Detection of Oncogenic Mutations in the EGFR Gene in Lung Adenocarcinoma with Differential Sensitivity to EGFR Tyrosine Kinase Inhibitors

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The complete sequencing of the human genome and the development of molecularly targeted cancer therapy have promoted efforts to identify systematically the genetic alterations in human cancer. By high-throughput sequencing of tyrosine kinase genes in human non-small-cell lung cancer, we identified somatic mutations in the kinase domain of the epidermal growth factor receptor tyrosine kinase gene (*EGFR*) that are correlated with clinical response to EGFR tyrosine kinase inhibitors (TKIs). We have shown that these mutant forms of *EGFR* induce oncogenic transformation in different cellular systems. Cells whose growth depends on *EGFR* with mutations in exons 19 and 21 are sensitive to EGFR-TKIs, whereas cells expressing insertion mutations in exon 20 or the T790M point mutant, found in tumor biopsies from patients that relapsed after an initial response to EGFR-TKIs, are resistant. Furthermore, by applying a novel, massively parallel sequencing technology, we have shown that clinically relevant oncogene mutations can be detected in clinical specimens with very low tumor content, thereby enabling optimal patient selection for mutation-directed therapy. In summary, by applying high-throughput genomic resequencing, we have identified a novel therapeutic target, mutant EGFR, in lung cancer and evaluated its role in predicting response to targeted therapy.

Lung cancer is the major cause of cancer-related deaths in the western world. In 2005, an estimated 163,510 Americans have died from lung cancer (Jemal et al. 2005). Although numerous novel drugs have been introduced, overall lung-cancer-specific survival for all patients has only increased by 5% in the last 30 years, supporting the notion that chemotherapy is largely ineffective against this cancer type. After 5 years, less than 15% of patients with lung cancer are still alive (Jemal et al. 2005). Lung cancer is generally subcategorized into small-cell lung cancer (SCLC, ~15% of cases) and non-small-cell lung cancer (NSCLC, ~85% of cases) comprising adenocarcinoma, squamous-cell carcinoma, large-cell carcinoma, and undifferentiated carcinoma (Brambilla et al. 2001). Adenocarcinoma is the most frequent subtype, followed by squamous-cell carcinoma.

The efforts to determine novel cancer-causing gene mutations in lung cancer were inspired by the clinical success of small-molecule inhibitors of tyrosine kinases. Almost all patients with chronic myeloid leukemia (CML) achieve complete remission by administration of imatinib, an inhibitor that is active against the BCR-ABL kinase—the fusion protein resulting from the translocation causing CML. This observation has created a paradigm that is the basis for many cancer genomics projects: Find a genetic lesion and find the agent that is active against the lesion (Druker et al. 1996, 2001a,b). This paradigm has been strengthened by the detection of mutations in genes encoding kinases that were strongly associated with clinical response to inhibitors targeting the respective kinase (Hirota et al. 1998, 2003; Demetri et al. 2002; Cools et al. 2003; Heinrich et al. 2003; Fiedler et al. 2005; Stone et al. 2005). The development of mutation-targeted therapy has necessitated systematic gene resequencing efforts in cancer. The complete sequencing of the human genome and the concurrent development of technologies that allow high-throughput generation of genomic data have opened avenues for such systematic resequencing approaches (Risch 2000; Lander et al. 2001; Venter et al. 2001).

In lung cancer, systematic resequencing efforts led to the discovery of mutations in the BRAF gene in approximately 2% of primary lung adenocarcinomas (Brose et al. 2002; Davies et al. 2002; Naoki et al. 2002; Tuveson et al. 2003) and in PIK3CA, the gene encoding the p110α subunit of the phosphoinositol-3 kinase (Samuels et al. 2004). Further analysis of the most frequent PIK3CA mutants, among them the E545K, revealed that these mutations are in fact oncogenic in vitro (Kang et al. 2005).

