patients "enriched" for possible genetic lesions. Thus, these patients were female never smokers with adenocarcinoma histology, who had large tumor blocks available for analysis and peripheral blood DNA banked as a source of "normal" germline DNA. Because the putative selective target of gefitinib and erlotinib was the EGFR, we decided to analyze the *EGFR* gene first. One of these patients had a multinucleotide in-frame deletion in exon 19, just adjacent to a critical lysine residue necessary for ATP-binding, and the other two patients had a point mutation in exon 21 leading to substitution of arginine for leucine at position 858 (L858R). All mutations were somatic.

EGFR mutations and sensitivity to EGFR TKIs

Following publications from the Massachusetts General Hospital and the Dana Farber Cancer Institute, both Cambridge, MA, USA [19, 24], we confirmed and extended the data on mutations associated with responses to gefitinib, and further demonstrated that the same types of mutations were associated with responses to erlotinib [25]. Our analysis of exons 2–28 in 96 untreated resected NSCLCs found mutations only in the kinase domain. Such mutations were much more common in never smokers with adenocarcinoma versus former or current smokers with NSCLC, and mutated tumors demonstrated features of BAC, consistent with the previously identified clinical predictors of response noted above. Since 2004, analyses of thousands of lung tumors examined for EGFR kinase domain mutations have further confirmed and extended these findings [13, 16, 19, 20, 24, 25, 30, 31, 34]. We have also performed a detailed analysis of smoking history in patients; in addition to never smokers, patients with lung adenocarcinoma who had smoked for less than 15 pack-years and quit smoking more than 25 years prior to diagnosis may also have a higher chance of having tumors with *EGFR* mutations [28].

Many mutations have now been reported in exons 18–21 in *EGFR*, including point mutations and small multinucleotide in-frame deletions or insertions. Up to 90% of such mutations are localized in two areas: the deletions in exon 19 that eliminate the four amino acids, LREA, and the exon 21 L858R point mutations. The overwhelming clinical and biochemical evidence suggests that among the various *EGFR* kinase domain mutations reported, there are only four that should be considered to be associated with sensitivity to gefitinib and erlotinib: G719X; exon 19 deletions; L858R; and L861Q [11]. Clinical testing for *EGFR* mutations is now widely available in the USA [9].

Although exon 19 deletions and L858R point mutations are both activating mutations, we and others have recently observed that the clinical outcome of patients on either gefitinib or erlotinib with the different types of mutations is distinct [22, 29] (and reported by Dr Bruce Johnson at this meeting). Analysis of 34 patients with mutations on EGFR TKI demonstrated a median survival of 34 months for patients with exon 19 deletions ($n = 23$) versus 8 months for patients with L858R ($n = 11$) ($P = 0.011$). The molecular basis for this clinical observation remains to be elucidated, but a difference in survival and response on a TKI with different types of mutations has also been observed in patients with gastrointestinal stromal tumors (GISTs) receiving imatinib [12].

Multiple reports have also recently suggested the importance of *EGFR* copy number analysis in tumors as a predictor of overall survival for patients on gefitinib [2, 32]. Full discussion of these data, are outside the scope of this report.

KRAS mutations and primary resistance to EGFR TKIs

Since the majority of patients do not respond dramatically to gefitinib or erlotinib, and since some patients whose tumors are reported to have wild-type EGFR do respond to treatment, we next investigated whether we could find molecular markers predictive of primary resistance to these drugs. As a first step, we examined the status of *KRAS* (exon 2) mutations in drug-sensitive versus drug-refractory tumors, since (1) KRAS is a known downstream signaling molecule in the EGFR pathway, and (2) *KRAS* mutations had previously been reported to occur in 15–30% of lung adenocarcinomas. Our analysis of 60 tumors revealed that *EGFR* mutations were found only in sensitive tumors while *KRAS* mutations were found only in resistant tumors [27]. Since *EGFR* and *KRAS* mutations are, with some rare exceptions, mutually exclusive [16, 30], and since others have reported similar findings [5, 7], *KRAS* mutation testing in conjunction with *EGFR* mutation testing could be used to help guide treatment decisions regarding the use of gefitinib or erlotinib (Table 1).

Secondary resistance

Unfortunately, virtually all patients who initially respond to erlotinib and gefitinib eventually develop progression of disease. Shortly after the identification of lung-specific *EGFR* mutations, we initiated a clinical trial to re-biopsy patients who, after an initial response to either drug, developed progressive disease. Our molecular analysis of the first five of these patients with acquired resistance demonstrated that, in addition to a primary drug-sensitive mutation in *EGFR*, two of five patients had a secondary mutation in exon 20, which leads to substitution of methionine for threonine at position 790 (T790M) in the kinase domain [26]. (Tumor cells from a sixth patient with a drug-sensitive *EGFR* mutation whose tumor progressed on adjuvant gefitinib after complete resection also contained the T790M mutation.) In all instances, this mutation was not detected in untreated tumor samples. Thus far, collectively, seven of ten patients with acquired resistance on EGFR-TKIs display the T790M mutation [10, 14, 15, 18]. In untreated NSCLC patients, the incidence of the T790M mutation is <0.1% [26], suggesting that treatment with gefitinib or erlotinib confers a selective growth advantage to tumor cells harboring the T790M change. Interestingly, this mutation is analogous to mutations found in other kinases (i.e. ABL, PDGFR, and CKIT) targeted by a related kinase inhibitor, imatinib (used in chronic myelogenous leukemia (CML), hypereosinophilic syndrome, and GIST, respectively). Since this specific threonine appears to be critical for the binding of competitive inhibitors to the ATP-binding pocket of various kinases, it has been dubbed a "gatekeeper" residue [4].

Table 1 Using molecular diagnostics to help guide treatment decisions regarding the use of gefitinib or erlotinib

Scenario	*EGFR* mutation	*KRAS* mutation	Treatment
1	+	–	Gefitinib or erlotinib
2	–	–	Trial of drug not excluded
3	–	+	Alternative agents
4	+	+	Extremely rare

Overcoming acquired resistance

In our studies, we found serendipitously that one NSCLC cell line, H1975, contained both the drug-sensitive L858R and the drug-resistant T790M mutations. In collaboration with Ambit Biosciences, San Diego, CA, USA, we screened 47 known kinase inhibitors for their ability to inhibit proliferation of the H1975 cells lines and identified three irreversible EGFR inhibitors—CI-1033, CL-387,785, and EKB-569—that could overcome acquired resistance, at least in vitro [3]. Similar findings were reported in [15, 18]. Based on these studies, a related compound, HKI-272, is being studied in part, specifically to determine whether it has activity for patients with acquired resistance to either gefitinib or erlotinib.

Future directions

Gefitinib and erlotinib have provided an unexpected tool to dissect clinically relevant molecular subsets of NSCLC. In the past 2 years, we have learned that primary *EGFR* kinase domain mutations are associated with drug sensitivity, that *KRAS* mutations are associated with primary resistance, and that second-site *EGFR* mutations are associated with acquired resistance.

Based upon these findings, we not only have molecular tests available in the clinic to help guide treatment decisions regarding the use of these drugs, but also have identified potential ways to overcome and suppress the development of acquired resistance. We are now using molecular, biochemical, and mouse modeling approaches to help further define molecular subsets of lung cancer that have relevance in the clinic.

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