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Molecular pathology of lung cancer: key to personalized medicine

Liang Cheng[1], Riley E Alexander[1], Gregory T MacLennan[2], Oscar W Cummings[1], Rodolfo Montironi[2], Antonio Lopez-Beltran[3,4], Harvey M Cramer[1], Darrell D Davidson[1] and Shaobo Zhang[1]

[1]*Departments of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA;* [2]*Department of Pathology, Case Western Reserve University, Cleveland, OH, USA;* [3]*Department of Pathological Anatomy and Histopathology, School of Medicine, Polytechnic University of the Marche Region (Ancona), Ancona, Italy and* [4]*Department of Pathology, Cordoba University, Cordoba, Spain*

The majority of lung adenocarcinoma patients with epidermal growth factor receptor- (*EGFR*) mutated or *EML4–ALK* rearrangement-positive tumors are sensitive to tyrosine kinase inhibitors. Both primary and acquired resistance in a significant number of those patients to these therapies remains a major clinical problem. The specific molecular mechanisms associated with tyrosine kinase inhibitor resistance are not fully understood. Clinicopathological observations suggest that molecular alterations involving so-called 'driver mutations' could be used as markers that aid in the selection of patients most likely to benefit from targeted therapies. In this review, we summarize recent developments involving the specific molecular mechanisms and markers that have been associated with primary and acquired resistance to *EGFR*-targeted therapy in lung adenocarcinomas. Understanding these mechanisms may provide new treatment avenues and improve current treatment algorithms.
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Approximately 85–90% of all cases of lung cancer are carcinomas of non-small cell type.[1–3] These tumors can be further classified into several major histological subtypes, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, adenosquamous cell carcinoma, and sarcomatoid carcinoma.[4] In recent years, attention has been paid to the role that 'driver mutations,' such as epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase (*ALK*), have in the tumorigenesis of adenocarcinomas, and their potential use as targets for therapy.[5–9] Recent data suggest *EGFR* may also serve as a prognostic factor, in addition to its role as a predictive factor, as patients-bearing *EGFR* mutations have shown favorable clinical outcomes even with conventional chemotherapy.[10–13]

Correspondence: Dr L Cheng, MD, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 350 West 11th Street, Room 4010, Indianapolis, IN 46202, USA.
E-mail: liang_cheng@yahoo.com
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EGFR and members of its family have an important role in carcinogenesis through their involvement in the modulation of cell proliferation, apoptosis, cell motility, and neovascularization.[12–16] *EGFR* alterations have been implicated in the pathogenesis and progression of many malignancies.[13,17–21] The incidence of *EGFR* mutations in unselected tumors with non-small cell histology ranges from 10 to 50%, depending upon the ethnic makeup of the patient population and the detection methods used for mutation analysis; 95% of such mutations have been found in adenocarcinomas.[12,13,16,22–34] Although the exact molecular mechanisms resulting from these somatic mutations are not completely understood, it seems clear that mutant *EGFR* has enhanced tyrosine kinase activity. Tyrosine kinase is an enzyme that transports phosphates from adenosine triphosphate (ATP) to a protein's tyrosine residue. Although these cases are most often attributed to *EGFR* mutations, they may also result from increased gene copy number or increased EGFR protein expression.[35,36] EGFR tyrosine kinase inhibitors (TKIs) competitively

block the binding of ATP to the catalytic site in the tyrosine kinase domain of EGFR, subsequently inhibiting autophosphorylation. The process blocks downstream signaling and results in dramatic antitumor activity for a subset of lung adenocarcinoma patients.

EGFR alterations have prompted the development of two classes of anti-EGFR agents: monoclonal anti-EGFR antibodies (such as cetuximab, panitumumab, etc) and small molecule TKIs directed against EGFR tyrosine kinase (such as gefitinib, erlotinib, etc). Clinical trials were initiated that employed novel agents targeting EGFR tyrosine kinase. The results of the clinical trials indicated that many of the tumors harboring mutant *EGFR* are highly sensitive to EGFR TKIs, with up to 70% demonstrating a significant clinical response.[5,28,29,37–39] Recent studies have provided more compelling evidence of the clinical benefits of anti-EGFR treatment in the appropriate setting.[13,15,22,38,40–48] Evidence from the large phase III randomized Iressa Pan-Asia Study trial and other phase III trials have prompted the American Society of Clinical Oncology to issue a provisional clinical opinion recommending the testing of *EGFR* mutational status in patients being considered for first line EGFR TKI therapy owing to their demonstrated benefit on progression-free survival.[22,41] Of note, they caution that no definitive benefit has been shown in patients treated with first-line TKIs in regards to overall survival.[22]

Biomarkers to predict which patients might benefit from targeted therapy are urgently needed. Pathologists have a central role in the process of determining appropriate testing of these tumors and in the interpretation of the test results. In this review, we summarize the most recent developments involving the specific molecular mechanisms and markers that have been associated with primary and acquired resistance to EGFR-targeted therapy, which may lead to new, more effective treatment possibilities and may augment the currently used treatment algorithms.

EGFR alterations in lung cancer

EGFR Mutations

EGFR is located at chromosome 7p11.2, spans about 200 kb, and contains 28 exons. The gene encodes a protein of 464 amino acids.[49,50] EGFR is composed of an N-terminal extracellular ligand-binding domain, a transmembrane lipophilic segment, and a C-terminal intracellular region containing a tyrosine kinase domain. The EGFR tyrosine kinase modulates cell proliferation and survival through autoactivation of EGFR itself, or through two downstream intermediate pathways: the PIK3CA/AKT1/MTOR pathway and the RAS/RAF1/MAP2K1/MAPK1 pathway.[51] Upon ligand binding to EGFR, the receptors form homodimers or heterodimers, which activate their intrinsic intracellular protein-tyrosine kinase. The ligand binding-induced dimerization results in cross-autophosphorylation of key tyrosine residues in the cytoplasmic domains, which function as docking sites for downstream signal transducers.[36] This activation of EGFR initiates signaling cascades involving several downstream pathways, which induce crucial cellular responses, such as proliferation, differentiation, motility, and survival[13,52–62] (Figure 1).

EGFR mutations, which are associated with objective responses to single-agent TKI therapy in lung adenocarcinomas, are preferentially observed in a specific subset of patients: females of East Asian ethnicity who have never smoked and who have adenocarcinoma with lepidic growth pattern (formerly bronchioloalveolar carcinoma).[5,6,14,63,64]

In adenocarcinomas, the majority of mutations have been identified in exons 18–21 of the *EGFR* gene.[9,65,66] These mutations can be roughly classified into three major categories: in-frame deletions in exon 19, insertion mutations in exon 20, and missense mutations in exons 18–21 (Figure 2). Different *EGFR* mutations have different signaling properties, but most mutations affect the ATP-binding cleft, where targeting TKIs compete for binding.[58] The most frequent mutations were located at exon 19 and exon 21. There are over 20 variant types of exon 19 deletions, with the most common including delE746-A750, delL747-T751insS, and delL747-P753insS. L858R, in exon 21, is the second most frequent mutation. Additional mutations are located at exon 18 including G719C, G719S, G719A, and S720F and mutations found in exon 21 including L861Q and L861R. The exon 20 insertions frequently associated with EGFR-TKI non-responsiveness, including D770-N771insNPG, D770-N771insSVQ, D770-N771insG, and point mutations, including T790M, V769L, and N771T.[15,67] The most important mutation in exon 20 is T790M, which is associated with a small fraction of adenocarcinomas with primary resistance to EGFR TKI and over one-half of the patients with acquired resistance to EGFR TKI (Figure 2).[12,67–71]

A comprehensive literature review by Yamamoto *et al*[33] indicated that 569 mutations were found in 2880 lung cancer patients (20%). The distribution of *EGFR* mutations was as follows: 48% in exon 19, 43% in exon 21, 4% in exon 20, and 3% in exon 18. *EGFR* mutations, except *EGFRvIII*, are rarely found in squamous cell and large cell carcinomas, thus EGFR TKI therapy may not be a relevant therapy for patients with those tumors. In a large series of lung carcinomas investigated for the presence of *EGFR* mutations in exons 18, 19, and 21, no *EGFR* mutations were found in the 454 squamous carcinomas and 31 large cell carcinomas investigated. In contrast, *EGFR* mutations were found in 10% of 375 adenocarcinomas and in 26% of the 86 cases designated as bronchioloalveolar carcinomas.[27]

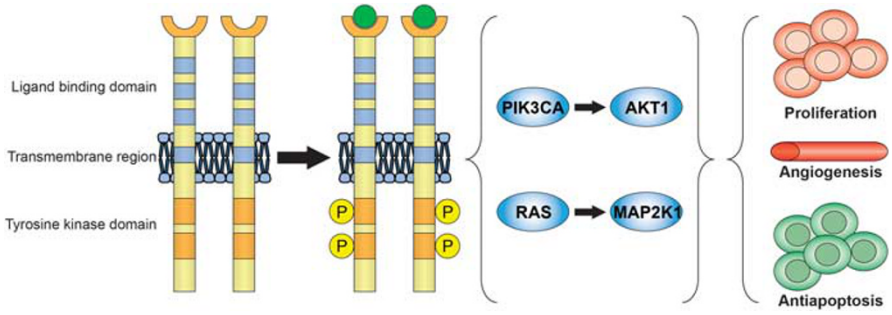

Figure 1 The EGFR-signaling pathway. EGFR is composed of an extracellular domain, a transmembrane lipophilic segment, and an intracellular region containing tyrosine kinase domains that occupy exons 18–24. The binding of ligands to EGFR results in autophosphorylation of key tyrosine residues in the tyrosine kinase domain and activates tyrosine kinase activity, which further activates the downstream PIK3CA/AKT1/MTOR and RAS/RAF1/MAP2K1 pathways. The aberrant signaling influences several key aspects including cell proliferation, apoptosis, migration, survival, and more complex processes such as angiogenesis.