Recently, small-molecule inhibitors targeting the epidermal growth factor receptor (EGFR) tyrosine kinase were introduced for second-line treatment of NSCLC. These inhibitors (further referred to as EGFR TKIs), erlotinib and gefitinib, specifically target the EGFR tyro-

sine kinase (Wakeling et al. 2002; Grunwald and Hidalgo 2003), with estimated 100-fold lower activity against other kinases. The introduction of EGFR TKIs was mainly based on the detection of recurrent overexpression of the EGFR protein in NSCLC (Rusch et al. 1993). In fact, results from phase II clinical trials showed responses in 10% of Caucasian and approximately 30–40% of Japanese patients treated with EGFR TKIs (Fukuoka et al. 2003; Kris et al. 2003; Soulieres et al. 2004). However, responses were not correlated with EGFR expression status because EGFR is overexpressed in almost all patients with NSCLC (Hirsch et al. 2003), indicating that the target of EGFR TKIs in the patients with responses was not the overexpressed EGFR protein. In contrast, clinical responses were mostly seen in Asian patients, in patients with adenocarcinoma, in women, and in never-smokers (Fukuoka et al. 2003; Kris et al. 2003; Miller et al. 2004). Despite the fact that several hundred thousand patients had been treated throughout the world by 2004, the actual target of EGFR TKIs remained elusive.

DETECTION OF EGFR KINASE DOMAIN MUTATIONS IN LUNG CANCER BY SYSTEMATIC KINASE GENE RESEQUENCING

When we systematically resequenced the juxtamembrane domains and the activation loops within the kinase domains of all receptor tyrosine kinases in NSCLC, we detected heterozygous mutations in the EGFR kinase domain in 16 of 119 unselected NSCLC samples (Paez et al. 2004). Included were 58 samples of Japanese origin and 61 samples of U.S. origin; 70 of the 119 samples were adenocarcinomas. We detected a mutation in the glycine-rich nucleotide triphosphate-binding P loop encoded by exon 18 leading to substitution of glycine 719 with serine (G719S), small heterozygous in-frame deletion mutations in exon 19 affecting the residues ELREA and nearby residues, and a heterozygous mutation leading to substitution of leucine 858 located directly adjacent to the DFG motif of the activation loop, with arginine (L858R). Interestingly, all types of mutations that we detected were located around the active site of the kinase, suggesting that they might affect catalytic activity. The residues affected by substitution mutations were all highly conserved within the protein kinases, also pointing toward a role in malignant transformation. Furthermore, all mutations were somatic, because they were detected only in the tumors and not in the corresponding normal DNA. These results imply that somatic mutations in the EGFR gene underlie malignant transformation in a subset of NSCLC patients. Numerous other reports have added to these initial findings and the distribution of different EGFR mutations. Approximately 45% of all EGFR mutations are exon 19 deletions, 40% are L858R mutations, 10% are in-frame duplications/insertions in exon 20, and 5% are other mutations (including G719S) (Huang et al. 2004; Kosaka et al. 2004; Marchetti et al. 2005; Shigematsu et al. 2005). In addition, the mutation frequencies vary significantly depending on ethnicity; whereas the frequency of EGFR gene mutations appears to be roughly 10% in Caucasian patient populations, in East Asian patients this number is about 30% (Jänne et al. 2005; Shigematsu et al. 2005). The reason for these differences remains elusive. It is conceivable that differences in exposure to environmental factors may act in concert with polymorphisms inherent to different genetic backgrounds.

Mutations were more frequent in adenocarcinomas, in women, in never-smokers, and in patients with East Asian ethnicity (Jänne et al. 2005). Interestingly, these features match those of patients who responded to EGFR TKI treatment in initial phase II trials (Fukuoka et al. 2003; Kris et al. 2003; Miller et al. 2004; Perez-Soler et al. 2004). We therefore sequenced the EGFR kinase domain in patients showing dramatic responses to gefitinib and in non-responders. Importantly, we detected mutations in 5/5 responders, but in none of 4 non-responders ($p = 0.0027$) (Paez et al. 2004). These results indicate that patients whose tumors harbor a mutation in the EGFR kinase domain have a high chance of experiencing at least a partial response (reduction of the tumor volume of at least 50%). Findings reported from other groups investigating EGFR gene mutations in EGFR TKI responders are in line with our findings, as they also found mutations in responders but not in non-responders (Lynch et al. 2004; Pao et al. 2004). Although the patients analyzed in these studies were highly selected (i.e., patients showing progressive disease were analyzed in the control group but no patients experiencing disease stabilization), results from several other studies are supporting these initial findings (Huang et al. 2004; Han et al. 2005; Mitsudomi et al. 2005; Tokumo et al. 2005).

