EGFR as therapeutic target in non-small cell lung cancer

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Junho de 2010
**Abstract**

Non-small cell lung cancer (NSCLC) therapeutic approach has been a priority in recent years, since conventional treatments seem to have reached a plateau in survival improvement. Epidermal Growth Factor Receptor (EGFR), a transmembrane receptor with tyrosine kinase activity, was identified as a promising target for NSCLC molecular therapy given its preponderant role in development and progression of some lung tumors. Unfortunately, the Tyrosine Kinase Inhibitors (TKI) that target the EGFR pathway, like Gefitinib or Erlotinib, are active only in a minority of patients. The aim is now to accurately identify patient’s clinical and biological characteristics that will allow prediction of response or resistance to these agents, therefore contributing to a comprehensive patient screening and also for important economical savings.

The most common clinicopathologic predictors of response to TKI are never-smoking history, female sex, Asian ethnicity and adenocarcinoma histology. At the molecular level, EGFR gene mutations and increased gene copy number are the most critical predictors of response, although with conflicting results with regard to survival. Several mutations involving not only the EGFR gene but also KRAS or HER2 have been consistently associated with resistance to those agents. This article intends to systematize the current knowledge on therapeutic targeting of EGFR in NSCLC, which provides an important and useful tool for the clinical management of a subset of lung cancer patients.

**Keywords:** EGFR, Non-small cell lung cancer, Tyrosine Kinase Inhibitors.

**Introduction**

Lung cancer remains the leading cause of cancer-related mortality worldwide, being responsible for more than a million deaths each year. Its peak incidence occurs between 55 and 65 years and about 85% of all patients will die within 5 years after diagnosis. With such a mortality and a number of new cases exceeding 1.5 millions every year (1, 2), new treatment strategies beyond conventional therapy are urgently needed to overcome such a poor prognosis.

Lung cancer has been associated to cigarette smoking several decades ago, although, like in other cancers, the accumulation of genetic abnormalities in a stepwise process is part of the malignant transformation mechanism. As smoking habits progressively decreased in men, lung cancer incidence was also reduced in the male sex. By contrast, given the increasing smoking habits in women, incidence and mortality are increasing in this group. Despite this marked causal effect, only 25% of all lung cancers develop in non-smokers, more commonly in women. Most of these are adenocarcinomas. On the other hand, cigarette smoke is more often associated with small cell and squamous cell carcinomas. Other risk factors for developing lung cancer include industrial exposures (uranium, radiation, asbestos) and air pollution, not only by atmospheric pollutants but also by indoor agents like radon.

At the molecular level, numerous genetic mutations (from 10 to 20) can be usually found by the time the patient develops clinical manifestations of lung cancer. Several oncogenes have been implicated in lung carcinogenesis, including c-MYC, KRAS, EGFR, c-MET, and c-KIT. Some tumor suppressor genes are also usually found deleted or inactivated in many of these cancers, more commonly TP53, RB1, p16(INK4a), and other located on chromosome 3p. Other molecular mechanisms that contribute to lung cancer emergence and progression include increased telomerase activity (more than 80% of cases) and mutations in the mTOR pathway, more precisely LKB1, PTEN, and TSC, in up to 30% of lung cancers (3-5).

Although a large variety of cancers may develop in the lung, more than 90% are carcinomas. The four most common histologic types of those are: adenocarcinoma, squamous cell carcinoma, small cell carcinoma and large cell carcinoma, with the first being now the commonest, owing to the increasing...
incidence over the last 20 years. Other rarer types include bronchial carcinoid, salivary gland type, among others. In about 10% of cases, an admixture of several histologic types may be present. World Health Organization (WHO) histologic classification of lung tumors is one of the most widely used nowadays (Table 1) (6).

### TABLE 1: World Health Organization Histologic Classification of Malignant Epithelial Lung Tumors

<table>
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<th>Classification</th>
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<tr>
<td>Squamous cell carcinoma</td>
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<tr>
<td>Small-cell carcinoma</td>
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<tr>
<td>Combined small-cell carcinoma</td>
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<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Acinar; papillary, bronchioloalveolar, solid, mixed subtypes</td>
</tr>
<tr>
<td>Large-cell carcinoma</td>
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<tr>
<td>Large-cell neuroendocrine carcinoma</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
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<tr>
<td>Carcinomas with pleomorphic, sarcomatoid, or sarcomatoid elements</td>
</tr>
<tr>
<td>Carcinoid tumor</td>
</tr>
<tr>
<td>Typical, atypical</td>
</tr>
<tr>
<td>Carcinomas of salivary gland type</td>
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<tr>
<td>Unclassified carcinoma</td>
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Clinically, the histological classification has been clustered into 2 main subtypes, according to the likelihood of metastases and the response to conventional therapy: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). However, the dawn of innovative cancer therapies has challenged this simplistic classification scheme as it precludes an accurate management of many lung cancer patients (3, 7).