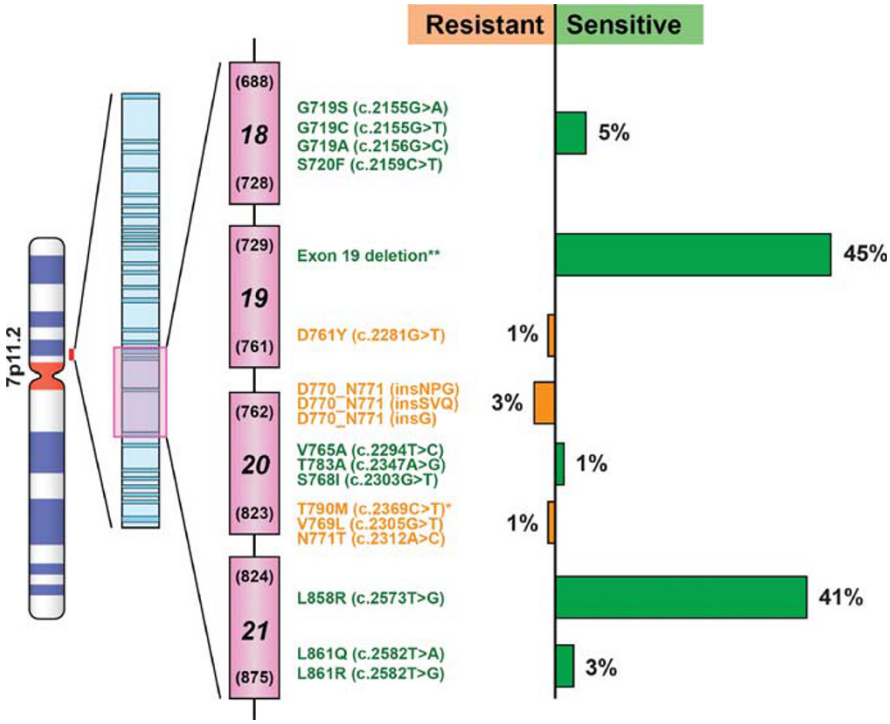

Figure 2 Frequency of mutations in exons 18–21 of the *EGFR* gene and the association with responsiveness to EGFR targeted therapy. The EGFR located in chromosome 7p11.2 contains 28 exons. Exons 18–21 in the tyrosine kinase region of the *EGFR* gene are scaled up; a detailed list of *EGFR* mutations in these exons associated with sensitivity (green) or resistance (orange) to EGFR TKI.[6,12,67–71,80–84,195] The frequency of the mutations is labeled to the side of the color-coded bars. The most prevalent *EGFR* mutations are in-frame deletions of exon 19 (45%), followed by L858R substitution in exon 21 (41%). Exon 18 mutations (G719A/C/S) account for ~5% of the overall mutations. The exon 19 deletions, L858R in exon 21, G719A/C/S in exon 18, the L861Q and L861R in exon 21, are mutations that predict the probability of benefit from EGFR TKI therapy of adenocarcinomas. The insertion mutations in exon 20 (D770_N771 (insNPG), D770_N771 (insSVQ), D770_N771 (insG)) are the second most common and are associated with EGFR TKI therapy resistance. D761Y in exon 19 is also associated with resistance to EGFR TKI although it occurs in low frequency. *T790M mutation represents ~1% of primary resistance but over 50% of acquired resistance in adenocarcinomas. **There are more than 20 exon 19 deletion forms in the lung adenocarcinomas, with the most common ones including delE746-A750, delL747-T751insS, and delL747–P753insS.

The most commonly used method to detect *EGFR* mutations is direct sequencing.[23,25] It is noteworthy that tissue slides frequently contain heterogeneous components of cells, a fact that sometimes hampers optimal analysis. In addition, some patients present with multifocal lung tumors.[72] Careful dissection of cells from a suitably representative area selected by a pathologist is essential to ensure a successful test result. Other methods include PCR–single-strand conformational polymorphism analysis[73,74] and high resolution-melting amplicon analysis.[75,76] Relative to the direct sequencing method, the other two

techniques allow for the rapid detection of *EGFR* mutations with high sensitivity and specificity. However, confirmation of mutations via direct sequencing is still necessary.[27,76,77] Though not of any current clinical use, an assay that provides a rapid assessment of *EGFR* mutation status in as little as 30 min using a 'smart amplification process' has been described. These may one day provide greatly improved turnaround times for this analysis.[78] Formalin-fixed and paraffin-embedded tissue is perfectly suitable for fluorescence *in situ* hybridization (FISH) and DNA-based tests, but tissue preservation is critical for a successful test. Decalcified and ethanol-fixed tissue, as well as tissues containing abundant necrosis, should be avoided.

The ability to detect multiple driver mutations in lung adenocarcinoma has revolutionized the medical management of this disease and multiplexed testing for all common driver mutations will provide physicians with a more precise guide for therapy.[9] Recently, Kris *et al*[79] identified 10 driver mutations in tumor samples from 1000 lung adenocarcinoma patients enrolled in the National Cancer Institute Lung Cancer Mutation Consortium. The mutations, involving *KRAS*, *EGFR*, *ERBB2* (*HER2*), *BRAF*, *PIK3CA*, *AKT1*, *MAP2K1*, and *NRAS*, were screened using standard multiplexed assays and FISH. Driver mutations were detected in 60% of tumors. The incidences of mutations were as follows: *KRAS* 25%, *EGFR* 23%, *ALK* rearrangements 6%, *BRAF* 3%, *PIK3CA* 3%, *MET* amplifications 2%, *ERBB2* 1%, *MAP2K1* 0.4%, *NRAS* 0.2%, and *AKT1* 0% (Figure 3).[12,67–71] It is noteworthy that 95% of molecular lesions were mutually exclusive.[79]

EGFR mutations are responsible for the constitutive activation of the tyrosine kinase receptor. These mutations are also most frequently associated with either sensitivity or resistance to EGFR TKIs (Figure 2).[6,80–84] The response-associated mutations are linked with response rates of >70% in patients treated with either erlotinib or gefitinib.[85,86] However, upto 25% of patients with TKI resistance-associated mutations will also respond to the therapy.[67] Pao *et al*[7] analyzed *EGFR* mutation of exons 18–24 in tumors from 10 gefitinib-responsive and from 7 erlotinib-responsive patients. The results demonstrated that *EGFR* mutations were present in 7 of 10 (70%) gefitinib-responsive and in 5 of 7 (71%) erlotinib-responsive tumors.

EGFR genotype was more useful than clinical characteristics for selection of appropriate patients for consideration of first-line therapy with an EGFR TKI.[85] *EGFR* mutations are generally associated with sensitivity to TKI therapy.[71,87] Both retrospective and prospective studies have demonstrated that lung adenocarcinoma patients carrying such an *EGFR* mutation and who were treated with TKIs had significantly higher response rates and longer progression-free survival than patients without an *EGFR* mutation,[5–7,25,29,71,83,85,87,88] with some patients experiencing rapid, complete, or partial responses

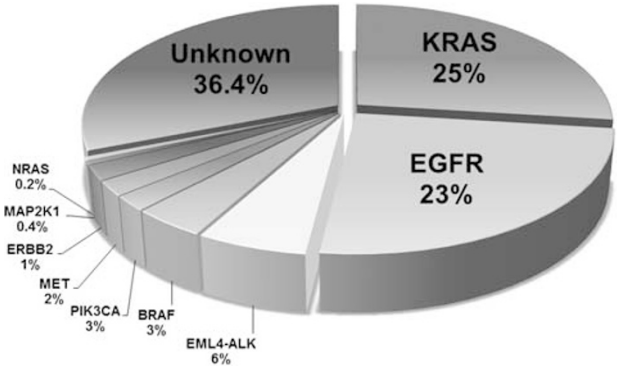

Figure 3 Frequency of major driver mutations in signaling molecules in lung adenocarcinomas. About 64% of all adenocarcinoma cases harbor somatic driver mutations. According to the National Cancer Institute Lung Cancer Mutation Consortium data,[79] ~23% of lung adenocarcinomas harbor *EGFR* mutations. The *EGFR* mutation status of the cancer is associated with its responsiveness or resistance to EGFR TKI therapy. *KRAS* mutations are more frequently found in adenocarcinomas (25%), which are mutually exclusive with *EGFR* mutations. Mutations in *KRAS* have been proposed as one of the mechanisms of primary resistance to gefitinib and erlotinib therapy. A subset of adenocarcinoma cases harbors a transforming fusion gene, *EML4–ALK* (6%), which mainly involves adenocarcinoma from non-smokers with wild-type *EGFR* and *KRAS* mutations. The mutation frequency of *BRAF* is 3%, *PIK3CA* 3%, *MET* amplifications 2%, *ERBB2(Her2/neu)* 1%, *MAP2K1* 0.4%, and *NRAS* 0.2%. Each of the molecular alterations has a role in the signal pathways, activating important cell functions, including cell proliferation and survival. Approximately 36.4% of lung adenocarcinomas do not harbor currently detectable mutations.

that were persistant.[55] Jackman *et al*[85] studied 223 chemotherapy-naïve patients with advanced lung cancer of non-small cell type, among which 86% were adenocarcinomas. Sensitizing *EGFR* mutations were found in 84 carcinomas, 89% of which were adenocarcinomas. The mutations were associated with a 67% response rate, with a time to progression of 11.8 months, and overall survival of 23.9 months.[85] Exon 19 deletions were associated with a relatively longer median time to progression and overall survival compared with L858R (exon 21) mutations. Wild-type *EGFR* was found in 139 patients (62%), and this finding was associated with poor outcomes (response rate, 3%; time to progression, 3.2 months), irrespective of *KRAS* status.