Most recently, it was found that EGFR copy number changes as determined by fluorescence in situ hybridization (FISH) and even EGFR protein expression may be better predictors of survival (Cappuzzo et al. 2005). Several factors should be taken into consideration when examining these data: A survival advantage may also exist for the patients experiencing stable disease, whereas EGFR mutations were found in patients experiencing major, occasionally dramatic, responses. Additionally, it was found that EGFR copy number changes are correlated with the presence of mutations (Tracy et al. 2004; Amann et al. 2005; Cappuzzo et al. 2005). Thus, the cases with amplification in the EGFR gene might also harbor yet unidentified mutations. Conversely, it might also be the wild-type allele that is amplified followed by subsequent mutation, and, thus, the mutant allele might have escaped detection due to the limited sensitivity of Sanger dideoxy sequencing. Finally, in most cases, sequencing is being performed on paraffin-embedded archival specimens. Because in most studies EGFR TKIs have been used at a very late clinical stage, the question remains whether these analyses actually represent the tumor cell clone that is being treated. Future prospective studies will help to determine which molecular alteration is most stringently associated with response to EGFR TKIs.

RELAPSE TO EGFR TKIs IS ASSOCIATED WITH A SECOND-SITE MUTATION IN THE EGFR GENE

Unfortunately, all NSCLC patients treated with the EGFR TKIs gefitinib or erlotinib will eventually relapse and succumb to their tumor. In CML, acquired resistance to Gleevec was found to be mainly caused by the emergence of secondary resistance mutations in the BCR-ABL kinase domain (Gorre et al. 2001; Shah et al. 2002). In some cases, these mutations were existent prior to treatment, indicating that they contribute to malignant growth and implying that continuous molecular monitoring of a patient's tumor cell clone should help guide the optimal therapy. The resistance mutations in CML fall into three major classes: mutations that impair binding of the inhibitor in the nucleotide-binding pocket of the kinase domain, mutations that impair acquisition of an inactive state (a prerequisite for binding of Gleevec), and mutations that lead to autonomous signaling. Interestingly, one of the residues frequently mutated in CML, T315, is conserved in EGFR (T790) and was shown crystallographically to bind erlotinib via a bridging water molecule (Stamos et al. 2002). When an EGFR expression construct harboring the mutation, T790M, which is analogous to the T315I mutation in CML, was introduced into CHO-K1 cells, they became resistant to EGFR TKI treatment (Blencke et al. 2003). Moreover, Kobayashi and colleagues, in collaboration with our group, sequenced the EGFR kinase domain in a patient who had relapsed after a complete response to gefitinib that lasted for 24 months. Whereas sequencing of the pre-therapy sample revealed the presence of a heterozygous exon 19 deletion (delL747-S752), sequencing of the sample obtained at relapse showed the presence of the T790M resistance mutation in addition to delL747-S752. These results indicated that the T790M is in fact a clinically meaningful resistance mutation (Kobayashi et al. 2005). In this study, too, the T790M resistance mutation rendered transfected cells resistant to gefitinib, underlining the initial findings by Blencke et al. Similar results were also reported by another group which found the T790M mutation in 3/6 patients who suffered acquired or primary resistance to gefitinib or erlotinib (Pao et al. 2005). We have introduced the T790M mutation into murine Ba/F3 cells in combination with a variety of lung cancer-derived *EGFR* mutations, including the various substitution mutations as well as various deletions, to determine the effect on sensitivity to EGFR TKIs (Fig. 1 and data not shown). Figure 1 shows the impact of the T790M mutation on EGFR TKI sensitivity of cells carrying the L858R mutation. Whereas L858R-only cells (dotted lines) were exquisitely sensitive to gefitinib (Fig. 1A) and erlotinib (Fig. 1B), cells harboring both the L858R and the T790M mutations (solid lines) were completely resistant to both inhibitors.

However, treatment with irreversible inhibitors of the EGFR kinase at concentrations that might be achievable in patients effectively killed cells carrying the T790M resistance mutation (Kobayashi et al. 2005; Kwak et al. 2005). One of these inhibitors, HKI-272, is currently in clinical trials.

ONCOGENIC TRANSFORMATION BY INHIBITOR-SENSITIZING EGFR MUTANTS WITH DIFFERENTIAL RESPONSE TO EGFR TKI

We have asked whether the EGFR mutations observed in lung tumor samples were oncogenic, capable of contributing to tumor development (Greulich et al. 2005). A representative mutation from each of the four major categories of EGFR mutations (exon 18 missense substitution, exon 19 deletion, exon 20 insertion, and exon 21 missense substitution) was recreated in the wild-type EGFR cDNA by site-directed mutagenesis and expressed in NIH-3T3 cells. Expression of these four mutants, G719S, L747_E749del A750P, D770_N771insNPG, and L858R, affected cell morphology and supported anchorage-independent growth, focus formation, and tumor formation in immunocompromised mice (Greulich et al. 2005). Anchorage-independent growth was also observed upon expression of the mutant EGFR in the more physiologically relevant human tracheobronchial epithelial cells. The mutant EGFR were shown to be constitutively active and lig-