NSCLC accounts for about 85% of all lung cancers. It includes several histotypes, with adenocarcinoma and squamous cell carcinoma being the most frequent (7). Other important histologic characteristics include the presence of bronchioloalveolar carcinoma (BAC) patterns, papillary features, solid growth, presence of necrosis and mucin production (8-12).

Since nearly 75% of NSCLC present at an advanced stage (stages III or IV) at the time of diagnosis, surgical resection is rarely an option, and, consequently, chemotherapy and/or radiotherapy are generally recommended as first line therapy. However, conventional treatment (surgery, radiation and/or platinum-based doublet chemotherapy) has apparently reached a plateau, since little increments above the overall 5-year survival rate of 15% have been achieved (1, 2).

The recent advances in cancer biology identified molecular pathways responsible for cancer cell survival and growth. This led to the development of new agents that specifically target the molecules involved in those pathways. Such targeted therapies should result, at least theoretically, in superior therapeutic efficacy and less toxicity (13). In NSCLC, epidermal growth factor receptor (EGFR) has become a major therapeutic target owing to the fact that it is frequently overexpressed, mutated or both. EGFR is one of a large number of similarly structured receptors containing an intracellular tyrosine kinase (TK) domain that activate several downstream signaling pathways that, ultimately, lead to cell growth and survival (13, 14).

Gefitinib and Erlotinib are selective EGFR-TK inhibitors (TKI) that are being used in clinical practice for advanced NSCLC treatment, as well as the monoclonal antibody Cetuximab against the extracellular portion of EGFR. Despite these and other new similar drugs in study have proved to be very active in certain patients, only a small proportion of all cases actually responds to these therapies. Because those therapies have important side effects and are very expensive, it is of major importance to accurately identify the patients that will benefit from them. It is now widely accepted that certain patient characteristics are correlated with improved response to these agents: never-smoking history, adenocarcinoma histology, female gender, East Asian origin, as well as other molecular predictors of response. However, these are not sufficient to accurately triage patients for targeted therapies, thus requiring the identification of molecular biomarkers predictive of response (13, 15).

The goal of this review is to critically summarize current knowledge on the response and resistance determinants of molecularly targeted therapies against EGFR in NSCLC, in an effort to understand its limitations, future directions and possible improvements.
**EGFR**

EGFR is one of many similar receptors that present an intracellular tyrosine kinase domain. The extracellular domain allows for the distinction between the different tyrosine kinase receptor (TKR) families.

The EGFR encoding gene is located on the short arm of chromosome 7, at 7p12, and comprises 28 exons and 27 introns. The extracellular domain is encoded by exons 1 to 16, the transmembrane domain by exon 17, and the intracellular domain by exons 18 to 28. The TK domain is encoded by exons 18 to 24, whereas exons 25 to 28 encode for the C-terminal domain.

The EGFR family is composed of 4 members: HER1 (EGFR), HER2, HER3 and HER4. When a ligand, such as EGF, binds to a single-chain EGFR, this receptor dimerizes with any of the four members of the EGFR family molecules. EGFR can dimerize with another EGFR single-chain, forming a homodimer, or form a heterodimer with another member of the family.

The assembly of these functionally active dimers is responsible for the ATP-dependent phosphorylation of tyrosine residues by the intracellular domain, leading to the activation of major intracellular pathways. The three major pathways activated by EGFR-TK are the Ras-Raf-MEK, ERK1 and ERK2 pathway (leading to cell growth), the mTOR pathway (leading to protein synthesis), and the PI3K-AKT pathway (leading to cell survival by blocking apoptosis). Abnormal activation of the EGFR pathway is responsible for the characteristic properties of cancer cells of proliferation, block of apoptosis, angiogenesis and metastatization. This abnormal activation may be initiated by one or several mechanisms including increased production of growth factors, overexpression of receptors in cells and mutation of these receptors and certain downstream enzymes, leading to constitutive activation of the intracellular pathways (14, 16, 17).

**EGFR as a therapeutic target**

The recognition of deregulation in the EGFR pathway as an important step for cancer development and the elucidation of molecular mechanisms such as somatic mutations in the TK domain of EGFR in NSCLC constituted a critical step in the development of new treatment strategies.

There are 2 main classes of EGFR antagonists in use in clinical practice for NSCLC treatment: monoclonal antibodies (Cetuximab) and tyrosine kinase inhibitors (TKI: Gefitinib and Erlotinib) (18, 19).

**Cetuximab**

Cetuximab is a human-mouse chimeric monoclonal antibody (IgG1 subtype) that acts by binding to the extracellular domain of the EGFR receptor, preventing ligand-receptor interaction, as it shows an affinity five times greater than natural ligands for the EGFR. When Cetuximab binds to EGFR, dimerization does not occur and the receptor-antibody complex is internalized and degraded, decreasing EGFR availability. In addition, Cetuximab also demonstrated the ability to mediate antibody-dependent cellular cytotoxicity (ADCC) against EGFR in vitro.