EGFRvIII Mutation

EGFR variant III (*EGFRvIII*), a mutation resulting from an in-frame deletion of exons 2–7 of the coding sequence (amino acids 6–273), has been associated with a subset of squamous cell lung cancers.[89–91] A number of functional differences between *EGFRvIII* and *EGFR* have been characterized.[90,91] *EGFRvIII* has been identified in an array of human solid tumors, including glioblastoma, breast cancer, ovarian cancer, prostate cancer, and lung caner. Although *EGFRvIII* fails to bind EGF, its intracellular tyrosine

kinase is constitutively activated, allowing the receptor to undergo tyrosine autophosphorylation.[92–94] *EGFRvIII* activates the phosphatidylinositol 3′ kinase (PIK3CA) signaling pathway, which provides critical information for cell survival, proliferation, and motility.[95,96] The true incidence and clinical significance of *EGFRvIII* mutations are not yet clearly defined. There have also been reports that *EGFRvIII* mutation in lung cancer correlates with increased *EGFR* copy number.[97]

Ji *et al*[98] determined that *EGFRvIII* mutations were present in 5% (3/56) of human lung squamous cell carcinomas, but not in adenocarcinomas (0/123). The information concerning whether *EGFRvIII* mutation is associated with specific histological types of lung cancer is conflicting, although most studies have indicated that *EGFRvIII* is associated with squamous cell carcinoma.[98,99] In the study by Sasaki *et al*,[97] *EGFRvIII* mutation was detected in 3% (8/252) of non-selected lung cancer patients. All patients bearing a *EGFRvIII* mutation were male, smokers, and seven had squamous cell carcinoma, whereas one had poorly differentiated adenocarcinoma. However, in the investigation of Ohtsuka *et al*,[99] *EGFRvIII* mutation was detected in one of seven squamous cell carcinomas with an adenocarcinoma component, in two of four adenosquamous carcinomas and in one of seven large cell carcinomas.

EGFRvIII-bearing squamous cell carcinomas were reportedly insensitive to gefitinib and erlotinib, but showed sensitivity to neratinib (HKI-272).[98,100]

EGFR Copy Number Alteration

Some, but not all, studies have shown that *EGFR* gene amplification is associated with significantly better survival after treatment with TKI.[10,30,101] Despite the fact that the majority of studies demonstrate that a high *EGFR* gene copy number correlates with better response and increased survival in adenocarcinoma patients treated with EGFR TKI, debate remains about its true prognostic value. Dahabreh *et al*[31] reviewed 59 publications concerning 1020 mutations in 3101 patients. *EGFR* mutations were detectable in 70% (15/21) of patients who had a gain of *EGFR* copy number. There are several methods for detecting and determining *EGFR* gene copy number, or dosage, including FISH,[25,30,102] chromogenic *in situ* hybridization,[32,103] and real-time quantitative PCR.[10,104,105] When compared with *EGFR* mutations, *EGFR* gene copy number gain was a less sensitive and less specific marker, and may therefore not be considered clinically suitable for patient selection.[31]

EGFR Protein Overexpression

There are three main types of immunohistochemical tests for EGFR protein: total EGFR, phosphorylated EGFR, and mutant-specific EGFR.

Total EGFR

Overexpression of total EGFR has been demonstrated in 40–80% of tumors representing various subtypes of lung tumors; however, the use of EGFR overexpression as a prognostic marker has been largely unsuccessful.[106–109] Many studies suggest that immunohistochemistry-based assays measuring EGFR expression do not serve as a robust predictors of response to TKI therapy.[110] The study from Li *et al*[111] further emphasized that EGFR overexpression appears to be independent of *EGFR* mutation. As total EGFR did not correlate well with *EGFR* mutations, it is not accepted as a marker for EGFR TKI treatment selection.

Phosphorylated Form of EGFR

Phosphorylations in the carboxyl-terminus of EGFR have a key role in the recruitment of signaling molecules and activation of downstream signaling pathways.[51,112,113] The utility of detecting phosphorylated EGFR remains questionable owing to concerns about its stability and its compatibility with routine pathology practice. Further studies are warranted to evaluate the potential clinical utility of antibodies that recognize phosphorylated EGFR.

EGFR Mutation-Specific Antibodies

The current commercially available antibodies recognize two of the most common *EGFR* mutations ((delE746_A750) in exon 19 and L858R in exon 21).[114] The antibodies successfully detected EGFR alterations in 51 of 217 adenocarcinomas and in 1 of 217 squamous carcinomas. These findings were confirmed by DNA sequencing.[114] Immunohistochemistry using mutation-specific antibodies could potentially be used to screen for patients who may be candidates for EGFR inhibitors.[115] However, there are concerns about the limited mutation types the antibodies recognize and a practical cutoff point for deeming a test positive or negative has yet to be established. In light of currently available data, it has been proposed that commercially available antibodies may be most useful for initial screening.

Other Alterations that Affect the EGFR TKI Response

Other gene mutations downstream of the *EGFR* signaling pathway are also involved in tumorigenesis of lung adenocarcinomas. Studies have indicated that any activating mutation in the *EGFR/RAS/RAF1* signaling pathway may be sufficient for the pathogenesis of certain lung cancers.[116,117] The findings of Yamamoto *et al*[33] indicated that *EGFR*-mutant cancers tend to have fewer downstream molecular alterations. If *EGFR*-mutant cancers acquire other critical molecular alterations, genetically

or epigenetically, they may survive by using alternative signaling pathways, even if the *EGFR* signaling is effectively inhibited. It is expected that in the presence of additional molecular alterations, the effectiveness of TKI therapy for *EGFR*-mutant tumors will be reduced. The most frequently encountered alterations include *KRAS* mutations,[118–121] *MET* amplification,[122–125] *ALK* gene fusion,[126–129] *PIK3CA* mutations,[130] *BRAF* mutations,[118,131–133] and IGF1R overexpression (Figure 3).[134,135]

Is *EGFR* mutation specific for lung adenocarcinoma?

Current data indicate that *EGFR* mutations are adenocarcinoma dominant, rather than adenocarcinoma specific. Clinically, most *EGFR* mutations are detected in adenocarcinomas;[27] with other types of lung carcinomas showing a much lower frequency of *EGFR* mutations: 5% in squamous cell carcinomas and virtually none in large cell carcinomas.[27,136] Lung adenocarcinomas frequently possess *EGFR* mutations and frequently exhibit increased *EGFR* copy number.[111] A study including 334 cases of lung adenocarcinoma using PCR-based assays to detect deletions within exon 19 and the L858R mutation in exon 21 of the *EGFR* gene found that 23% of these tumors contained a mutation. Of those, 29% were exon 19 deletions and 29% were the L858R mutation in exon 21. In addition, *EGFR* amplification, defined as greater than five *EGFR* signals per nucleus, was detected in 52% of *EGFR*-mutated tumors, but in only 6% of those lacking the *EGFR* mutations.[111] Among adenocarcinomas, *EGFR* mutations are more prevalent in cases formerly subtyped as bronchioloalveolar carcinomas.[27,41,99] However, *EGFR* mutations in squamous cell carcinoma appear to occur much less frequently, with a reported incidence as high as 14% from a group of seven patients to as low as 0% in a group of 454 squamous cell lung cancers.[27,41,99] The National Comprehensive Cancer Network recommends erlotinib as the first-line therapy for patients who have an *EGFR* mutation and who have advanced, recurrent, or metastatic adenocarcinoma.[3]

Adenosquamous carcinomas appear to have an *EGFR* mutation incidence that is similar to that of adenocarcinomas. Furthermore, adenosquamous carcinomas have been treated in a manner similar to adenocarcinomas as these tumors harbor comparable *EGFR* mutations. In a study that included 23 adenosquamous carcinomas with separately microdissected glandular and squamous components analyzied for *EGFR* and *KRAS* mutations, *EGFR* mutations were observed in 13% of cases (3/23), two of which had identical mutations in the glandular and squamous elements.[137]

Although *EGFR* mutations in lung cancers other than adenocarcinoma type are not common, occasionally reported cases bearing *EGFR* mutation have indicated that EGFR TKI therapy could still be a potentially effective approach for those patients (Figure 4). Large cell carcinoma, appears to harbor *EGFR* mutations very rarely.[27,138,139] In one study, only one L858R mutation was found in 60 large cell carcinomas of lung.[139] In the study of Marchetti *et al*,[27] *EGFR* mutations were identified in 39 (10%) of 375 adenocarcinomas, but no *EGFR* mutations were found in 454 squamous carcinomas and 31 large cell carcinomas. De Pas *et al*[140] reported a 66-year-old woman with metastatic large cell lung cancer harboring *EGFR* mutation. A positron emission tomography scan performed 2 months after the initiation of gefitinib therapy showed a dramatic response to treatment in both the patient's primary tumor and her metastatic deposits.

Sarcomatoid lung cancer is rare and highly malignant. Whether *EGFR* mutation is involved in its tumorigenesis is unclear. Jiang *et al*[141] investigated a group of 33 patients with sarcomatoid lung cancer for *EGFR* mutations by direct sequencing. *EGFR* mutations were detected from 9 of 32 patients and only one patient had a *KRAS* mutation.

