Epidermal Growth Factor Receptor Mutation Testing in the Care of Lung Cancer Patients

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Abstract

As the literature about epidermal growth factor receptor (EGFR) mutations grows and screening for mutations becomes increasingly integrated into clinical care, it is important to examine how best to do somatic mutational analyses and how best to use the test results in clinical decision making. We began offering mutation screening by comprehensive direct sequence analysis of exons 18 to 24 of the tyrosine kinase domain of EGFR in August 2004 as part of clinical cancer care and protocol therapy at our institutions. All identified potential mutations are confirmed with three to five independent PCRs of the original genomic DNA sample and, if not previously noted in the literature, are compared with the patient’s germ-line DNA to ensure the finding is somatic. We formally analyzed the first 100 patients to undergo EGFR sequence analysis and found that testing was feasible and significantly affected the treatment of patients with non–small cell lung cancer (NSCLC). Patients harboring EGFR mutations were significantly more likely to receive recommendations for therapy with EGFR tyrosine kinase inhibitors (i.e., gefitinib or erlotinib) than patients without mutations. However, negative EGFR test results did not prevent physicians from administering these agents to selected patients. Ideally, a standardized technique for mutation testing could be developed, with demonstrated reproducibility and validity. Clinical trials incorporating molecular diagnostics are ongoing to assess the efficacy of EGFR tyrosine kinase inhibitors as first-line therapy for metastatic NSCLC and as adjuvant therapy for early-stage resected NSCLC. It is likely that mutation testing and other molecular analyses will be most useful in these two clinical situations.

Agents that target the epidermal growth factor receptor (EGFR) have made a major impact on the treatment of advanced non–small cell lung cancer (NSCLC). The small-molecule EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib elicit a dramatic response in some patients with relapsed NSCLC, with an objective response rate of 9% to 19% (1, 2). Patients previously treated with chemotherapy live longer when given erlotinib compared with those given placebo (3). Certain patient characteristics are associated with increased response to TKIs and may guide treatment decisions. These characteristics include adenocarcinoma tumor histology, female gender, nonsmoking history, and Asian race (1–5).

Somatic activating mutations in the tyrosine kinase domain of EGFR also correlate with improved response (6–11) and, in some series, improved survival (12–15) with TKI therapy. Compared with wild-type EGFR, mutant receptors exhibit increased activation following ligand binding as well as enhanced inhibition by TKIs (16, 17). EGFR mutations are more common in patients with adenocarcinoma tumor histology, female gender, nonsmoking history, and Asian race, likely underlying the association of these clinical characteristics with treatment response.

Due to increasing interest on the part of both clinicians and patients, we began offering mutation screening by comprehensive direct sequence analysis of exons 18 to 24 of EGFR as part of clinical cancer care and protocol therapy at our institutions in August 2004. More than 350 patients with NSCLC have been screened in the past year, with 23% of samples positive for EGFR mutations. Recently, we formally analyzed the first 100 patients to undergo EGFR testing to describe the feasibility of mutation testing in NSCLC clinical care, to characterize the patients referred for testing, and to evaluate the effect of mutation status on the clinical decision-making process.

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We did a retrospective cohort analysis of the initial 100 NSCLC patients referred for somatic EGFR kinase domain sequencing at Massachusetts General Hospital (Boston, MA), Dana-Farber Cancer Institute (Boston, MA), and Brigham and Women’s Hospital (Boston, MA) from approximately August 2004 to February 2005. Clinicians could select which patients to refer for testing; however, patients needed to have sufficient tumor specimens available. Samples could be either paraffin-embedded or frozen tissue. Due to the low incidence of EGFR mutations in squamous cell tumors (18), patients with this diagnosis were not eligible for testing. Sequencing was done and interpreted at the Laboratory for Molecular Medicine of the Harvard Medical School/Partners HealthCare Center for Genetics and Genomics (Boston, MA; CLIA# 22D1005307).