After demonstrating promising results in preclinical trials, Cetuximab was introduced into clinical trials. Despite being generally well tolerated, some common side effects reported in phase I trials included skin toxicity (flushing, acne-like rash and folliculitis), fever, chills, asthenia, transient elevations in aminotransferase levels and nausea.

After its addition to platinum-based regimens (paclitaxel and carboplatin) in phase II studies had shown clinical benefit, Cetuximab was introduced in a phase III trial (FLEX). In this trial, clinical benefit of cisplatin/vinorelbine plus Cetuximab was compared to cisplatin/vinorelbine alone. The basis for patient selection in this trial was positive immunohistochemical (IHC) staining for EGFR. Results showed statistically significant improvement in overall survival for the Cetuximab group, which led to approval of this scheme by the National Comprehensive Cancer Network (NCCN) in patients who meet the criteria for Cetuximab therapy: NSCLC stage IIIB with pleural effusion or stage IV, EGFR expression detected by IHC, age ≥ 18, ECOG PS 0-2, no known brain metastasis and no prior chemotherapy or anti-EGFR therapy.

Several trials are ongoing to determine the benefits of Cetuximab in second-line therapy or in a combination
regimen with radiotherapy. Moreover, the role of K-ras mutations in predicting the benefits of Cetuximab use in NSCLC is also under study (18, 19, 20).

**Small Molecule TKI: Gefitinib and Erlotinib**

Gefitinib was the first anti-EGFR agent to show clinical activity in NSCLC. Phase II studies (IDEAL 1 and 2) showed that Gefitinib was active as monotherapy in patients with advanced stage lung cancer previously treated with conventional chemotherapy. In the same study, 40% of all patients experienced symptom improvement and a 1-year overall survival rate ranging from 25 to 35% was observed. These data and the observation that a few patients had dramatic responses led to the approval of Gefitinib as second-line therapy, even before a phase III study (21, 22).

Interestingly, the subsequent phase III trials, ISEL and INTACT 1 and 2, failed to show an improvement in overall survival (23, 24). However, when a pre-planned subgroup analysis of ISEL data was performed, patients in the Gefitinib group who were never-smokers and of Asian ethnicity showed statistically significant longer survival than the patients in the placebo group (23). In addition, a subset analysis of all Asian patients from the ISEL trial identified other patient characteristics associated with improved survival: adenocarcinoma histology, never smokers and female gender (25). A possible explanation to these results is that a high number of chemo-resistant patients have been directed to the Gefitinib arm of the ISEL trial. The need to select patients for TKI therapy has been, therefore, evidenced.

More recently, in a much larger international trial (INTEREST), Gefitinib was compared to Docetaxel for second-line treatment of NSCLC. Similar overall survival was observed in both arms of the study, but Gefitinib showed better tolerability as well as better quality of life, given the ability of oral administration (26). These results were also demonstrated in other studies testing Gefitinib versus Docetaxel in patients who failed first-line platinum-based chemotherapy (SIGN, V-15-32 and ISTANA) (27, 28, 29).

Based on the available data, the current FDA approval is limited to patients that are currently using the drug and benefiting from its use or patients who have previously used and benefited from this treatment. In Europe, CHMP approved Gefitinib for the treatment of adult patients with locally advanced or metastatic disease harboring activating mutations of EGFR-TK across all lines of therapy (30).

Erlotinib is another TKI that, like Gefitinib, inhibits the EGFR pathway by binding to the intracellular TK domain, thus interfering with the phosphorylation of critical tyrosine residues that are responsible for signal transduction through EGFR (15).

It has also been approved for NSCLC treatment after showing anti-tumor activity in phase II trials (31, 32, 33). However, unlike Gefitinib, subsequent phase III trials demonstrated improved survival. In the BR21 trial, this TKI was tested against placebo in unselected previously treated patients with advanced NSCLC. Not only overall survival (median of 2 months), but also the duration of response and the progression-free survival were improved (9, 34). These results were similar to those obtained by Docetaxel in a second-line setting (35). Once again, the response rate was higher in Asians, women, patients with adenocarcinoma and lifetime non-smokers. Although response was better when 10 or more percent of tumor cells expressed EGFR, the presence of EGFR gene mutations was not predictive of survival benefit. Given the results of this phase III trial, Erlotinib was approved for second or third line therapy of NSCLC (9). The TRUST study is now evaluating the efficacy and tolerability of Erlotinib (150mg/day) under conditions of daily practice (36). Erlotinib was also tested against placebo as maintenance treatment for non-progressing patients who were submitted to first line platinum-based chemotherapy (SATURN trial). Once again, this TKI improved progression-free survival and overall survival as well. Although the detection of activating EGFR mutations was associated with a much greater reduction of in the risk of progression in this study, this benefit did not correlate with a correspondingly greater benefit in overall survival (37).