In contrast to the *EGFR* mutations that are often found in adenocarcinomas, pure squamous cell carcinomas exhibit mutations in the discoidin domain receptor tyrosine kinase 2 with a frequency of 3.8% (11/290).[142] Discoidin domain receptor 2, a tyrosine kinase receptor that binds collagen as its endogenous ligand, has been previously shown to promote cell migration, proliferation, and survival when activated by ligand binding and phosphorylation. *EGFRvIII* mutations were found in 5% of human lung squamous cell carcinomas (also see prior discussion) (Figure 4).[98,99]

The advent of targeted therapy based on driver mutations in lung adenocarcinoma has countered the notion that non-small cell lung cancer (NSCLC) is a distinct clinical entity. Current information indicates that distinguishing a tumor as NSCLC alone is no longer sufficient for patient management and the term 'non-small cell lung cancer (NSCLC)' should be abandoned. Recently, a panel of experts proposed a major revision of the lung cancer classification system.[143] These changes primarily affect the classification of adenocarcinoma and its distinction from squamous cell carcinomas (Table 1).[143] The new classification system from the International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society further classifies lung carcinomas into more precise subtypes based on a multiparameter approach that incorporates and integrates clinical, molecular, and histological features (Table 1).[143] The advent of mutation-specific therapies has dramatically changed the landscape of lung cancer treatments.[3,13] Patients without *EGFR* mutations seldom respond to EGFR-targeted therapy. Targeted therapy drugs are inherently costly as these carefully designed

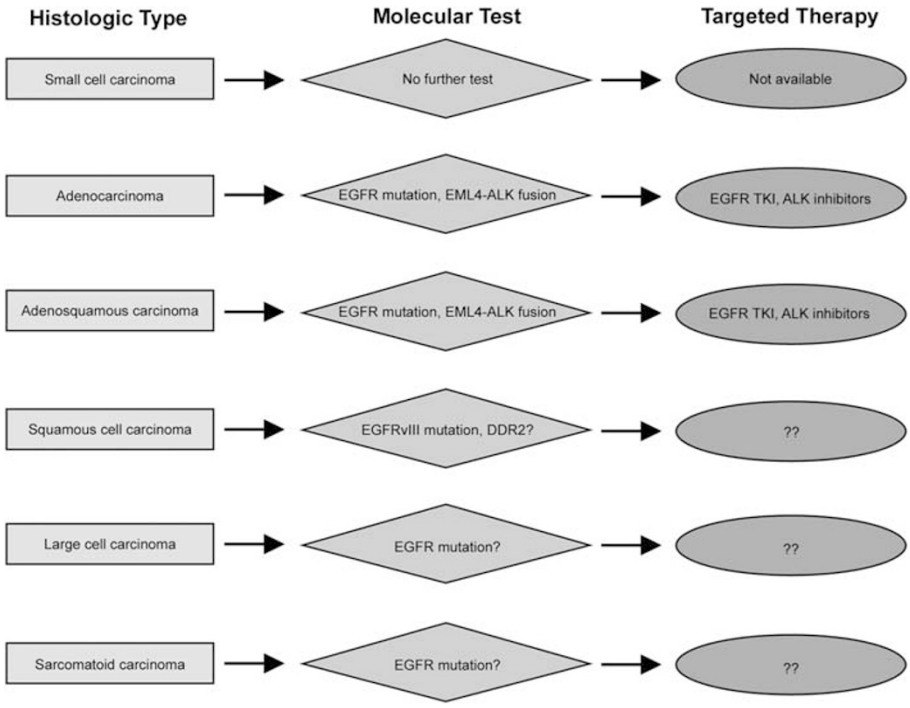

Figure 4 Current molecular tests and options for targeted therapies. Adenocarcinomas and adenosquamous carcinomas have a relatively high incidence of *EGFR* mutations or *EML4–ALK* rearrangement. Patients with such tumors could potentially benefit from targeted therapies using EGFR TKI and ALK TKI. *EGFRvIII* is associated with a small subset of squamous cell carcinomas, but the rationale for the therapy targeted to this mutation has not yet been established. Large cell and sarcomatoid carcinomas are not considered suitable tumors for the EGFR TKI therapy although a recent article reported 28% EGFR mutations from a group of 32 sarcomatoid lung cancers.

Table 1 International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS). Classification of lung adenocarcinoma in resection specimens

Preinvasive lesions
Atypical adenomatous hyperplasia
Adenocarcinoma *in situ* (≤ 3 cm formerly bronchioloalveolar carcinoma)
Non-mucinous
Mucinous
Mixed mucinous/non-mucinous
Minimally invasive adenocarcinoma (≤ 3 cm lepidic predominant tumor with ≤ 5 mm invasion)
Non-mucinous
Mucinous
Mixed mucinous/non-mucinous
Invasive adenocarcinoma
Lepidic predominant (formerly non-mucinous bronchioloalveolar carcinoma, with >5 mm invasion)
Acinar predominant
Papillary predominant
Micropapillary predominant
Solid predominant with mucin production
Variants of invasive adenocarcinoma
Invasive mucinous adenocarcinoma (formerly mucinous bronchioloalveolar carcinoma)
Colloid
Fetal (low and high grade)
Enteric

molecules are patented, yet aimed at a limited patient population. Inappropriate use of these and other targeted therapies run the risk not only of clinical failure, but also of unnecessary expense or even life-threatening toxicity.

EML4–ALK rearrangement: driver mutation of lung cancer

The *ALK* gene encodes a receptor tyrosine kinase found in a number of fusion proteins consisting of

the intracellular kinase domain of *ALK* and the amino terminal portions of different genes.[144,145] *EML4–ALK* fusion is formed as the result of a small inversion within the short arm of chromosome 2 that joins intron 13 of echinoderm microtubule associated protein-like 4 (*EML4*) to intron 19 of *ALK* [*inv*(*2*)(*p21*;*p23*)], generating an oncogenic fusion encoding a constitutively activated protein tyrosine kinase.[8,146] A subset of lung adenocarcinoma cases harbor within the genome this transforming fusion gene, *EML4–ALK* (Figure 5).

The *EML4–ALK* fusion is a rare abnormality detected in ~3–13% of patients with adenocarcinomas.[8,146–152] The most common fusion results from the joining of exons 1–13 of *EML4* to exons 20–29 of *ALK*. At least seven *EML4–ALK* variants (V1–V7) have been identified in lung adenocarcinomas.[153] All seven variants are formed through the fusion of the intracellular tyrosine kinase domain of *ALK* with a variably truncated *EML4* gene.[8,154–156] Activated *ALK* is involved in the inhibition of apoptosis and the promotion of cellular proliferation through activation of downstream *PIK3CA/AKT1*- and *MAPK1*-signaling pathways.[157] Fusion of the *EML4–ALK* gene and its associated *EML4–ALK* product may further lead to constitutive activation of the *RAS/RAF1/MAP2K1/MAPK1* pathway.[54] Additionally, two other less frequent *ALK* fusions in lung cancer have been reported, but not yet studied for their downstream consequences.[158] The key downstream effectors on the ALK pathway include the RAS-activated protein, extracellular signal regulated kinase (MAPK1), phosphoinositide 3-kinase (PIK3CA), and STAT3 signaling pathways.[151] *RAS/MAP2K1/MAPK1* pathways are critical for cell proliferation, whereas the PIK3CA/AKT1 and STAT3 pathways are important for cell survival. The histology of these tumors is typically characterized by mucin production and either a solid growth pattern containing signet ring cells in western patients or an acinar growth pattern in Asian patients.[159–162] Compared with patients with wild-type *ALK* and *EGFR*, patients with the *EML4–ALK* fusion gene tend to be younger (median, 52 *vs* 64 years), of Asian ethnicity, diagnosed at an advanced clinical stage at presentation, male dominant (58 *vs* 32%), and more likely to be never-smokers (74 *vs* 26%), but with a comparable response rate to chemotherapy and overall survival.[8,148,159,160] The *EML4–ALK* fusion gene was detected in 19 of 141 (13%) tumor samples by FISH.[148] None of the 10 patients with an *EML4–ALK* rearrangement achieved an objective response to EGFR TKIs. In contrast, 24% of responding patients in the *EML4–ALK*-negative cohort showed an objective response with an EGFR TKI.[148,163] Tiseo *et al*[126] reported a 48-year-old Caucasian never-smoker man with lung adenosquamous carcinoma harboring *EML4–ALK* fusion and exon 19 deletion in *EGFR* gene. The patient manifested resistance to the erlotinib therapy. The authors concluded that *ALK* status should be investigated in unexplained cases of EGFR TKI-resistance lung cancers. In the study of Shaw *et al*,[148] none of the 10 patients with *EML4–ALK* fusion had a documented clinical response to erlotinib. As the presence of the *EML4–ALK* fusion gene is mutually exclusive with the *EGFR* mutation, it is unclear if EGFR TKI resistance is owing to *EML4–ALK* mutation itself or because of the *EGFR* wild-type.