Patient age, gender, and race were collected from the electronic medical record system. Smoking status, cancer history, EGFR kinase domain sequencing results, and subsequent TKI treatment plans were documented using structured physician chart review. Former smokers were defined as having quit smoking at least 1 year before their diagnosis of NSCLC and never smokers were defined as having smoked <100 cigarettes in their lifetime. Pack-years of smoking were calculated by multiplying the number of packs smoked daily by the number of years of smoking. Tumor histology and EGFR kinase domain sequencing results were obtained from pathology reports. All pathology specimens were reviewed by pathologists at either Massachusetts General Hospital or Brigham and Women’s Hospital, and histology was categorized using the WHO classification system (19). This study was approved by our Institutional Review Board.

Among the first 100 patients with NSCLC referred for somatic EGFR kinase domain sequencing as part of clinical cancer care, the mean age was 60.7 years and 63% were female. The majority of patients were white (76%) and had metastatic disease at the time the test was ordered (66%). Nearly all patients (94%) tested for EGFR mutations had adenocarcinoma, adenocarcinoma with bronchioloalveolar carcinoma features, or pure bronchioloalveolar carcinoma. Approximately one third of the patients were never smokers (29%).

The average length of time from referral for testing to result availability was 12 business days. The majority of specimens submitted were paraffin embedded (74%). Six of the 74 (8%) paraffin-embedded specimens failed PCR amplification, whereas all of the 26 frozen specimens were successfully amplified. Among the 94 patients with interpretable results, 23 (24%) were found to have at least 1 mutation in the EGFR kinase domain, with 2 of these patients showing 2 point mutations each, for a total of 25 mutations identified. Among the 23 patients with mutations, 9 (39%) had one or more point mutations, including 5 (22%) with the substitution mutation 2573T>G (L858R), 12 (52%) had in-frame overlapping deletions in exon 19, and 2 (9%) had duplications in exon 20. As has been seen in other series, exon 19 deletions and the L858R point mutation in exon 21 accounted for the majority of mutations. No mutations were detected in exons 22, 23, or 24.

In our sample, the only clinical characteristic that predicted mutations was smoking status. None of the 17 current smokers were found to have a mutation. Never smokers were significantly more likely to have an EGFR mutation than former smokers (odds ratio, 3.08; 95% confidence interval, 1.09-8.76). The mean number of pack-years smoked was significantly lower among EGFR mutation-positive patients (0.7 pack-years) compared with EGFR mutation-negative patients (25.0 pack-years; P < 0.001). For each additional pack-year smoked, there was a 4% decrease in the likelihood of having a mutation (odds ratio, 0.96; 95% confidence interval, 0.93-0.99). The number of pack-years of smoking remained a significant predictor of mutation status after controlling for gender, race, and tumor histology (odds ratio, 0.96; 95% confidence interval 0.93-0.99).

Medical records of EGFR mutation-positive patients were significantly more likely to include documented plans to receive subsequent TKIs (83%) than EGFR mutation-negative patients (11%; P < 0.001). Clinicians documented that the EGFR results affected their prioritization of recommended therapies in 40% of all cases. Mutation status was more likely to change prioritization of treatment options in patients with metastatic disease (54%) than in local or locally advanced disease (19%; P = 0.002). Given this finding, we further analyzed the observed treatment patterns in patients with metastatic NSCLC (Fig. 1). Among the 31 patients with metastatic NSCLC whose test results affected treatment recommendations, 5 mutation-positive patients were offered first-line TKIs and 5 mutation-positive patients were offered second-line TKIs in lieu of chemotherapy. Twenty-one mutation-negative patients were encouraged to defer TKIs until third-line treatment or beyond based on their negative EGFR test results. Among the 26 patients with metastatic disease whose test results did not affect treatment recommendations, 2 mutation-negative patients received first-line TKIs despite their negative results (1 through a clinical trial and 1 unfit for chemotherapy), 9 patients, including 4 mutation-positive patients, received second- or third-line TKIs, and 15 patients, including 2 mutation-positive patients, did not receive TKIs (1 mutation-positive patient expired before treatment and 1 refused). Three of the patients with metastatic disease were participating in trials evaluating first-line TKI therapy. Ten of the patients with metastatic disease had previously received or were receiving TKIs at the time of EGFR testing.

Implications of Clinical EGFR Testing

As the literature about EGFR mutations grows and screening for mutations becomes increasingly integrated into clinical care, it is important to examine how best to perform somatic mutation analyses and how best to use the test results in clinical decision making.