Neither of these two TKI has led to significant improvement in overall survival or response rate when used in combination with doublet-based first line chemotherapy (38-41). The addition of Erlotinib to Carboplatin and Paclitaxel has produced
significant results in a subgroup analysis only in lifetime non-smokers (40). Possible explanations for this fact include interactions between TKI and conventional chemotherapy (TKI induce G1 cell arrest in the presence of wild-type EGFR), blocking chemotherapy actions, and/or lack of drug or patient selection (42, 43).

A comprehensive approach to all these data leaves no doubt that patient selection is mandatory in order to obtain significant treatment responses and also to avoid unnecessary treatments in patients who will not respond to these targeted therapies. Predictors of response and of resistance will be assessed next in this article.

Clinical Predictors of Response to EGFR TKI

When the results of the first phase II trials of Gefitinib and Erlotinib were available, it became obvious that there were certain clinical and histological patient characteristics that were predictive of enhanced response to these TKI (table 2). Even though the intention when introducing a new therapy is to offer an effective treatment to the largest possible number of patients, it soon became evident that only some selected subgroups actually responded to TKI.

Adenocarcinoma histology, Asian ethnicity, female gender and never-smoking history were consistently reported as predictors of response to these therapies (9, 23, 25). The development of cutaneous side effects, like a rash, has also been reported as a good predictor of response (44, 45). Based on the elucidation of these predictive patient features from several phase II and III trials, further studies were developed to test TKI against first line chemotherapy in clinically selected cohorts. The iPASS trial included Asian patients with adenocarcinoma who were never or light smokers and the results showed that the ones receiving Gefitinib had significant advantage in progression-free survival when compared to the study arm in which Carboplatin/Paclitaxel was administrated (46). Similar results were reported after the First-SIGNAL trial, in which never-smokers with adenocarcinoma were randomized to Gefitinib or to Cisplatin/Gemcitabin. Although overall survival improvement was not achieved in the Gefitinib arm, progression-free survival was prolonged and better quality of life over chemotherapy was confirmed (47).

Non-smoking history has been consistently reported as the most likely predictor of TKI activity, besides the improved survival of non-smokers (prognostic factor). Although tobacco smoke is known to be associated with specific molecular processes in carcinogenesis, another possible explanation for the inferior response to TKI in smokers is that it accelerates the drug elimination by CYP1A1 and CYP1A2 enzymes induction. Consequently, TKI bioavailability would be reduced when compared to non-smokers (48). Since phase II studies showed that dosages may be escalated, taking toxicity into account, a phase III randomized trial, currently underway, is comparing conventional 150mg/day dosage with escalated dosage up to 300mg/day of Erlotinib in current smokers with NSCLC (Protocol MO22162). Other recent studies are also presenting promising results in using this escalated dosage approach, with manifestations of patient response to TKI, without apparent increase in toxicity (49, 50).

Although clinical predictors are of great usefulness for patient selection, the molecular mechanisms behind that enhanced response are far more important, since, ideally, they will allow a much more accurate identification of responders. Ultimately, clinical predictive factors are no more than the reflection of molecular predictive characteristics.

Histopathologic Predictors of Response to EGFR TKI

Response to EGFR TKI has also been associated with certain histological features (table 3). Adenocarcinoma has consistently been reported as a predictor of response since the first available trials. But subsequent studies noted that, among adenocarcinomas, the ones presenting BAC or papillary features are more commonly associated with a positive response. Other important predictive histological characteristics include little heterogeneity, good to moderate differentiation, absence of necrosis and solid growth and, clearly, absence of mucin production. This last feature has been strongly correlated with an absent response
to TKI, even when other positive predictors were present (8-12, 51-56).

Another peculiar type of adenocarcinoma that has first been described by Yatabe et al. is the terminal respiratory unit (TRU)-type adenocarcinoma. This entity includes most nonmucinous BAC’s, mixed acinar and BAC subtypes and also some papillary subtypes; it exhibits immunohistochemical markers of peripheral airways, namely thyroid transcription factor-1 (TTF-1) and surfactant apoproteins. This type of adenocarcinoma is more frequent in non-smoking women and, since it is associated with the presence of EGFR mutations, it is predictive of response to TKI (57-60).

Lung adenocarcinomas may also be classified according to cell type. This classification includes hobnail, columnar, polygonal, goblet and mixed cell type (61). The hobnail cell type has been significantly more associated with EGFR mutations, as well as with low mutation frequency of TP53. On the other hand, columnar and the other cell types have been associated with higher mutation frequencies of TP53, previously related to environmental carcinogens like tobacco smoke (61, 62).

The morphological features described above are now known to more likely harbor EGFR mutations and, for that reason, to significantly predict response to TKI. Since molecular analysis of tumor samples is not always available or may be too expensive for generalized use, patient stratification based on clinicopathologic features may be a valuable alternative for accurate use of EGFR TKI.

<table>
<thead>
<tr>
<th>Table 2: Clinical predictors of response to EGFR tyrosine kinase inhibitors</th>
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<tbody>
<tr>
<td>Smoking History</td>
</tr>
<tr>
<td>Female gender</td>
</tr>
<tr>
<td>Asian Ethnicity</td>
</tr>
<tr>
<td>Adenocarcinoma histology</td>
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<tr>
<td>Cutaneous side effects with treatment (rash)</td>
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</table>

Abbreviations: EGFR, epidermal growth factor receptor.