It has been reported that although *ALK*-fusion-positive lung cancers are resistant to the EGFR TKIs, gefitinib, and erlotinib, they are sensitive to small molecule TKIs against ALK.[152] ALK TKIs (ALK TKI), including crizotinib, are effective treatments in preclinical models for patients with ALK-fusion cancers.[164] In a pivotal phase 1 clinical trial, the ALK tyrosine kinase inhibitor (TKI) crizotinib (PF-02341066) demonstrated impressive antitumor activity in the majority of patients with adenocarcinomas harboring *EML4–ALK* fusions. However, despite these remarkable initial clinical responses, these cancers eventually developed resistance to crizotinib, usually within 1 year, thereby limiting the potential clinical benefit of this drug. Katayama *et al*[165] found that cells resistant to intermediate doses of crizotinib develop either amplification of the *EML4–ALK* gene or a gatekeeper mutation, L1196M, within the kinase domain. Sasaki *et al*[164] proposed two mechanisms of ALK TKI resistance based on evidence from a crizotinib-treated ALK-positive lung cancer patient and in a cell line generated from the resistant tumor. The crizotinib-resistant DFCI076 cell line harbored a unique L1152R *ALK* secondary mutation and was also resistant to the structurally unrelated ALK TKI, TAE684. In contrast, the TAE684-resistant (TR3) H3122 cell line did not contain an *ALK* secondary mutation, but instead harbored coactivation of EGFR signaling. Therefore, the authors suggested that dual inhibition of both *ALK* and *EGFR* was the most effective therapeutic strategy for the DFCI076 and H3122 TR3 cell lines.[164]

In a recent analysis of 82 *ALK*-positive lung cancer patients, ALK tyrosine kinase inhibitor (crizotinib) therapy was associated with improved survival compared with that of crizotinib-naive controls.[166] Survival among ALK-positive patients who were given crizotinib in the second-line or third-line setting was significantly better than those patients given any second-line therapy (2-year survival, 55 *vs* 12%). However, unlike *EGFR* mutation, *ALK* rearrangement was not a favorable prognostic factor.[166]

Dual-color–split-apart FISH is the recommended method for the *EML4–ALK* test. A positive test result in >15% of 50 analyzed tumor cells is the cutoff point recommended by the College of American Pathologist and the Association of Molecular Pathology. The results of ALK FISH interpretation should be verified by two independent personnel. RT-PCR is not recommended due to repeated failures of RNA-based testing using formalin-fixed

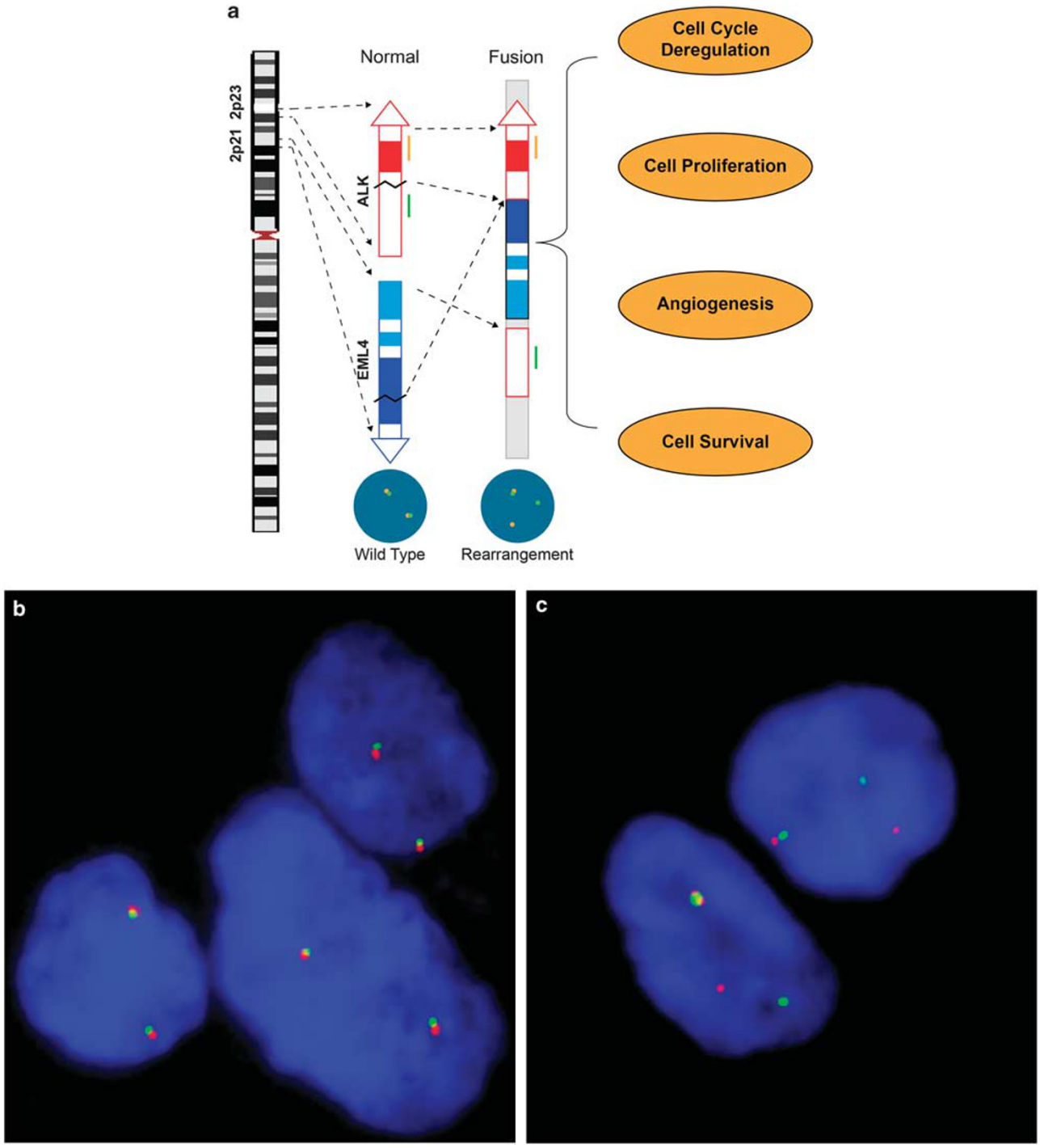

Figure 5 Schematic of *EML4–ALK* rearrangement, its detection by FISH, and its downstream effects. Both *EML4* and *ALK* genes are located on the short arm of chromosome 2. The *EML4–ALK* rearrangement results from a chromosomal inversion, t(2;5) (**a**). Green and orange bars represent DNA probes corresponding to the 5′ and 3′ fragments of the *ALK* gene. The *EML4–ALK* fusion gene is mainly found in adenocarcinomas that arise in non-smokers with wild-type *EGFR* and *KRAS*. The EML4–ALK fusion protein activates canonical signaling pathways, including STAT3, RAS/MAP2K1, and PIK3CA/AKT1 cascades, which further affect cell cycle regulation, cell proliferation, neovascularization, and cell survival. At least nine variants have been identified. FISH detection of *ELM4–ALK* uses break-apart technology, which detects the adjacently located *EML4* and *ALK* genes in wild-type signals (overlapping green-red) (**b**), and break-apart signals (separated green-red in one set of green-red) caused by chromosomal inversion (**c**).

paraffin-embedded tissue. Immunohistochemistry is also not currently recommended as an alternative to FISH testing, due to the low expression level and resulting suboptimal sensitivity and specificity.[167,168]

EGFR targeted therapy approaches

There are two major approaches for inhibiting *EGFR* signaling: (1) prevent ligand binding to the

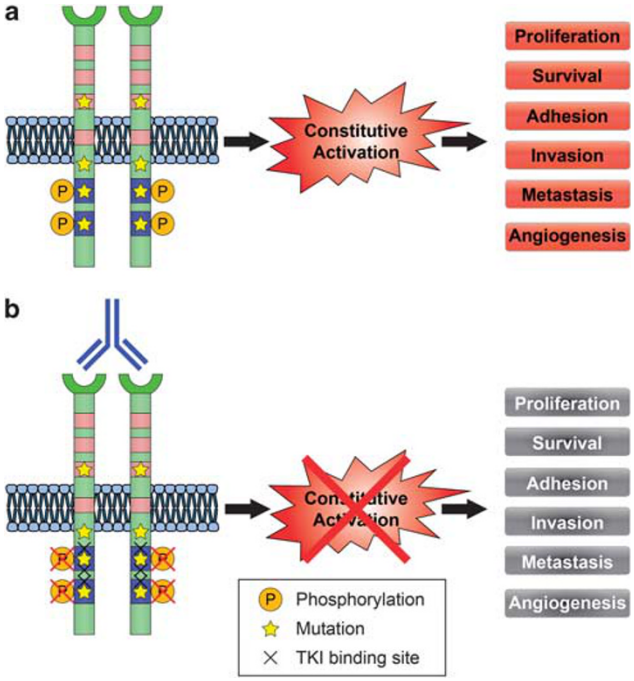

Figure 6 Mechanism of constitutive activation of EGFR results from *EGFR* mutation and strategies of anti-EGFR therapy. (**a**) *EGFR* mutations provoke autophosphorylation of key tyrosine residues (P) in the tyrosine kinase domain, thus activating tyrosine kinase activity constitutively and initiating downstream effectors. (**b**) Two strategies are used for inhibiting *EGFR* signaling: humanized antibodies and small molecule TKIs. The antibodies inhibit the ligand-dependent activation of *EGFR* by blocking the ligand-binding site and preventing *EGFR* from activation. In contrast, TKIs block the magnesium-adenosine triphosphate-binding pocket of the intracellular tyrosine kinase domain, further inhibiting autophosphorylation. This inhibition disrupts tyrosine kinase activity and abrogates intracellular downstream signaling.

extracellular domain with a monoclonal antibody and (2) inhibit the intracellular tyrosine kinase activity with a small molecule TKI. Ligand binding to the extracellular domain of EGFR promotes receptor dimerization, which in turn leads to activation of the cytoplasmic tyrosine kinase. The activated EGFR kinase phosphorylates tyrosines in the EGFR C-terminal, initiates signaling cascades, and stimulates cell growth and differentiation (Figure 6).[13,169,170]

Monoclonal Antibodies

Monoclonal antibodies, such as cetuximab and panitumumab, are either chimeric mouse-human or fully humanized antibodies targeting the EGFR extracellular domain, leading to blockade of ligand-activated signal transduction and receptor dimerization. Fully humanized antibodies, such as panitumumab, have a high affinity for EGFR and a longer half life.[171]

The binding of the antibody initiates EGFR internalization and degradation, which leads to signal termination.[110,172–174] The treatment has shown consistent benefit to clinical outcome when added to chemotherapy.[173] However, this class of treatment only inhibits ligand-dependent activation of EGFR and not autophosphorylation of the tyrosine kinase domain via constitutive activation. These mutations may still activate the downstream pathways, and upregulate cell cycle progression, cell growth, and angiogenesis.