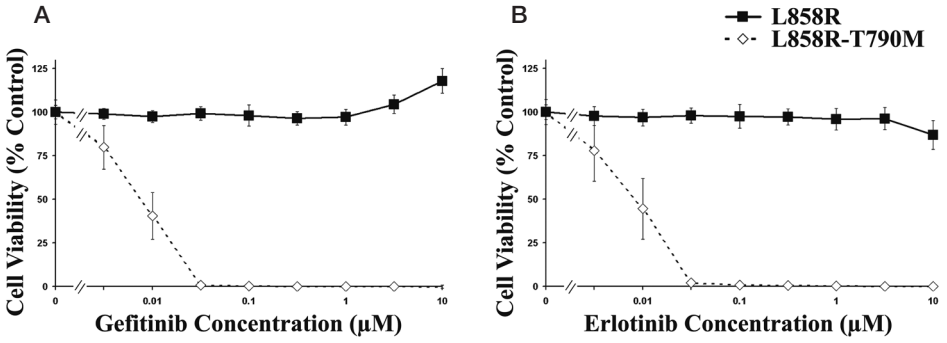

Figure 1. Resistance to EGFR tyrosine kinase inhibitors caused by a second-site mutation in the EGFR kinase domain. The sensitivity of EGFR-transformed Ba/F3 cells to EGFR inhibitors in the absence of IL-3 was assayed using the MTS assay. Percentage of cell viability, as assayed by MTS assay, is shown relative to untreated controls. Results are indicated as mean ± standard deviation. (*A,B*) The L858R-T790M double mutant EGFR (IC_{50} >10 μM) showed very strong resistance to both gefitinib (*A*) and erlotinib (*B*), whereas the L858R mutant EGFR was highly sensitive to both TKIs (IC_{50} ~10 nM).

and-independent, to associate with endogenous Shc, and to constitutively activate the Stat3 and Akt signaling pathways. We have more recently confirmed that other exon 19 deletions and exon 20 insertions, including E746_A750del, L747_S752del,E746V, S752_I759del, V769_D770insASV, and H773_V774insH also support anchorage-independent growth in the murine fibroblast model (Fig. 2 and data not shown).

The sensitivity of transformation to EGFR inhibitors was determined by treating the NIH-3T3 cells with gefitinib, erlotinib, and the irreversible inhibitor CL-387,785. Addition of exogenous EGF to cells expressing the wild-type EGFR causes these cells to become transformed. However, transformation of cells expressing the L858R mutant EGFR was more sensitive to gefitinib treatment than the EGF-stimulated wild-type EGFR-expressing cells (Greulich et al. 2005). Whereas NIH-3T3 cells expressing G719S and L747_E749del A750P behaved similarly to cells expressing L858R EGFR, cells expressing the exon 20 insertion mutant D770_N771insNPG were surprisingly relatively resistant to reversion of transformation by gefitinib and erlotinib (Fig. 3) (Greulich et al. 2005). However, the irreversible inhibitor CL-387,785 was significantly more effective against the insertion mutant than gefitinib and erlotinib, similar to the situation with the previously described T790M resistance mutation, also in exon 20 (Fig. 4) (Greulich et al. 2005).

The sensitivity of lung adenocarcinoma cell lines to gefitinib has also been extensively studied. Functional experiments showed that the H3255 adenocarcinoma cell line harboring the L858R mutation was extremely sensitive to treatment with gefitinib, with an IC_{50} of only 40 nM compared to wild-type cell lines that were growth-inhibited only at concentrations that were 100-fold higher (Paez et al. 2004). Additionally, levels of phosphorylated EGFR, ERK1/2, and Akt were strongly reduced by gefitinib treatment in H3255 cells but not in wild-type cells, implying a role for these proteins in mutant EGFR-dependent cell survival. Similar enhanced apoptosis results were reported for treatment of HCC827, a NSCLC cell line expressing mutant EGFR encoding an exon 19 deletion, with the EGFR-targeted monoclonal antibody cetuximab (Amann et al. 2005). Cos-7 cells genetically engineered to express one of the deletion mutations or the L858R mutation exhibited enhanced EGFR phosphorylation only upon addition of EGF, and this autophosphory-