<table>
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<tr>
<th>Table 3: Histopathologic predictors of response to EGFR tyrosine kinase inhibitors</th>
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<tr>
<td>Adenocarcinoma with Bronchioloalveolar or Papillary features and TRU-type</td>
</tr>
<tr>
<td>Good to moderate differentiation</td>
</tr>
<tr>
<td>Little histologic heterogeneity</td>
</tr>
<tr>
<td>Absence of solid growth</td>
</tr>
<tr>
<td>Absence of necrosis</td>
</tr>
<tr>
<td>No mucin production</td>
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</table>

Abbreviations: EGFR, epidermal growth factor receptor; TRU, terminal respiratory unit.

Molecular Predictors of Response to EGFR TKI

The discovery that the EGFR pathway was somehow implicated in lung cancer development and progression has soon led to an attempt to elucidate the molecular mechanisms capable of explaining the differential patient responses to EGFR TKI (table 4).

Since then, several molecular predictors of response to these agents have been announced, although without achieving a complete consensus among the scientific community.

EGFR protein expression, EGFR gene mutations and EGFR copy number have been the most widely studied molecular predictors of response, although others have been proposed.

EGFR Protein Expression

The first biomarker to be analyzed in TKI trials was EGFR expression, assessed by IHC. The large majority of trials showed no correlation between IHC-positive tumors and response to TKI (63-66). Nevertheless, two studies identified this biomarker as a possible predictor of survival improvement if TKI were used (67, 68). In the BR21 trial, EGFR IHC-positive patients had a significant improvement in survival when using Erlotinib in comparison to placebo, and IHC-negative patients showed no difference between the two study groups (67).
In presence of these conflicting results, IHC assessment of EGFR expression has not been considered an optimal approach to patient selection, although EGFR IHC-positive patients should not be denied TKI therapy.

**EGFR Gene Mutations**

When the EGFR gene was completely sequenced, the discovery that the majority of tumors responding to EGFR TKI harbored mutations in the tyrosine kinase domain was determinant to the elucidation of the reasons for differential TKI activity. Obviously, those mutations were significantly correlated with the clinicopathologic features previously known to be associated with enhanced TKI response: non-smoking history, female sex, Asian ethnicity and adenocarcinoma histology (69-71), with particular emphasis on exposure to tobacco smoke, since an increase in exposure is associated with a decrease in the likelihood of the presence of mutations (72).

These somatic mutations are thought to be primordial genetic events in lung cancer development, given their high frequency in NSCLC (5 to 20%, depending on the population studied) (73, 74). The fact that the tumors in which they are present reveal susceptibility to EGFR TKI, with response rates near 75%, suggests that they are also responsible for the maintenance of the malignant phenotype (74, 75).

All somatic activating mutations occur in the ATP-binding pocket of the TK domain of the receptor, the binding site of EGFR TKI. These are called ‘activating mutations’ since they are responsible for ligand-independent activation of TK activity (76).

EGFR gene activating mutations may occur in the first four exons of the TK domain (18 to 21), although about 85% are in-frame deletions in exon 19 (44%) or L858R single-nucleotide substitution in exon 21 (41%) (69-71, 77, 78). Other rarer mutations include G719, V765A and T783A substitutions (79), as well as in-frame duplications and/or insertions in exon 20. When present, such mutations are thought to be responsible for structural alterations in the kinase domain leading to its constitutive activation, preventing normal auto-inhibition in the absence of ligands (69-71, 77-80). This is the process by which the neoplastic cells become oncogene-addicted to EGFR (79, 81).

The prevalence of these activating mutations is significantly higher in patients with clinical predictors of response (30 to 50%) when compared to those not included in this group (~10%) (56, 67, 82-85). Furthermore, it is important to keep in mind that not all mutations are activating and that some activating mutations are associated with resistance to TKI, particularly insertions and certain substitutions in exon 20 (86).

Most studies had no difficulty in demonstrating an association between EGFR gene mutations, particularly exon 19 deletions, and response to TKI (84, 87-95). But when survival analysis of some large trials of patients receiving TKI was performed, no significant survival improvement was apparent (67, 96, 97). Furthermore, the fact that patients with mutant EGFR treated with chemotherapy showed longer survival than wild-type EGFR patients receiving the same treatment points to a better natural history of EGFR mutated tumors (INTACT trial) (96). Once again, contrasting results were also revealed in other trials, like the INTEREST trial, suggesting a predictive value for the EGFR mutations regarding response to TKI (26, 46). Another possible conclusion from several studies, some of them ongoing, is that different mutations may have different prognostic value. Indeed, some studies suggested that patients treated with TKI harboring exon 19 mutations may have a longer survival than those presenting with an exon 21 mutation (74, 75, 84, 98, 99).