Tyrosine Kinase Inhibitors

TKIs are synthetic small molecules that block the magnesium-ATP-binding pocket of the intracellular tyrosine kinase domain.[110] TKIs prevent the intracellular tyrosine kinase domain of the EGFR from autophosphorylation through binding to its ATP-binding site. Several TKIs, such as gefitinib and erlotinib, are specific for EGFR, whereas others inhibit other receptors in addition to EGFR, such as ERBB2 and VEGFR2. TKIs block ligand-induced receptor autophosphorylation by binding to the tyrosine kinase domain and disrupting tyrosine kinase activity, thereby abrogating intracellular downstream signaling. Somatic activating mutations of the *EGFR* gene in exons 19 and 21 increase gene copy number. Certain clinical and pathological features have been associated with dramatic tumor responses and favorable clinical outcomes with these agents in patients with lung cancer.[30,55,175]

Mechanisms of Resistance to EGFR Targeted Therapy

In a population of unselected patients, response to EGFR TKIs has been reported to be only 10%.[176] These disappointing outcomes may reflect the fact that the majority of lung cancers are *EGFR*-mutation negative. Moreover, the outcomes may also be related to evidence that some *EGFR* mutations not only activate EGFR tyrosine kinase and drive cancer cells to grow, but are also associated with resistance to current EGFR TKI therapy.[82,84,177,178] Current strategies have been focused on detection of EGFR mutation and EML4–ALK rearrangement in lung adenocarcinomas (Figure 7).

The most important mutation associated with acquired EGFR TKI resistance is T790M, a point mutation located at exon 20, resulting in the substitution of methionine for threonine.[70,179–181] Clinically, patients with *EGFR* exon 20 mutations do not respond to gefitinib.[67] Moreover, the appearance of a secondary mutation in exon 20 (T790M) accounts for ~50% of acquired drug resistance.[71,182] Screening for the emergence of such mutations on circulating tumor cells from the blood of patients during the course of treatment may allow earlier identification of acquired resistance.[178,180] Other TKI-resistant mutations include insertions in D770 at exon 20 and D761Y at exon 19 (Figure 2).

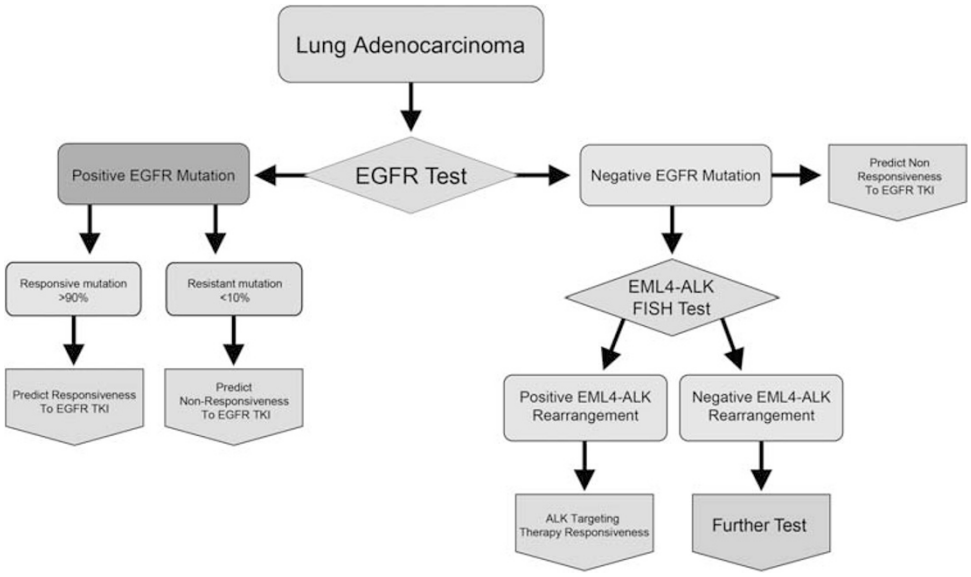

Figure 7 Suggested algorithm for molecular testing for patients with lung adenocarcinoma. The algorithm defines the rationale in selecting patients who could benefit from *EGFR* and *EML4–ALKA* targeted therapy. Adenocarcinoma cases are subjected to testing for *EGFR* mutations. The *EGFR* mutation-positive cases (25%) are further divided into responsive and resistant groups according to their mutation profiles. A responsive mutation predicts a response rate of 91% and a resistant mutation predicts a response rate of 9%. The presence of wild-type *EGFR* characterizes about 75% of the adenocarcinomas, and predicts the likelihood of non-responsive to EGFR TKI. Tumors with wild-type *EGFR* are further tested for *EML4–ALK* rearrangement. Although *EML4–ALK* rearrangement is found in only 3% of patients with lung adenocarcinoma, its presence predicts a 53% probability of response to targeted therapy.

Results of some preclinical studies suggest that the clinical benefit observed with EGFR TKIs is not restricted to those patients harboring *EGFR* gene mutations. This may be due to molecular factors other than *EGFR* mutation. *EGFR* gene amplification and receptor/ligand overexpression, both allowing for a 'gain of function' to occur, may account for some cases of tumor sensitivity to single-agent *EGFR* inhibitors.[30,36] Recent evidence suggests that the approach to accurately predicting response to TKI therapy in adenocarcinomas should combine the status of *EGFR* mutation and its copy number. Although multiple parameters have been used to predict tumor responsiveness to TKI, *EGFR* mutation detection remains one of the most important determinants for the prediction of clinical responsiveness and survival benefit.[183]

The *EGFR* pathway has a central role in a subset of adenocarcinomas through converging signals for cell proliferation, motility, and other cancer cell behaviors.[5,184] However, the mechanisms underlying tumor resistance to EGFR-targeted therapy are still not completely known. Genetic alterations frequently occur during lung cancer progression owing to genomic instability. Biological stress may also modulate multiple signaling pathways and trigger epigenetic alterations. Despite impressive initial clinical responses, patients with *EGFR*-mutated adenocarcinomas almost inevitably develop drug resistance after ~1 year of TKI treatment.[185,186] Studies have revealed several molecular mechanisms that may contribute to the development of tumor resistance to TKI therapy,[71,84,186,187] including acquired secondary *EGFR* mutation, activation of alternative signaling pathways that bypass the *EGFR* pathway, overexpression of HGF,[122] tyrosine protein kinase MET amplification,[53,124] epigenetic factors,[188] constitutive activation of signaling pathways downstream of *EGFR*,[13,189] tumor stromal and extracellular matrix alterations,[190,191] or host-related mechanisms such as rapid drug inactivation and ATP-binding cassette transporters efflux.[192]

Primary resistance to *EGFR* targeted therapy

Recent clinical trials of gefitinib or erlotinib therapy report response rates in *EGFR*-mutated lung cancer cases ranging from 75 to 90%.[13,15,22,38,193] A subset of lung adenocarcinomas show primary resistance to EGFR TKI therapy, even in the presence of an activating mutation in *EGFR*.[194] The most commonly found mutations associated with TKI drug sensitivity include exon 19 deletions downstream of the lysine residue at position 745 (ΔE746-A750), point mutations in exon 21 (L858R and L861Q and L861R), in exon 18 (G719A/C/S), and in exon 20 (V765A, T783A, and S768I).[67–71] However, insertion mutations of exon 20, D770_N771 (insNPG), D770_N771 (insSVQ), D770_N771 (insG), and point mutations in exon 20 (V769L, N771T) were associated with EGFR TKI resistance.[12,195] This observation has been confirmed in an *in vitro* model in which insertion mutations in exon 20 rendered transformed cells less responsive to EGFR TKIs

compared with the sensitizing mutations of exons 19 and 21.[195] Insertion mutation in exon 20 at 770 renders the EGFR 100-fold less sensitive to TKIs when compared with the sensitizing mutations.[195] The mutation T790M, which is associated with ~50% of acquired resistance, has also been linked to primary resistance, although infrequently (<5% of such cases) (Figure 2).[179]

Approximately 10–25% of *EGFR*-mutant lung adenocarcinomas do not respond to an EGFR TKI.[15] In addition to the previously mentioned mutations, even rarer primary mutations, such as D761Y, G719C, and E709A mutations, have also been shown to be insensitive to EGFR TKIs, and even more so when co-occurring with other genetic alterations.[196]

Primary TKI resistance may also be mediated by the presence of other genetic alterations that affect signaling downstream from EGFR, such as mutation of *KRAS*, *PIK3CA*, and loss of PTEN expression.[197–199] Mutations in *KRAS,* which are frequently found in adenocarcinomas with wild-type *EGFR*, are a mechanism of primary resistance to gefitinib and erlotinib.[63] PTEN is one of the key downstream components of the EGFR pathway and has a significant role in cell survival, proliferation, and growth. Knockdown of PTEN expression in cells results in drug resistance to gefitinib and erlotinib.[200] Loss of PTEN expression results in overactivation of the Akt pathway and confers resistance to EGFR TKI.[198] Study results suggest that the loss of expression of PTEN may be mediated by an epigenetic mechanism, as genetic alterations on the *PTEN* gene are found in fewer than 10% of cases.[201,202] *BRAF* mutations may also be associated with primary resistance to EGFR TKIs.[203]

Wild-Type *EGFR*

The wild-type *EGFR* appears to be a significant marker for the primary EGFR TKI resistance. The Iressa Pan-Asia Study clinical trial demonstrated that most tumors without detectable *EGFR* tyrosine kinase domain mutations were insensitive to gefitinib.[41] Tumors with wild-type *EGFR* often harbor somatic mutations in other genes that affect the key pathways in lung adenocarcinoma. Thus, primary drug insensitivity is likely linked to the absence of drug-sensitizing mutations in *EGFR* and is more likely to be a result of mutations in other genes.[15,85] Further complicating matters are the findings that even in tumors with *EGFR* mutations, certain mutations appear to confer greater sensitivity to treatment than others.[33] This emphasizes that predicting response to treatment is a complex process that is not completely understood at this time.