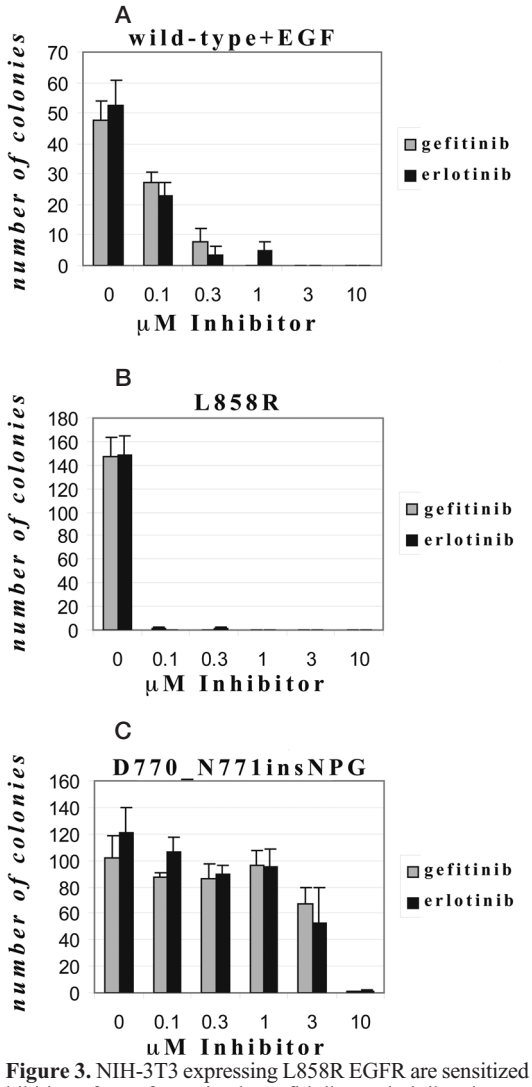

Figure 3. NIH-3T3 expressing L858R EGFR are sensitized to inhibition of transformation by gefitinib or erlotinib, whereas NIH-3T3 expressing an exon 20 insertion mutant are resistant to gefitinib or erlotinib. Panels *A–C* show numbers of colonies of different EGFR mutants following treatment with EGFR tyrosine kinase inhibitors gefitinib and erlotinib. Cells were treated with inhibitors and suspended in soft agar as described previously (Greulich et al. 2005). (*A*) EGF-stimulated cells expressing wild-type EGFR (unstimulated cells expressing the wild-type EGFR are not transformed and do not form colonies in soft agar). (*B*) Unstimulated cells expressing L858R EGFR. (*C*) Unstimulated cells expressing the exon 20 insertion mutant D770_N771insNPG.

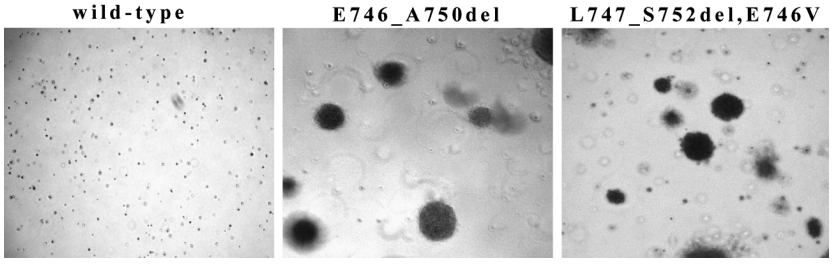

Figure 2. Expression of EGFR exon 19 deletion mutants in NIH-3T3 cells enables cells to grow in an anchorage-independent manner. Wild-type or mutant EGFR was stably expressed in NIH-3T3 cells by retrovirus-mediated gene transfer and suspended in soft agar as described previously (Greulich et al. 2005). Shown are images from soft agar colonies after 14 days of culture.