Based on these data, the distinction between prognostic and predictive values of EGFR mutations needs to be sought by additional prospective studies, as well as the role of different mutations in patient survival and tumor response to TKI (76).

**EGFR Gene Copy Number**

Given the fact that response to TKI is not linear when based on the presence of EGFR mutations, it is clear that other molecular mechanisms have to be involved. Increased EGFR gene copy number has been consistently associated with enhanced sensitivity to TKI (67, 82, 100). Several methods are available for gene copy number evaluation, namely, fluorescent in situ hybridization (FISH), chromogenic in situ hybridization (CISH)
and quantitative PCR, with the first one being the most commonly used (89, 101).

Tumors displaying high polysomy or gene amplification (referred to as EGFR FISH-positive) have recurrently revealed higher response rate, longer survival and time to progression. FISH-positive status has also been associated with clinical and biological features predictive for TKI response, namely female sex, never-smoking history and presence of EGFR mutations (82, 99). Once more, conflicting results were obtained concerning survival, response rates and association with other predictors of response to TKI. Recently, EGFR gene gain was shown not to be a prognostic marker (102).

A problem that has commonly been raised when using FISH to evaluate EGFR gene numerical status is the absence of standardized procedures which allow for the correct distinction between EGFR amplification and chromosome 7 polysomy, given their distinct role in predicting susceptibility to TKI. A recent proposed solution includes further evaluation of 7q31 region to help distinguish between these two events (103).

Gene copy number has proved to be an accurate predictor of response to TKI and, although further prospective studies are required, this method should be used for patient selection. FISH technology is by far the preferred method for its evaluation, even in the need for standardization, given the conflicting results of the other available technologies.

Other Predictors of Response

An obvious approach to identify molecular predictors of response is to evaluate the state of activation of the EGFR pathway, as well the mechanisms that may be activating it. Thus, it is not surprising that the evaluation of activated members of the pathway has been suggested to be predictive markers of response to TKI. Although only a few studies are available, it seems that the combined evaluation of phosphorylated members of the EGFR pathway (increased pEGFR, pAKT, and pSTAT3 and decreased pERK1/2) might be good predictors of response (104).

HER2 is another member of the HER receptor family that has some particular characteristics. This monomer has intrinsic kinase activity and it is the preferred co-receptor for EGFR in the process of EGFR heterodimerization, since they form more stable complexes (105). Overexpression of HER2 by amplification is commonly associated with poor prognosis, and the addition of EGFR overexpression may even worsen the prognosis (106). Conflicting results about the predictive value of HER2 overexpression for TKI response have been reported. When FISH analysis is performed, positivity (gene amplification or high polysomy) seems to be related to higher response rate and time to progression, with a trend towards longer survival in response to TKI. A significant correlation was also found between HER2 FISH-positivity and EGFR FISH-positivity and the presence of EGFR mutations (107). Importantly, when an increased HER2 gene copy number is present simultaneously with EGFR mutations or increased gene copy number, a better response and improved survival with TKI is achieved, suggesting an important role for HER2 gene gain in increasing sensitivity to EGFR TKI (107-109). HER2 mutations have also been reported in NSCLC and, although they offer a potential target for therapy, they seem to be related with an absent response to EGFR TKI. These mutations were more frequently observed in never-smokers and in adenocarcinomas, mainly consisting of exon 20 insertions or duplications (110-113).

In summary, HER2 seems to be a promising complementary biomarker with an important role in patient selection for EGFR TKI, as well as a promising target for therapy, even in combination with therapy against EGFR.

<table>
<thead>
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<th>Table 4: Molecular predictors of response to EGFR tyrosine kinase inhibitors</th>
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<tr>
<td>EGFR mutations</td>
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<tr>
<td>EGFR gene gain</td>
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<td>HER2 gene gain</td>
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<tr>
<td>Increased pEGFR, pAKT and pSTAT3</td>
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<tr>
<td>Decreased pERK1/2</td>
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Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; p, phosphorylated; STAT3, signal transducer and activator of transcription 3
Molecular Predictors of Resistance

Despite the urgent need to identify the patients that may benefit from EGFR TKI, it is of no lesser importance to accurately identify patients who will not respond to these therapies (table 5), since as much as 30% of patients with NSCLC are within this group (114). It is also important to retain that resistance to EGFR TKI may be intrinsic (primary) or acquired (secondary), with different mechanisms of development and, consequently, requiring different types of alternative therapeutic approach (115).

Intrinsic Resistance

The first and most obvious factor for predicting resistance to EGFR TKI is the lack of EGFR expression. Logically, the absence of the target through which these agents act leads to therapeutic failure. Unfortunately, it is not always clear which patients will fail to respond because, in some studies, even patients with wild-type EGFR, EGFR FISH-negative status or IHC negative sometimes demonstrated marginal response. Hence, TKI should sometimes be considered even in patients not showing any clear predictor of response, in the absence of other therapeutic options (115).