KRAS Mutations

One of the most important discoveries for the clinical management of colorectal carcinoma has been the association of mutations in *KRAS* and the usual failure of monoclonal antibodies targeting EGFR, such as panitumumab and cetuximab. The *KRAS* protooncogene encodes *KRAS* G-protein, which has a critical role in the *RAS/MAPK1* signaling pathway downstream of many growth factor receptors, including *EGFR*. Some tumors harbor somatic mutations in exon 2 of *KRAS* that compromise the hydrolysis of RAS-bound GTP to GDP, resulting in constitutive activation of the RAS pathway.[204] In the presence of a *KRAS* mutation, the EGFR pathway activation is not significantly inhibited by cetuximab or panitumumab, which acts upstream of the KRAS protein, diminishing the efficacy of the agents. This pathway is identical to the EGFR pathway targeted by TKIs in adenocarcinomas.

KRAS has a key role in the EGFR signaling network. An activating mutation of *KRAS* is present in ~25–35% of TKI non-responsive cases.[63] *EGFR* and *KRAS* mutations are rarely detected in the same tumor, suggesting that they may perform functionally equivalent roles in lung tumorigenesis.[205,206] *KRAS* mutation is a negative predictor of response to anti-*EGFR* monoclonal antibodies and is also an important mechanism of resistance to TKIs in lung adenocarcinomas.[55] A meta-analysis by Linardou *et al*[207] provided empirical evidence that somatic mutations of the *KRAS* oncogene are highly specific negative predictors of response to single-agent EGFR TKIs in advanced lung cancers, mostly adenocarcinomas. Mao *et al*[121] reviewed 1470 lung cancers from 22 publications, of which 231 had *KRAS* mutations (16%). The mutations were more common in adenocarcinoma than in other histological types of lung cancer (26 *vs* 16%). The objective response rate of patients with mutant *KRAS* was 3% (6/210), whereas the objective response rate of patients with wild-type *KRAS* was 26%. As most *KRAS* mutations are detected in codons 12 and 13 of exon 2, an alternative-screening algorithm is to perform *KRAS* mutation analysis first, followed by *EGFR* mutation test if *KRAS* mutations are not found (Figure 8).

Mutations in *KRAS* are one mechanism of primary resistance to gefitinib and erlotinib.[208] *KRAS* mutations are almost exclusively detected in codons 12 and 13 of exon 2, resulting in *EGFR* independent intracellular signal transduction activation. In the study of Eberhard *et al*,[11] *EGFR* exons 18 through 21 and *KRAS* exon 2 mutations were investigated via sequencing in tumors of 274 patients. *KRAS* mutations were present in 21% of tumors, which were associated with significantly decreased time to progression and survival in patients treated with erlotinib plus chemotherapy.

KRAS mutations were detected in 21/215 (10%) of adenocarcinomas but were not found in 15 squamous cell carcinomas or 11 lymphoepithelioma-like carcinomas.[196] Santis *et al*[209] observed a similar

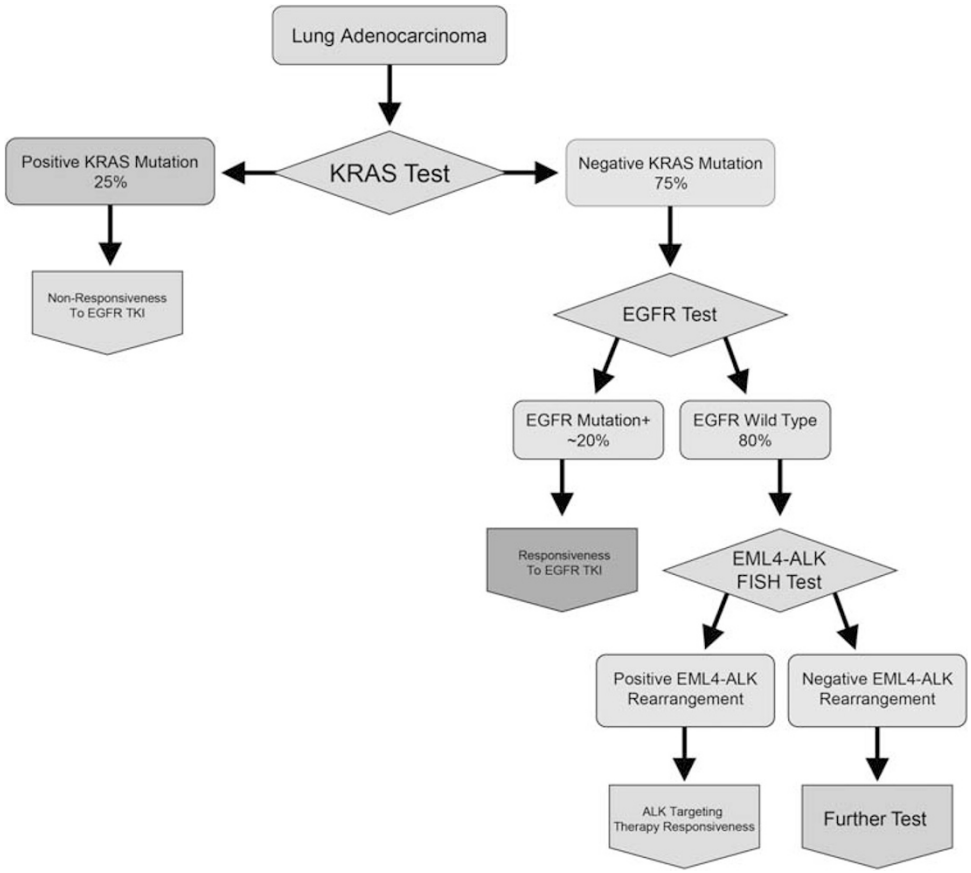

Figure 8 Alternative algorithm for molecular testing for patients with lung adenocarcinomas. Approximately 25% of lung adenocarcinomas harbor *KRAS* mutations, which predict non-response to EGFR TKI therapy. Of the remaining *KRAS*-negative lung adenocarcinomas, ~20% harbor *EGFR* mutations, which are associated with responsiveness to EGFR TKI therapy. *EGFR* mutation-negative cases may benefit from additional testing for the *EML4–ALK* rearrangement, which will be helpful in selecting patients potentially eligible for *ALK* targeted therapy.

pattern. In a study including 121 adenocarcinomas from African–American patients, *KRAS* mutations were compared with data from Caucasian patients ($n=476$).[210] The *KRAS* mutations were found in 17% (21/121) of African–American patients compared with 26% (125/476) of Caucasian patients.[210] *KRAS* mutations in adenocarcinoma are usually associated with wild-type *EGFR* and non-responsiveness to EGFR TKI therapy. Therefore, it is difficult to determine whether the resistance is due to the presence of mutated *KRAS* or to the absence of mutated *EGFR*. Mao *et al*[121] reviewed 22 studies, finding that *KRAS* mutations were detected in 150 of 718 (21%) patients with lung cancer. Mutations were more common among adenocarcinomas than in other types of cancer (26 *vs* 16%). The objective response rate of patients to EGFR TKI with mutant *KRAS* was 3% (6/210), compared with 26% (287/1125) of patients without *KRAS* mutations. Having a wild-type *KRAS* is very important if benefit is to be derived from EGFR inhibition, but is not the sole determinant of this outcome as other mechanisms of resistance to EGFR inhibitors exist.

BRAF Mutation

The *BRAF* gene encodes a protein that has a key role downstream of KRAS in the cell signaling pathway activating important cell functions, including cell proliferation and survival.[132,133] Both *KRAS* and *BRAF* genes are part of the signaling cascade for the EGFR family proteins.[211] The BRAF protein is a serine/threonine protein kinase that is activated by KRAS in a GTP-dependent manner.[118] Mutant BRAF proteins have elevated kinase activity and can transform NIH3T3 cells.[212] *KRAS* function is not required for the growth of cancer cell lines with *BRAF* mutations.[212] Among 697 patients with lung adenocarcinoma, *BRAF* mutations were present in 18 (3%) of the patients. The *BRAF* mutation frequencies were V600E (50%), G469A (39%), and D594G (11%), in exons 15, 11, and 15, respectively.[213] All patients with *BRAF* mutations were current or former smokers. The major *BRAF* functions are believed to be mediated by phosphorylation and thus activate the *MAPK1*, *MAP2K1*, and *MAP2K2* pathways.[118,214] Mutations in *BRAF* have been shown to impair responsiveness to

panitumumab or cetuximab in patients with colorectal carcinomas.[215] *BRAF* mutations are found in 1–3% of lung cancers, most of which are adenocarcinomas.[206,212] *BRAF* mutations are found in a mutually exclusive pattern with *KRAS* mutations, suggesting that these genetic events activate a set of common downstream effectors of transformation. However, *BRAF* exon 15 mutations were tested on 96 paired samples of primary lung adenocarcinomas and corresponding locoregional lymph node metastases.[133] *BRAF* mutations were observed in two patients with *KRAS* mutations demonstrating the possibility of both mutations in *BRAF* and *KRAS* occurring in the same tumor.

Mutations in the *KRAS*/*BRAF* pathway were recently shown to predict clinical response to MEK inhibition in lung adenocarcinoma.[56,216] The histological phenotype of *BRAF* mutant adenocarcinomas has not been well described, but was reported to be a mixed type adenocarcinoma with a high incidence of papillary (80%) and lepidic growth (50%) patterns.[217]

De Oliveira Duarte Achcar *et al*[218] investigated 15 primary micropapillary lung adenocarcinomas for *KRAS*, *EGFR*, and *BRAF* mutations. *BRAF* was found in three (20%) of these cases. The tumors had diverse histological characteristics including mucinous, lepidic, acinar, and solid growth pattern.

