Predictive molecular markers for EGFR-TKI in non-small cell lung cancer patients: new insights and critical aspects

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Abstract

In recent years, a number of novel agents have been investigated that target specific molecular pathways in non-small cell lung cancer (NSCLC). A great deal of effort has been focused on identifying specific markers that predict treatment response, given that a tailored approach would maximize both the therapeutic index and the cost-effectiveness. The epidermal growth factor receptor (EGFR) pathway has emerged as a key regulator of cancer cell proliferation and invasion, and several specific EGFR inhibitors have been examined. Gefitinib and erlotinib are selective EGFR tyrosine kinase inhibitors (EGFR-TKIs), demonstrating good results in selected cases both in terms of objective response rate and of overall survival. At present, EGFR gene mutations are the best positive predictive factors for TKI therapy, and a number of other potential biomarkers are being investigated as additional positive or negative predictors of response. The correct selection of patients that could benefit from these innovative therapies, based on an accurate molecular characterization, is mandatory to provide the best clinical management. Currently, the main factor limiting the characterization of metastatic NSCLC patients is the small quantity of tumor cells available for molecular analysis. In this paper we provide an overview of the most important molecular predictive markers for EGFR-TKIs therapy in NSCLC patients, and focus attention on biological samples suitable for analysis and alternative sampling approaches such as plasma- or serum-derived DNA.

Introduction

The epidermal growth factor receptor (EGFR) signaling pathway has emerged as a key signal transduction pathway in promoting cancer cell proliferation and tumor invasion. EGFR is normally found on the surface of epithelial cells and its overexpression is commonly observed in several malignancies including lung cancer. The tyrosine kinase domain of EGFR consists of an N- and a C-lobe, with ATP binding to the cleft formed between these two lobes. Activation by specific ligands or mutations leads to homodimer and heterodimer formation (with other members of the ERBB protein family). Dimerization consequently stimulates intrinsic EGFR tyrosine kinase activity and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic regulatory domain. Several signal transducers are then activated that initiate multiple signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase/AKT, and the signal transducer and activator of transcription (STAT) 3 and STAT5 pathways. All these events trigger an increase in cell proliferation, migration, metastatization, angiogenesis, and evasion of apoptosis (Figure 1).

Inhibition of the EGFR pathway with tyrosine kinase inhibitors (TKIs) has proven to be an effective treatment strategy for advanced non-small cell lung cancer (NSCLC). TKIs are a class of drugs that act on the EGFR ATP-binding site, leading to the reversible blocking of downstream signaling pathway activation. In view of results reported by the IPASS study, gefitinib (IRESSA, AstraZeneca Pharmaceuticals, Wilmington, DE, USA) was the first TKI approved by the European Medicines Agency (EMEA) for all lines of therapy in adults with locally advanced or metastatic NSCLC with activating EGFR tyrosine kinase mutations. Erlotinib was the next TKI to be developed (TARCEVA, Genentech, Inc, South San Francisco, and OSI Pharmaceuticals, Inc, Melville, NY, USA), receiving FDA approval for salvage use in unselected patients with locally advanced or metastatic NSCLC who had progressed after standard chemotherapy. Despite the fact that these drugs act specifically on EGFR, there is no direct correlation between receptor expression and therapeutic drug efficacy. Indeed, many EGFR-positive tumors do not respond to EGFR TKI therapy, while a large number of EGFR-negative tumors have been reported to respond. Moreover, although EGFR mutation status is the best predictor of response to TKIs, many NSCLC EGFR mutated patients do not respond. For this reason, a number of alternative predictive markers are currently under investigation.

In this review we focus on the most promising predictive markers for EGFR-TKIs, and discuss how this knowledge could help to improve treatment approaches. We also consider the correlation between primary tumor and metastatic lesion alterations, and discuss other biological samples suitable for the study of predictive markers, with particular attention on those obtained from non-invasive procedures such as plasma or serum-derived DNA.