More recently, retrospective data analysis has advanced that the concerted use of two EGFR status analysis methods (IHC with FISH or mutation) could result in a more accurate approach to patient selection (114).

Another example in which the target is modified is the type III EGFR mutation (EGFRvIII) that consists on the deletion of exons 2 to 7, producing a truncated receptor, lacking a portion of the extracellular ligand binding domain. This mutation confers relative resistance to Gefitinib (116).

But even when molecular predictors of response to TKI are present, resistance can occur because of inadequate inhibition of this pathway or due to concomitant presence of different pathways intervening in the process, originating target by-pass (115).

One the most common and well known tumor resistance marker is KRAS mutation. KRAS is a critical downstream effector of the EGFR pathway that is often mutated in lung adenocarcinomas (15 to 30%). KRAS mutants have consistently been associated with poor prognosis and also with a history of tobacco smoke exposure, male gender, and high-grade mucin-producing tumors (117-123). Activating mutations occur preferentially in codon 12 of exon 2, but also in codon 13 (118, 119). KRAS mutations have been traditionally associated with intrinsic resistance to TKI (120-124). Nevertheless, some recent studies have identified some cases of prolonged disease stabilization and even modest survival benefit in KRAS mutant patients receiving TKI (125). These data, along with the evidence that KRAS and EGFR mutations are mutually exclusive (84, 86), suggests that resistance to TKI may be a consequence of the absence of predictors of response and not a direct result of KRAS mutations. Furthermore, when EGFR gene copy number is increased in KRAS mutant patients (126), a modest benefit may be observed with this targeted therapy (125). A recent study emphasized the critical role of KRAS mutations in TKI resistance: even when these mutants are present in only minor cell clones of the tumor, resistance to EGFR TKI is observed (127).

More recently, a new molecular mechanism of resistance associated with never-smoking status has been reported and this EML4/ALK inversion and EGFR/KRAS mutations are mutually exclusive (128). This inversion origins a fusion transcript that is responsible for constitutive ALK signaling induction and, for this reason gives rise to a mechanism of target by-pass. Specific inhibition of this mechanism is now being studied (129, 130).

Other rarer molecular intrinsic resistance mechanisms have been reported, such as the previously mentioned HER2 mutations (110-113). The most important of those resistance mechanisms are the rare EGFR mutations (T790M, exon 20 insertions) (131), PTEN loss (132) and MET activation (133), either by increased copy number or owing to increased production of HGF, its ligand. The T790M substitution in exon 20 of the EGFR gene, responsible for the intrinsic resistance to TKI, is thought to have germline transmission, therefore resulting in inherited susceptibility to lung cancer. However, this mutation is far more prevalent in the context of acquired resistance following treatment with TKI (77, 84, 134).
Acquired Resistance

The development of acquired resistance is evidenced when tumor progression occurs after a certain time period of disease stabilization.

The acquisition of the T790M substitution is, by far, the most common mechanism of secondary resistance to EGFR TKI, which is present in about 50% of EGFR mutant tumors (135). Once thought to determine resistance by affecting drug binding to the receptor due to conformational changes, this mutation has been recently shown to increase ATP affinity at the ATP-binding pocket, preventing optimal TKI activity on their target (136). This finding would explain why the newly irreversible EGFR TKI may overcome resistance in vitro (136, 137).

Study models of acquired resistance have also identified MET amplification as a mechanism of permanent activation of the PI3K pathway, therefore escaping EGFR inhibition (138, 139). IGF1R overexpression is also a probable independent predictor of resistance to TKI (140).

The accurate identification of these resistance factors is of great value not only to predict TKI therapy failure but also to develop new target therapies that might help overcome this resistance.

A New Generation Drugs for the EGFR Pathway Inhibition

In recent years, an immense amount of knowledge on translational oncology has become available, opening avenues to a whole new field of opportunities for the development of innovative cancer therapies.

There is already a large number of targeted agents under investigation for the treatment of NSCLC, although many of them are still far from being introduced in clinical practice. The most important are summarized on table 6 (141-143).

As it became clear from the description of the action and resistance mechanisms of the EGFR pathway inhibitors, future targeted therapies must take into account the possibility to overcome these mechanisms of resistance and also to inhibit other pathways which are concomitantly activated, probably using multiple combinations of therapeutic agents.