We perform our mutation analysis with the following procedure. Serial sections of either frozen or formalin-fixed, paraffin-embedded tumor tissue are cut and placed on a glass slide, and an appropriate region of tumor tissue is identified by a pathologist. Formalin-fixed, paraffin-embedded samples are extracted with xylene and ethanol to remove paraffin. Both formalin-fixed, paraffin-embedded and frozen tissue samples are digested with proteinase K overnight. Genomic DNA is extracted from tissue and peripheral whole blood using standard procedures.
The kinase domain of EGFR (exons 18-24 and flanking intronic regions) is amplified in a set of individual nested PCRs. The primers used in the nested PCR amplifications have been described previously (7), with the addition of universal linker sequences to the 5'-ends of the internal primers used for sequencing (forward, 5'-tgtaaaacgacggccagt; reverse, 5'-aacagcatgtgaccatg). PCR is done in a 384-well plate in a volume of 15 μL containing 5 ng genomic DNA, 2 mmol/L MgCl₂, 0.75 μL DMSO, 1 mol/L betaine, 0.2 mmol/L deoxynucleotide triphosphate, 20 pmol primers, 0.2 μL AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 1× buffer (supplied with AmpliTaq Gold). Thermal cycling is done in two cycles under the following conditions: 95°C for 10 minutes; 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute for 30 cycles; and 72°C for 10 minutes. PCR products are purified with AMPure magnetic beads (Agencourt, Beverly, MA).

The PCR products are directly sequenced bidirectionally by dye terminator sequencing. Sequencing products are purified using CleanSEQ magnetic beads (Agencourt) and separated by capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems). Sequence analysis is done by Mutation Fig. 1. Observed treatment patterns for patients with metastatic NSCLC undergoing EGFR mutation testing.
Surveyor (SoftGenetics, State College, PA) and manually by two reviewers.

Because the tumor samples obtained for testing are inherently variable in size and are usually a mix of tumor and other cell types, the sensitivity of mutation detection may vary from sample to sample. By mixing genomic DNA obtained from tissues with normal genotype with those of heterozygous genotype, we have determined that a heterozygous mutation can be reliably detected by our methodology if it represents 20% of the total signal present in the sample. Therefore, tissue samples with <40% tumor cells and heterozygous mutations have a risk of yielding false-negative results. Although more sensitive methodologies for mutation detection exist, the biological relevance of mutations present in very low percentages has not yet been established.

The tumor samples obtained often yield very little intact genomic DNA. Low copy number DNA template has been shown to produce PCR error (20). In addition, formalin fixation has been shown to produce artifacts during PCR amplification that are detectable during DNA sequence analysis with an error rate as high as 1 in 500 bases (21, 22). All of the DNA sequence variants we have observed have been confirmed with three to five independent PCRs of the original genomic DNA sample. This is essential to ensure that DNA sequence variants detected are truly represented in the tumor tissue sample examined.

Although response data have been reported for >29 EGFR kinase domain mutations, we continue to uncover new variants. When a novel mutation is detected (one for which no response data have been reported), we sequence genomic DNA derived from a normal tissue sample from the individual to determine whether the variant is unique to tumor tissue. We have identified one patient in whom a novel variant was present in both the tumor and the normal tissue (P848L). The patient was a never-smoking female with adenocarcinoma, who had stable disease for 15 months on gefitinib treatment and ultimately developed progressive disease around the time she underwent EGFR testing. This variant detected in her case may represent a rare normal polymorphism and not an activating mutation. Additionally, the recently described T790M mutation is associated with resistance to TKIs (23, 24), showing that not all somatic EGFR mutations behave similarly. For these reasons, novel variants should continue to be interpreted with caution.

Regarding the use of mutation testing in clinical decision making, we studied the first 100 patients with NSCLC to undergo EGFR screening as part of clinical cancer care at our institution and found that testing was feasible and could be used as a factor in clinical decision making. This cohort of patients was all tested immediately after EGFR mutations were discovered to be associated with dramatic response to EGFR TKI therapy and before significant further research was available. Patients harboring EGFR mutations were significantly more likely to receive recommendations for TKIs than patients without mutations. Physicians adjusted their treatment recommendations based on the test results in nearly half of the cases and were more likely to do so in patients with metastatic disease. In our patient sample, physicians used positive EGFR test results to help make the decision to prioritize TKIs over chemotherapy for some patients, especially for first- or second-line treatment. However, negative EGFR test results did not prevent physicians from administering TKIs to selected patients. Some clinicians viewed the test results as experimental and made clinical decisions without regard to the mutation results.