Predictors of response to tyrosine kinase inhibitors

Epidermal growth factor receptor overexpression

EGFR protein expression, evaluated by immunohistochemistry (IHC), was the first putative predictive marker to be retrospectively explored in EGFR-TKI-treated NSCLC patients. Several studies have reported no correlation between EGFR levels and response to gefitinib or erlotinib. Conversely, two other studies have suggested that EGFR IHC assessment could help to identify a subset of patients achieving survival improvement. In the BR21 trial, individuals with high EGFR expression were associated with response to erlotinib (P=0.03). The univariate analysis showed a significant overall survival (OS) advantage of erlotinib compared with placebo in IHC-positive patients (HR, 0.68 (95% CI, 0.49-0.95); P=0.02), but not in IHC-negative cases (HR, 0.93 (95% CI, 0.63-1.36); P=0.70). However, the multivariate analysis did not reveal any correlation between EGFR expression and survival. These conflicting results, together with the availability of many different commer-

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cial anti-EGFR antibodies, indicate that IHC may not be the best method to determine a patient’s eligibility to receive EGFR TKI therapy.

Epidermal growth factor receptor copy number

High EGFR gene copy number (amplification or high polisomy), using fluorescent in situ hybridization (FISH), has been detected in approximately 30% of NSCLC patients, and is usually associated with poor clinical outcome. Furthermore, significant survival benefits have been observed in NSCLC patients treated with EGFR-TKIs in both phase II and phase III trials. In the ISEL trial, a double-blind randomized phase III study evaluating the efficacy of gefitinib in 1,692 individuals with locally advanced or metastatic NSCLC, high EGFR copy number was associated with a significantly longer OS than low copy number (P=0.045). Moreover, high EGFR copy number patients treated with gefitinib were associated with a 39% lower risk of death than those receiving placebo. Response rates (RR) and time to progression (TTP) were also improved in high EGFR copy number patients treated with EGFR-TKI, although these results have not been demonstrated in other published studies (Table 1). In contrast, in the study by Crinò et al., individuals receiving gefitinib, with EGFR FISH-positive tumors, appeared to have poorer outcomes than those with EGFR-FISH-negative tumors. Moreover, individuals who were EGFR-FISH-positive benefited more from vinorelbine than from gefitinib, although the latter showed an improved toxicity profile.

Somatic epidermal growth factor receptor mutations

In 2004, three different research groups showed that EGFR TK domain mutations are associated with the response of NSCLC patients to gefitinib or erlotinib. Somatic mutations were more frequently observed in patients with features known to be associated with TKI sensitivity, such as female gender, adenocarcinoma histology, Asian ethnicity, and no smoking history (“never smokers”). Following these initial observations, the majority of EGFR mutations have been reported to be found in the first four TK domain exons (Figure 2). The most common EGFR-sensitizing mutations, accounting for 85-90% of all those found in NSCLC, include the exon 19 deletion (loss of codons 746-750, ELREA amino acid sequence) and the exon 21 L858R substitution. Both mutations have been shown to enhance EGFR kinase activity and activate its downstream signaling, playing a pivotal role in NSCLC cell survival. EGFR-TKIs are thought to neutralize the excessive survival signals that cancer cells are “addicted to”, leading to dramatic apoptosis. Moreover,
activating EGFR mutations have also been shown to enhance gefitinib affinity by increasing its activity.13 Point mutations in exon 18 (G719A/C) occur in about 5% of cases, are associated with oncogenic potential in both cell culture and transgenic mouse studies,32,33 and are also correlated with moderate TKI sensitivity.13,14 A large number of studies have reported a significantly higher overall response rate (ORR >80%), OS and time to progression (TTP) in patients with activating EGFR mutations compared to wild-type individuals (ORR <10%) (Table 1).

EGFR kinase domain mutations have also been associated with acquired resistance to EGFR TKI, approximately 50% of cases being explained by the presence of a secondary mutation involving the methionine to threonine substitution in codon 790 (T790M) of exon 20.15-17 However, although the presence of T790M does not preclude a response to EGFR TKI, it is associated with significantly shorter progression free survival (PFS) compared to wild-type patients (7.7 vs. 16.5; P<0.001).18 Recently, a novel, irreversible covalent pyrimidine inhibitor that is specific for T790M has shown promising results, underlining the importance of the strategy to identify new classes of mutant-selective kinase inhibitors.19 Other less common mutations conferring modest resistance to EGFR-TKIs include the D761Y substitution and insertions in exon 20.20,21

Somatic mutations have frequently been correlated with high EGFR copy number, but supporting data on this point are still discordant (Table 1).