<table>
<thead>
<tr>
<th>Table 5: Molecular predictors of resistance to EGFR tyrosine kinase inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrinsic Resistance</strong></td>
</tr>
<tr>
<td>No EGFR expression</td>
</tr>
<tr>
<td>EGFRvIII</td>
</tr>
<tr>
<td>KRAS mutations</td>
</tr>
<tr>
<td>HER2 mutations and overexpression</td>
</tr>
<tr>
<td>EML4/ALK inversion</td>
</tr>
<tr>
<td>EGFR exon 20 mutations (insertions and substitutions)</td>
</tr>
<tr>
<td>PTEN loss</td>
</tr>
<tr>
<td>MET activation</td>
</tr>
<tr>
<td>Gene amplification</td>
</tr>
<tr>
<td>HGF production</td>
</tr>
<tr>
<td><strong>Acquired Resistance</strong></td>
</tr>
<tr>
<td>EGFR exon 20 mutations</td>
</tr>
<tr>
<td>T790M</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>MET activation</td>
</tr>
<tr>
<td>Gene amplification</td>
</tr>
<tr>
<td>HGF production</td>
</tr>
<tr>
<td>IGF1R overexpression</td>
</tr>
</tbody>
</table>

Abbreviations: ALK, anaplastic lymphoma receptor tyrosine kinase; EGFR, epidermal growth factor receptor; EGFRvIII, type III EGFR mutation; EML4, Echinoderm microtubule-associated protein-like 4; HGF, hepatocyte growth factor; IGF1R, insulin-like growth factor 1 receptor; PTEN, phosphatase and tensin homolog.
Table 6: Targeted therapies in NSCLC

<table>
<thead>
<tr>
<th>Class</th>
<th>Agent</th>
<th>Target</th>
<th>Binding/Type</th>
<th>Stage of development in NSCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Generation TKI</td>
<td>Gefitinib</td>
<td>EGFR</td>
<td>Reversible</td>
<td>Approved for a restricted group of patients</td>
</tr>
<tr>
<td></td>
<td>Erlotinib</td>
<td>EGFR</td>
<td>Reversible</td>
<td>Approved</td>
</tr>
<tr>
<td>Second Generation TKI</td>
<td>EKB-569</td>
<td>EGFR</td>
<td>Irreversible</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>CL-387,785</td>
<td>EGFR</td>
<td>Irreversible</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Multi-Targeted TKI</td>
<td>HKI-272</td>
<td>EGFR, HER2</td>
<td>Irreversible</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td>Canertinib</td>
<td>EGFR, HER2, HER4</td>
<td>Irreversible</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>BIBW 2992</td>
<td>EGFR, HER2</td>
<td>Irreversible</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td>HKI-357</td>
<td>EGFR, HER2</td>
<td>Irreversible</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>Vandetanib, ZD-6474</td>
<td>EGFR, HER2, FLT1, KDR</td>
<td>Reversible</td>
<td>Phase III</td>
</tr>
<tr>
<td></td>
<td>XL647</td>
<td>EGFR, HER2, KDR, EPHB4</td>
<td>Reversible</td>
<td>Phase II</td>
</tr>
<tr>
<td>HER2 Heterodimerization</td>
<td>BMS-599626</td>
<td>EGFR, HER2</td>
<td></td>
<td>Phase I</td>
</tr>
<tr>
<td>Macrolide Derivatives</td>
<td>RAD001</td>
<td>mTOR</td>
<td></td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>CCI-779</td>
<td>mTOR</td>
<td></td>
<td>Phase II</td>
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<tr>
<td></td>
<td>AP23573</td>
<td>mTOR</td>
<td></td>
<td>Phase I</td>
</tr>
<tr>
<td>Monoclonal Antibodies</td>
<td>Cetuximab</td>
<td>EGFR</td>
<td>Chimeric mAB</td>
<td>Approved</td>
</tr>
<tr>
<td></td>
<td>Matuzumab</td>
<td>EGFR</td>
<td>Humanized mAB</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>Panitumumab</td>
<td>EGFR</td>
<td>Humanized AB</td>
<td>Phase II/III,</td>
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<tr>
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<td>Trastuzumab</td>
<td>HER2</td>
<td>Humanized mAB</td>
<td>Approved</td>
</tr>
<tr>
<td></td>
<td>Bevacizumab</td>
<td>VEGF-A</td>
<td></td>
<td>Approved</td>
</tr>
<tr>
<td>VEGF Inhibitors</td>
<td>Sorafenib</td>
<td>VEGFR2, FLT3, PDGFR, fibroblast growth factor receptor-1</td>
<td></td>
<td>Phase III</td>
</tr>
</tbody>
</table>
Conclusion and Future Perspectives

Despite the massive investment and its consequent encouraging results, lung cancer treatment still remains a complex and incomplete theme nowadays. Although countless new agents are rapidly emerging to reinforce the treatment options available, data analysis shows that they seem to have a limited spectrum of action and patients need to be accurately stratified and selected to obtain benefit and prevent unnecessary harm from the therapy.

Targeted therapies against EGFR have consistently shown that it is mandatory to identify predictors of response and of resistance, not only for clinical reasons, but also for economic ones.

Thus, in the near future, it will be fundamental to develop clinically applicable tools for patient screening to achieve a more customized approach. Eventually, the combined use of several targeted therapies directed against different targets will provide a solution to overcome some limitations and achieve optimal treatment results. Finally, although conventional therapeutic modalities will still be necessary in the upcoming years, the initial steps towards a patient-tailored therapeutic intervention have already taken place and are likely to be successful.

Disclosure of competing interests

The authors state they have no competing interests.

References