We continue to learn from the collective experience using EGFR mutation analysis in the clinical care of patients. However, there are still crucial questions to be answered before we can prescribe the optimal method of integrating molecular diagnostics into clinical decisions. Which subgroup of patients is most likely to benefit from TKIs? EGFR mutations are most widely associated with potential TKI benefit in the literature, but there is also interest in examining EGFR amplification and EGFR protein expression as biomarkers for tumors that are dependent on EGFR activation (11, 25). Retrospective analyses and prospective studies are ongoing to try to understand the predictive role of each of these biomarkers.

Secondly, at what point in the course of NSCLC treatment should TKIs be used for the subgroup deemed likely to benefit? Neither gefitinib nor erlotinib has benefited unselected patients with advanced NSCLC when used as first-line treatment in combination with chemotherapy (26–29), but either TKI alone may be effective in a patient population selected for EGFR mutations or clinical characteristics of responsive disease. For example, a subset analysis of never smokers receiving first-line chemotherapy with or without erlotinib in a randomized trial showed increased survival of 22.5 months with erlotinib compared with 10.1 months with placebo (P = 0.01; ref. 29). Similarly, a phase II study of gefitinib as first-line treatment in a clinically enriched Korean population of never-smoking patients with metastatic adenocarcinoma (30) showed a promising response rate of 69% and an estimated 1-year overall survival rate of 73%. Median survival has not yet been reached at a median follow-up of 11 months. To estimate the response rate in a molecularly defined population, a multicenter phase II study using gefitinib as first-line therapy in patients with advanced NSCLC known to harbor somatic EGFR mutations is now under way in the United States. Additional ongoing studies in the United States include first-line TKI treatment in a population defined by clinical characteristics and phase II and III clinical trials of TKIs in combination with chemotherapy as adjuvant and neoadjuvant therapy for early-stage disease. These types of studies will provide needed evidence about the clinical benefit of TKIs in rationally selected populations.

Based on the accumulating international experience with mutation testing, we suspect that mutational status may be most useful in patients with newly diagnosed, treatment-naive metastatic NSCLC and in patients considering adjuvant therapy for early-stage NSCLC. Currently, at Massachusetts General Hospital and Dana-Farber Cancer Institute, we encourage patients to participate in clinical trials that incorporate molecular profiling of tumors whenever possible. Outside the setting of clinical trials, we are not routinely offering TKIs as part of adjuvant therapy but do frequently obtain EGFR sequencing in patients with metastatic disease and encourage early use of EGFR TKIs to those who are mutation positive.

Conclusions

Translation of the molecular biology underlying NSCLC into the clinical decision-making process of caring for NSCLC patients has advanced tremendously in the last year. As further
research clarifies the clinical implications of identifying mutations in EGFR and other alterations, such as KRAS mutations and EGFR amplification, a new paradigm for decision making may emerge. However, at this time, data are not sufficient to make such testing the standard for clinical practice. General acceptance of novel treatments or biomarkers appropriately requires a certain weight of evidence. This is exemplified by the HER-2/neu story in breast cancer, as the initial report correlating amplification of HER-2/neu with prognosis was published in 1987, but the guidelines of the National Comprehensive Cancer Network and the American Cancer Society did not recommend HER-2/neu testing for all newly diagnosed patients with invasive breast cancer until 2002 (31, 32).

Nevertheless, our experience shows the feasibility and use of comprehensive screening of the tyrosine kinase region of EGFR for somatic mutations as part of clinical NSCLC care. The result of the test provides useful information that adds to the previously described clinical predictors of TKI response and significantly influenced clinical decisions for patients in our series. Current smokers are less likely to harbor a mutation, as are former smokers with a high number of pack-years of smoking history. Further research is needed to more completely define the appropriate population for EGFR testing and to determine the effect of the results on patient care.