**KRAS mutations**

ERBB signaling pathways include downstream GTPases encoded by RAS genes. It has been estimated that 15-30% of lung adenocarcinomas contain activating mutations in the RAS family member, KRAS, most of which are found in codons 12 and 13 in exon 2.2,19,22 As a rule, EGFR and KRAS mutations are mutually exclusive, and, furthermore, it has been suggested that activation of either the EGFR or KRAS signaling pathways has similar effects on lung tumorigenesis.23 Moreover, EGFR mutations are common in tumors from patients who have smoked less than 100 cigarettes in their lifetime (“never smokers”),24 while KRAS mutations more frequently occur in individuals with a history of substantial cigarette use.25

The presence of KRAS mutations is associated with resistance to EGFR-TKI treatment,26 probably due to the fact that constitutive activation of the pathway by mutated KRAS neutralizes the inhibitory effects exerted by EGFR inhibition. However, a recent report by Jackman et al.27 demonstrated no apparent difference in survival between KRAS mutant/EGFR wild-type and KRAS wild-type/EGFR wild-type NSCLC patients. Furthermore, considering the mutually exclusive nature of KRAS and EGFR mutations, the presence of a KRAS mutation merely indicates the absence of an EGFR mutation, the main predictor of sensitivity. Taking these considerations into account, the clinical usefulness of KRAS mutations as a selective marker for EGFR-TKI sensitivity in NSCLC appears to be limited.

**MET**

MET is a high affinity tyrosine kinase receptor for hepatocyte growth factor (HGF). Interaction with its ligand has been shown to induce autophosphorylation at multiple tyrosine residues, activating downstream pathways involved in cell growth, motility, survival, invasion and metastasis.28 MET amplification has been observed in about 10-20% of NSCLC cases and is associated with shorter survival.29,30 Moreover, high MET copy number seems to correlate with shorter time to treatment failure in patients with gefitinib-sensitive activating EGFR mutations,31 although these results have not been confirmed in other studies.32 An increase in MET gene copy number is also reported to be a mechanism of acquired EGFR-TKI resistance, by driving ERBB3-dependent activation of PI3K, allowing tumor cells to bypass the activated mutant EGFR pathway.33,34 Furthermore, the acquired resistance due to MET amplification seems to occur independently of the T790M alteration.35 For these reasons, combination therapies with MET and EGFR kinase inhibitors should be considered for patients whose tumors have become resistant to gefitinib or erlotinib.36,37

**EML4-ALK**

The EML4-ALK fusion oncogene is one of the most recently identified molecular targets for the treatment of NSCLC. Consisting of a chimeric tyrosine kinase, the N-terminal of echinodermal microtubule associated protein-like 4 (EML4) is fused to the intracellular kinase domain of anaplastic lymphoma kinase

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**Figure 2. Schematic figure of EGFR mutations reported in NSCLC.** The principal mutations are located in exons 18-21, in the tyrosine kinase domain. Mutations associated with sensitivity and resistance are represented in green and orange, respectively. In frame deletions of exon 19 and the exon 21 point mutation (L858R) are the most frequent alterations, accounting for 85-90% of EGFR mutations. Nucleotide substitutions in exon 18, in particular G719C and G719S account for a further 5% of EGFR mutations, and alterations in exon 20 for another 5%.
(ALK), and the resulting fusion protein has shown oncopgenic activity in both in vitro and in vivo models. The frequency of this rearrangement is very low in NSCLC patients, about 6.7%, and is more common in young, never-smokers with adenocarcinoma. In addition, the presence of EML4-ALK is strongly associated with resistance to EGFR-TKIs and sensitivity to ALK inhibitors. Promising results have been achieved in a phase I study using the oral ALK inhibitor PF02341066 with FISH-detected ALK rearrangements, representing a new therapeutic target for this molecularly-defined subset of NSCLC patients.

p-AKT
AKT, a downstream mediator of phosphatidylinositol 3-kinase (PI3K), is a signal transduction protein that plays a central role in tumorigenesis. Moreover, its overexpression has been shown to confer resistance to chemotherapy and radiation. AKT is activated by PI3K, and it can be dysregulated because of frequent inactivation of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor gene, which negatively regulates PI3K levels. Phosphorylated-AKT has been reported to be expressed in lung cancer and it is correlated with a better response to gefitinib, EGFR gene gain and protein expression. Other studies, p-AKT expression has not shown a correlation with a better outcome of patients to EGFR-TKI. Conversely, PTEN loss, and subsequent p-AKT activation, has been associated with EGFR-TKIs resistance, by decreasing cell apoptosis.