The initial retrospective work on mutant *BRAF*'s effect on EGFR-targeted therapy was performed on a cohort of 132 metastatic colorectal cancer patients.[215] The results showed that none of the patients who experienced a response displayed *BRAF* mutations, whereas 11 of 79 (14%) nonresponders carried a *BRAF V600E* allele.[215] As *BRAF* mutations are mutually exclusive to *EGFR* and *KRAS* mutations, it is likely to be associated with lack of response to EGFR TKIs.[138]

Other Genomic Alterations

Genetic alterations other than *EGFR*, *KRAS*, and *EML4–ALK* alterations are relatively rare in lung adenocarcinomas and available data concerning such alterations are limited. However, these less common alterations may have significant clinical importance. Current literature indicates that 30–40% of EGFR TKI-resistant *EGFR*-mutated tumors do not carry secondary resistance mutations.[123,134,200,219] The role of oncogenic activation of *EGFR* downstream effectors such as *KRAS*, *BRAF*, *PIK3CA*, and *PTEN* in response to therapy has been discussed extensively in a series of studies.[54,62] The *RAS/MAPK1* and *PIK3CA/AKT1* pathways are the major signaling networks linking EGFR activation to cell proliferation and survival.[220] Activating mutations in these downstream effectors of EGFR signaling could lead to resistance to EGFR inhibitors.[221–223] The discovery of other molecular resistance aberrations, such as MET kinase amplification or mutations of *EML4–ALK* fusion, which cause constitutive activation of *RAS/RAF1/MAP2K1*, has provided further insight and validation into factors limiting the therapeutic efficacy of *EGFR* inhibitors.[53,148,224] The mystery as to why all tumors harboring drug-sensitive *EGFR* mutations do not respond to treatment with EGFR TKI inhibitors is yet to be resolved. Clinical investigations have shown that the presence of other genetic alterations affecting signaling pathways downstream of EGFR may have a crucial role. Mutation of *PIK3CA* confers gefitinib resistance and loss of PTEN expression also contributes to erlotinib resistance.[198,225]

Heterogeneity within cancer cell populations in the response to anticancer therapy is another factor to be considered.[72] However, some studies demonstrate that heterogeneity is not likely a significant factor that affects the EGFR targeted therapy. Yatabe *et al*[226] compared *EGFR* mutation patterns between primary and metastatic sites and between primary and recurrent tumors. There were no discordant mutation patterns among 77 paired primary and metastatic site samples or among 54 primary and recurrent tumor pairs.

A recent study evaluated whether abundance of *EGFR* mutations in tumors predicts treatment outcome in 100 cases of advanced lung cancer, among which 93 were adenocarcinomas.[205] Of the 100 samples studied, 51 and 18 samples harbored high and low abundances of *EGFR* mutations, respectively, and 31 carried wild-type *EGFR*. Differences in overall survival and objective response rate in patients with high and low abundances of *EGFR* mutations were not significant.[227] This study also pointed out that heterogeneity caused by factors other than *EGFR* mutation could also affect EGFR TKI response. Sharma *et al*[228] reported detecting a small subpopulation of reversibly 'drug-tolerant' cells showing more than 100-fold reduced drug sensitivity. The drug tolerant phenotype is maintained via engagement of IGF1 receptor signaling. Treatment against IGF1 could selectively ablate the drug-tolerant subpopulation, thereby potentially improving the therapeutic effectiveness of EGFR TKI.[228]

MET Amplification

MET also contributes to primary and acquired resistance to EGFR TKIs.[194,229] *MET* is located on chromosome 7q21, which encodes the tyrosine kinase, hepatocyte growth factor receptor.[230] Amplification of *MET* is associated with acquired resistance to EGFR TKIs through a mechanism termed kinase switch.[134] Overall, *MET* amplification has been reported in about 20% of tumors from patients with acquired resistance.[9,231,232] *MET* amplification occurs in both squamous cell carcinoma and adenocarcinoma.[232] *In vitro* studies have shown that *MET* amplification is associated with increased

concentrations of phosphorylated hepatocyte growth factor receptor.[53] Amplification of *MET* correlated with a poor prognosis in a study of surgically resected lung cancers, including 241 adenocarcinomas, 139 squamous cell carcinomas, and 67 other types of tumors.[231]

Aberrant *MET* signaling may have a key role in the development of acquired resistance to therapy with an EGFR TKI.[53] The clinical relevance of *MET* amplification has been investigated by examining tumor biopsies from patients who developed acquired resistance to gefitinib or erlotinib. The *MET* copy status was assessed in rebiopsy samples from 18 lung cancer patients at the time of secondary resistance development following an initial partial response.[53] *MET* amplification was detected in four patients (22%). In another study, *MET* amplification was identified in 9 of 43 (21%) patients who had developed secondary resistance to an EGFR TKI in contrast to 2 of 62 (3%) patients with known sensitizing *EGFR* mutations who also had amplification of *MET*.[233] The identification of MET amplification has led to the development of hepatocyte growth factor receptor-targeted TKIs.[234] Clinical trials are underway with hopes that this may provide another form of targeted in therapy in EGFR TKI nonresponders.

ERCC1 Expression

ERCC1 (excision repair cross-complementing 1) is a DNA repair gene located at 19q13.32, which encodes a protein consisting of 297 amino-acid residues. Defects in *ERCC1* are the cause of cerebro–oculo–facio–skeletal syndrome type 4. The ERCC1 enzyme has a key role in the nucleotide excision repair pathway, and also removes cisplatin-induced DNA adducts.[235] The prognostic significance of ERCC1 was accessed by Simon *et al*[236] with real time quantitative-PCR in surgical specimens from chemotherapy-naïve patients.

Investigational results of 1207 lung cancer patients by Gandara *et al*[237,238] on the relationship between *EGFR* mutation status and *ERCC1* gene expression indicated that *EGFR* mutant cancers are more likely to be categorized as ERCC1 low and, therefore, platinum sensitive. Immunohistochemistry evaluation of ERCC1 expression in tumors from 130 patients revealed that ERCC1 was expressed in 10% of *EGFR*-mutated tumors and in 70% of *EGFR* wild-type tumors.[238] Patients with low ERCC1 expression had a longer overall survival compared with the patients with high ERCC1 expression.[239] Although most studies indicate a consistent association between ERCC1 expression level and responsiveness to cisplatin-based therapy, another study reported discordance of ERCC1 expression between primary and metastatic tumors. This discordance was found in about 40% ($n=49$) of cases, potentially indicating challenges in the clinical application of ERCC1.[240]

Acquired resistance to EGFR TKI

Published data indicate that 70–80% of mutation-positive adenocarcinomas are EGFR TKI sensitive, whereas response rates of tumors with wild-type EGFR are only 10–20%.[28,39,41,241,242] There are two well-established mechanisms of acquired resistance: additional mutations in the *EGFR* gene, acquired during the course of treatment, which change the protein coding sequence; and amplification of other oncogene signaling pathways, such as those involving the *RAS* and *MET* oncogenes.[84,221–223]

Kobayashi *et al*[182] reported a gefitinib-resistant advanced adenocarcinoma patient who had a relapse after 2 years of complete remission with gefitinib. The DNA sequence of the *EGFR* at relapse revealed the presence of a second point mutation, resulting in threonine–methionine amino-acid change at position 790 of *EGFR* (T790M). Structural modeling and biochemical studies showed that this second mutation led to gefitinib resistance.[182] The same mutation was confirmed by Pao *et al*[208] through molecular analysis of *EGFR* in patients with acquired resistance to gefitinib or erlotinib. These gefitinib-resistant cases contain the same secondary mutation (T790M) in the kinase domain as those reported by Kobayashi *et al*.[182] Codon 790 of *EGFR* is considered to be the 'gatekeeper' residue, which is an important determinant of inhibitor affinity in the ATP-binding pocket of *EGFR*.[110] Substitution of this residue in *EGFR* with a bulky methionine may cause resistance by steric interference with binding of TKIs, including gefitinib and erlotinib.[182,208,243] This mutation confers a survival advantage to the tumor and is selected while the patient is receiving anti-EGFR TKI treatment.[82,179] This secondary mutation is quite prevalent, being found in up to 50% of EGFR-mutant tumors treated with first-generation EGFR TKIs.[182,208]

Arcila *et al*[244] recently analyzed sensitizing *EGFR* mutations in 121 lung cancer patients, but only 104 (86%) cases were tested successfully. Most test failures were related to low-tumor content in the tested samples. All tested cases (61/61) with matched pretreatment and resistance specimens showed concordant sensitizing *EGFR* mutations. T790 M mutation was identified in 51 of 99 patients (52%) of cases analyzed. Retesting of 30 T790M-negative patients by the highly sensitive locked nucleic acid-based method detected 11 additional mutants for an overall prevalence of 68%.[244]

To address this particular cause of resistance, current third-generation EGFR TKIs are in development that irreversibly bind the ATP-binding socket in the presence of T790M.[15] This compound, WZ4002, has a selective affinity for doubly mutated cells and, therefore, may represent a second-line treatment in cases of acquired resistance due to T790M. Further trials are needed to assess the potential efficacy of this approach before its clinical

application. Because of the rarity of secondary mutations other than T790M in the EGFR tyrosine kinase domain, routine analysis of cases with acquired resistance is not recommended.

Conclusions

In summary, the advent of EGFR and ALK TKI therapy has provided a powerful new treatment modality for patients diagnosed with lung adenocarcinoma. Yet, primary and acquired resistance to targeted therapy continues to be a major obstacle to satisfactory clinical outcomes. Identification of the specific molecular alterations that contribute to response to EGFR targeted therapy will become a critical part of the process of selecting patients for appropriate treatments. Along with a growing understanding of the mechanisms of pharmacotherapy and the evolution of molecular resistance, it is anticipated that in order to maximize therapeutic effect and improve overall survival lung cancer treatments will be specifically tailored for the individual patient based on the presence or absence of critical molecular alterations.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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