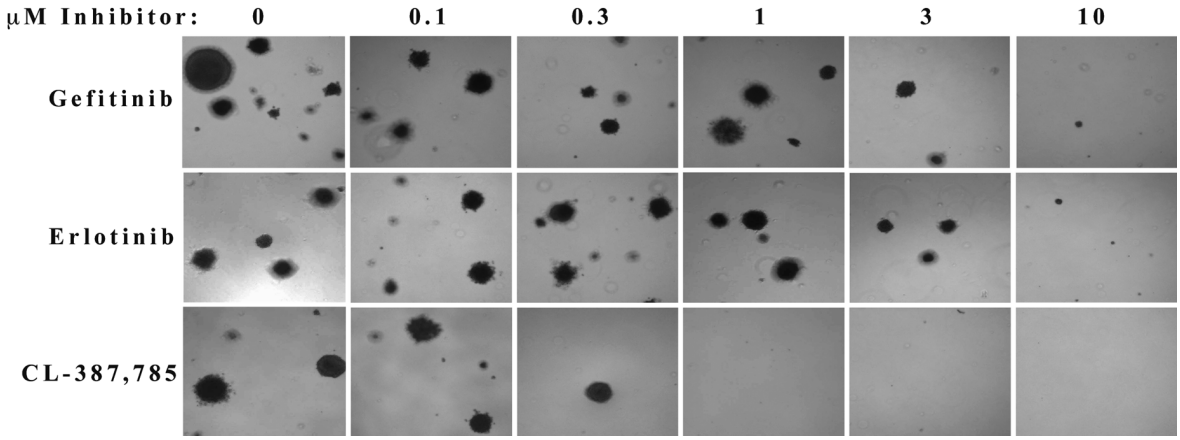

Figure 4. Transformation induced by the exon 20 insertion mutant is more sensitive to the irreversible inhibitor CL-387,785 than to gefitinib or erlotinib. Cells were treated with inhibitors and suspended in soft agar as described previously (Greulich et al. 2005). Shown are images of NIH-3T3 cells engineered to carry the exon 20 insertion mutant after 14 days of culture.

lation was 10-fold more sensitive to gefitinib than EGF-stimulated phosphorylation of the wild-type receptor (Lynch et al. 2004). In a subsequent analysis, Sordella and colleagues showed that lung-cancer-derived EGFR mutations activate the antiapoptotic Akt and STAT pathways, providing a link between these mutations and sensitivity of cell survival to EGFR inhibition (Sordella et al. 2004). This activation of the PI3K/Akt pathway appears to be mediated by ErbB3, a heterodimerization partner of EGFR in NSCLC cell lines (Engelman et al. 2005). Furthermore, by specifically silencing the mutant EGFR allele using RNA interference, Sordella and colleagues demonstrated induction of apoptosis in EGFR mutant but not wild-type cells and, thus, a dependency of NSCLC cells harboring EGFR mutations on this oncogene. It is still an unresolved issue whether the mechanism by which the mutations confer response to EGFR TKIs involves changing the biochemical properties of the kinase, or exploiting oncogene addiction, or some combination of these two mechanisms.

DETECTION OF HIDDEN EGFR MUTATIONS BY MASSIVELY PARALLEL SEQUENCING FROM CLONALLY AMPLIFIED SINGLE MOLECULES

Given the low sensitivity of Sanger sequencing in detecting mutations in clinical samples with low tumor content, other technologies that are capable of interrogating a locus of interest with greater depth are essential. We therefore used a novel ultradeep, massively parallel technology (further on referred to as "picotiter plate sequencing") to search for mutations in the EGFR kinase domain in patients with NSCLC. Picotiter sequencing involves the following steps (Margulies et al. 2005): clonal PCR amplification of single DNA molecules on beads in a water-in-oil emulsion; deposition of the beads into high-density picotiter plates together with enzymes required for pyrophosphate-dependent chemiluminescence signal generation; sequencing-by-synthesis dependent on flow-cell addition of individual nucleotide triphosphates, with concomitant generation of pyrophosphate upon nucleotide incorporation; and detection of the resultant chemiluminescence with a charge-coupled device. Since in excess of 200,000 high-quality reads can be generated during one round of sequencing from one sequencing plate, it is possible to cover a single locus of interest to a depth that allows statistically significant low-level mutation detection. Moreover, given the fact that each sequencing read represents a single template, analyzing complex mutations such as heterozygous deletions could easily be performed.

PCR products were generated covering the EGFR kinase domain from 22 patients with known mutational status. The mutations in this sample set comprised the substitution mutations G719S ($n = 1$) and L858R ($n = 2$), a 15-bp deletion (E746_A750del, $n = 5$), and a 24-bp deletion mutation (S752_I759del, $n = 1$). Traces were generated using 454 sequencing, and sequence variants were scored as real when they appeared in sequencing reads from both directions, when they were present in overlapping amplicons, and when they did not appear in the matched normal. All mutations that we had previously detected by Sanger sequencing could reliably be called by 454 sequencing. In addition to the known mutations, we were also able to detect three mutations that had not been detected by Sanger sequencing. All three mutations appeared at a relative allele frequency of less than 15%. One mutation was an E709A mutation in a sample with a known G719S mutation. Interestingly, this mutation was located on the same allele as the G719S. The other mutations were a 15-bp deletion and a trinucleotide insertion mutation. Analysis of the original Sanger traces revealed a low-level peak in the case of E709A that had been missed by the analysis software in combination with manual scoring and peaks that appeared as noise at the site of the deleted or inserted nucleotides in the case of the 15-bp deletion or the insertion, respectively.