Open Discussion

Dr. Thomas Lynch: So it is 3 to 4 weeks to get a test result. Are there faster ways to do this? Do we have to be sequencing exons 18 to 24? Are there ways of turning the test around within a timeframe that is more reasonable from a clinical care standpoint?

Dr. Paul Bunn: It is counting the FISH and the immuno-histochemistry, that’s what takes the time. Mutational analysis is not the hold up. You put it on your sequencer and run your sequencer overnight.

Dr. Matthew Meyerson: You can run your sequencer overnight, but you are not going to get accurate results because they are not validated. I think that time is really making sure it is done accurately.

Dr. Kwok-Kin Wong: I think the rate-limiting factor is actually finding the pathologic samples to send out. It’s not the actual PCR sequencing that is taking up most of the time.

Dr. Sequist: There are different rate-limiting steps depending on the patient. Sometimes there is a hold up in the pathology department, sometimes the sequencing has to be done twice for confirmation.

Dr. Daniel Haber: If you decide to have a first-line test looking for the two hotspot mutations, then you might accelerate the process tremendously. If you count the fraction of mutation-negative patients who do respond, the fraction of mutations that you miss by sequencing, all together that may be a comparable number to the novel mutations outside of those two hotspots. So aiming to identify 80% of mutations in a first pass with a relatively rapid turnaround makes a lot of sense.

Dr. Lynch: About 5 months ago, we had a meeting to ask whether we were ready to move to a system like that to look for mutations. Dr. Jänne, at the time you were someone who felt we were not ready. You still felt there was value to sequencing exons 18 to 24. Where are you now on that question?

Dr. Pasi Jänne: If you can test for common mutations and exclude people quickly for whom you don’t need to do further sequencing, that would be very valuable. I’m not sure at this point that we need to sequence beyond exon 21. That was one of the issues that was discussed: there is no convincing evidence of mutations in 22 or 23.

Dr. Sequist: In the 350 patients who have been tested in our institutions, we haven’t found anything in those higher exons either.

Dr. Glenwood Goss: I know this runs in the face of science, but just putting the patient on the drug will give you your answer as quickly. The majority of them will respond within 28 days. That is how long it is going to take you to get your test.

Dr. Lynch: There is some validity to that thinking. The flip side of it says, do you then miss the chance to put them on the carboplatin, paclitaxel, and bevacizumab regimen, what I call the "Sandler" regimen? Are you concerned that you may miss a chance to give someone a 12-month survival by putting them on an ineffective therapy for a month?

Dr. Goss: If you have clinical trials open, you should always enroll to the clinical trials, definitely. In front-line therapy, it is still an open question. But in the setting of third-line or fourth-line treatment, is it necessary to go and sequence these patients, when you haven’t got a lot of other options to the EGFR inhibitors? Now I realize that it contributes to the body of scientific information, but on a practical level, is there a reason to test?

Dr. Sequist: Many of the tests in this series were done in the setting of second-line treatment or beyond because it was a brand new opportunity and there was a lot of research interest in our group. Going forward, I think that testing will not have a role in second or third line. This is really more for first line.

Dr. Lynch: For the clinicians here, I would like your thoughts on what a clinically acceptable timeframe would be for a diagnostic test to choose a first-line therapy.

Dr. Panos Fidias: A week.

Dr. Rogerio Lilenbaum: I would feel comfortable with 2 weeks, perhaps a little longer. This is actually a refined version of a window-of-opportunity trial based on a molecular test. We have seen those in advanced non–small cell lung cancer before. In most of those studies, unlike small cell lung cancer, there was not a huge difference in outcome. I would not feel uncomfortable waiting 2 weeks for most of my patients, especially if they have a good performance status and they don’t have rapidly progressive disease-related symptoms.

Dr. Lynch: I don’t either. We have noticed with Dr. Sequist’s study, the TARGET trial, that we are putting people on trial who are selected for a better prognosis just by the fact that the physician is willing to wait a month for the test. So we are going to see a better survival than one would expect.

Dr. Lilenbaum: Because those who can’t wait, you know right away.

Dr. Lynch: Those who can’t wait are going to get the Sandler regimen right away.

Dr. Alan Sandler: Right, and it’s going to make mine look bad!
References


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