These results encouraged us to search for EGFR kinase domain mutations in clinical specimens with low tumor

content that had been extracted from the pleural effusion of patients with relapse to EGFR TKI. Since many cancer patients present with pleural effusion at time of relapse, and since drainage of the fluid often becomes necessary to assist the patients' vital functions, such material is frequently available without the possibility of sophisticated molecular analysis. The first patient that we tested, a 47-year-old male Caucasian, never-smoker, was originally diagnosed with lung adenocarcinoma in December 2003. He initially responded strongly to erlotinib with more than 50% of his original tumor burden disappearing. This response lasted for 12 months. In April 2005, the patient presented with massive pleural effusion. Drainage of the fluid and pathological examination of the cells in the fluid revealed relapse of lung adenocarcinoma. DNA was extracted from this specimen. A pathology report on this specimen confirmed the rare presence of scattered tumor cells. Using picotiter plate sequencing on this sample, we detected an 18-bp deletion mutation (L747-752del_P753S) in exon 19 at a frequency of approximately 3%. We had originally detected this mutation in a patient responding to gefitinib (Paez et al. 2004), suggesting that this mutation had rendered the patient's tumor cells sensitive to EGFR TKI treatment. Furthermore, picotiter plate sequencing revealed a T790M mutation in exon 20 occurring at a frequency of 2% (Fig. 5). This mutation has been found in tumors explanted from patients experiencing relapse to EGFR TKI (Kobayashi et al. 2005; Pao et al. 2005) suggesting that in the patient studied here, too, this mutation had led to resistance to treatment and to the occurrence of relapse. Importantly, attempts to detect the two mutations by conventional Sanger sequencing failed.

It has now been shown, both from in vitro experiments and from analysis of clinical samples, that the various exon 19 deletion mutations predict response to EGFR TKI whereas the emergence of the T790M mutation leads to acquired resistance to these drugs. Thus, using picotiter plate sequencing, we were able to recapitulate, on a molecular level, the clinical course of a patient who initially responded to EGFR TKI and then relapsed. More importantly, this monitoring was performed on clinical samples with a very low tumor percentage and, in the case of the pre-therapy sample, on a paraffin-embedded sample with extremely low tumor content. These results show that cancer gene mutation detection for both clinical and research purposes may now be feasible without the need for sample purification. Thus, picotiter plate sequencing could significantly help to guide patient selection for tailored treatment on a molecular basis in the near future.

CONCLUSIONS

The discovery of mutations in the EGFR gene in lung adenocarcinoma is a significant advance in this disease. These mutations are oncogenic and are associated with differential clinical response to EGFR TKI. Whereas most of the kinase domain mutations, including exon 19 deletion mutations and the L858R mutation, are associated with responsiveness to EGFR TKIs in vitro and in vivo, one insertion mutation in exon 20 was found to render genetically engineered cells resistant to EGFR TKIs. Another mutation, T790M, was found to emerge during therapy with EGFR TKIs and to lead to resistance. Importantly, irreversible inhibitors of the EGFR kinase were able to overcome resistance by T790M and the insertion mutant. By making use of a novel, extremely sensitive high-throughput sequencing technology, we were able to detect clinically meaningful EGFR kinase domain mutation in samples with very low tumor content. These latter results demonstrate that clinical sequencing is feasible in low-tumor-content samples without the need for sample purification. Thus, the clinical course of lung cancer patients treated with TKIs may now be predicted and monitored using picotiter plate sequencing.

Taken together, our findings have an impact on the understanding of the molecular biology of lung cancer as well as on clinical decision-making. The fact that lung tumors carrying particular EGFR mutations are exquisitely sensitive to EGFR inhibitory strategies (EGFR TKIs and RNAi) suggests that this subset of tumors represents a biologically distinct subentity where mutant EGFR is the

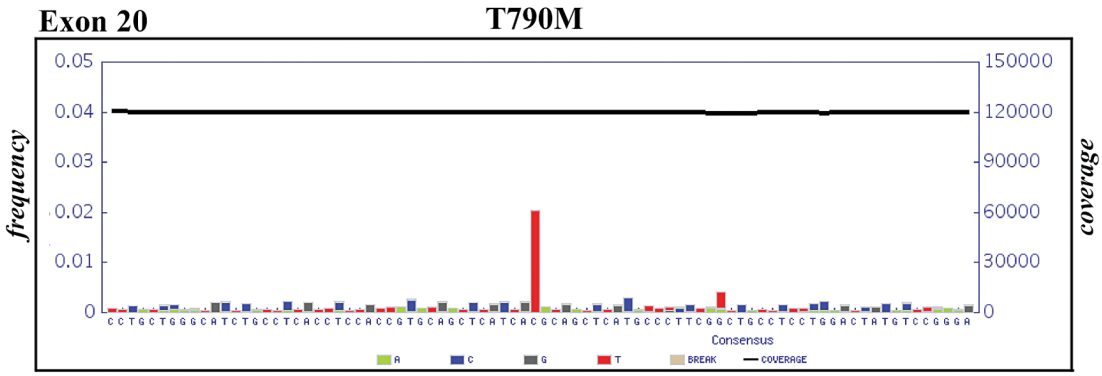

Figure 5. Detection of a T790M resistance mutation in pleural effusion fluid from a lung cancer patient who relapsed after a 1-year response to erlotinib. Shown is a variation plot analysis of a picotiter plate sequencing run of EGFR exon 20 amplified from DNA extracted at the time of relapse from a pleural effusion specimen with low tumor content. Nucleotide reads deviating from the reference signal appear as bars with the height indicating the relative frequency of the variant alleles (left Y-axis). The black bar indicates the coverage at the position of each nucleotide (right Y-axis).

driving oncogene. Apparently, these mutations occur in a specific context (women, non-smokers, adenocarcinomas, etc.), indicating that differential exposure to carcinogens might act in concert with a particular genetic context inherent to certain ethnicities. This observation emphasizes the need to broaden ongoing cancer-genome resequencing efforts to take into account patient ethnicity. Furthermore, EGFR and KRAS mutations were found to occur in a mutually exclusive fashion in lung cancer, suggesting that the net effect on activation of central downstream signaling elements might be equivalent. This might be of importance when considering gene families for resequencing efforts since genetic lesions (e.g., gene mutations, copy number changes) might be present in other, yet unidentified members of the EGFR/KRAS signaling pathway. Clinically, our results suggest that patient selection based on the presence of EGFR mutations might help to increase response rates and overall survival in a subgroup of NSCLC patients. A clinical trial is currently under way at the DFCI/Harvard Cancer Center that aims at prospectively assessing the predictive value of EGFR mutations for response and overall survival in patients with NSCLC treated with EGFR TKIs. Additionally, patients might be monitored on a molecular level to detect the occurrence of resistance mutations at an early time point. The development of a massively parallel sequencing technology, picotiter plate sequencing, that allows in-depth analysis of clinical specimens with low tumor content might help to guide cancer patient selection based on the presence or absence of certain mutations. We anticipate that this technology will permit clinical sequencing not only of lung cancer patients, but also of patients with other tumors that are known to harbor mutations associated with clinical response. We envision that treating a cancer patient based on the genetic makeup of his tumor and switching his treatment to other (e.g., irreversible) inhibitors upon the occurrence of resistance mutations detected early by ultradeep sequencing will become a clinical reality soon.

However, despite these encouraging findings, the overall response rates to these inhibitors are generally low. Thus, other targeted strategies are needed to improve outcome of NSCLC patients. We believe that among the most promising strategies to achieving this goal are genomic approaches such as SNP array-based genome-wide analyses of copy number changes as well as endeavors aiming at resequencing entire cancer genomes in the most common cancer subtypes, including NSCLC. These efforts may lead to identification of more "druggable" mutations in lung cancer. Finally, one even more inspiring conclusion from these findings might be that the discovery of EGFR mutations causing clinical responses to EGFR TKIs, second-site mutations such as T790M causing relapse, and, finally, irreversible kinase inhibitors overcoming resistance are an impressive example of how high-throughput genomic technologies can be successfully applied in collaborative projects involving clinicians, genome biologists, and basic scientists, finally leading to improvement in lung cancer patient care.

ACKNOWLEDGMENTS

Roman Thomas holds a Dr. Mildred Scheel Fellowship for Cancer Research from the Deutsche Krebshilfe. Our own work cited in this paper was supported by the Claudia-Adams Barr Foundation and the Novartis Foundation.

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