MicroRNAs
MicroRNAs (miRNAs) are a new class of non-coding RNAs of 21-25 nucleotides implicated in cancer biology. MiRNAs post-transcriptionally regulate gene expression by binding to complementary sequences in the 3’ untranslated region (3’ UTR) of target messenger RNAs (mRNAs), suppressing protein translation and downregulating protein expression. MiRNA deregulation is fast becoming an important area of study in carcinogenesis because it can drastically influence cell physiology. Some miRNAs, for example miR-21, have been shown to be more highly expressed in patients with EGFR mutations than in those without. It has been hypothesized that aberrant miR-21 expression might contribute to lung cancer development in “never smokers” through EGFR signaling pathway activation, and that miR-21 silencing might enhance EGFR-TKI induced apoptosis. In addition, miR-128b seems to be directly implicated in EGFR regulation. In particular, miR-128b loss of heterozygosity is frequently found in tumors and correlates significantly with clinical response and survival following gefitinib treatment. The identification of miRNA oncogene regulators could therefore have far-reaching implications for lung cancer treatment, including improved patient selection for targeted agents, and the development of novel therapeutics and early disease biomarkers.

Multivariate approaches
Some studies have tried to identify specific gene expression profiles able to discriminate between patients responsive or not to EGFR-TKIs. It has been demonstrated that a gene expression signature of 180 genes has sufficient robustness and accuracy to predict sensitivity, both in cell lines and in lung adenocarcinomas. Other studies have identified specific serum proteomic profiles able to distinguish between EGFR-TKI sensitive or resistant patients. In the paper by Carbone et al., a protein expression profile was identified that is able to discriminate patients treated with bevacizumab and erlotinib that have a good or poor prognosis. Median OS of 61 and 24 weeks, and median PFS of 36 and 8 weeks, were reported in the good and poor prognosis groups, respectively. These studies have highlighted the possibility of multiparametric approaches, encompassing many members of the EGFR signaling cascade.

Correlation between primary tumor and metastases alterations
Although there is a clear and consolidated need to screen NSCLC patients for EGFR mutations, the best type of biological sample for this characterization has not yet been elucidated. Recent experience in colorectal cancer has established that KRAS mutations in the primary tumor and the metastatic lesions are identical, simplifying patient characterization for cetuximab treatment. Conversely, lung cancer studies have demonstrated substantial differences between primary and metastatic sites. Moreover, the vast majority of studies have only reported EGFR status in the primary tumor even though the main targets of NSCLC therapy are the metastases themselves. Italiano et al. were the first group to question the stability of EGFR expression during the NSCLC metastatic process. EGFR status, confirmed by IHC and FISH, was found to vary significantly between primary NSCLC and distant metastasis. Subsequent studies have confirmed these results, in particular for lung cancer brain metastases. Further investigations have extended this analysis to other downstream signaling pathway markers, such as phosphorylated Akt and MAPK, ERCC1, VEGFR and Ki67.

There is no shortage of evidence supporting the discordance in EGFR and KRAS mutations between primary tumors and the corresponding metastases. In the study by Schmid et al. on 96 paired samples of primary lung adenocarcinoma and corresponding locoregional lymph node metastases, a correspondence of EGFR and KRAS alterations in the two biological samples was observed in 14% and 31% of patients, respectively, demonstrating a substantial discordance between metastases and primary tumor that may be important for the selection of patients for EGFR-TKI therapy. Similarly, Monaco et al. demonstrated a substantial discordance in KRAS mutations between the primary tumor and corresponding synchronous or metachronous metastases, with a concordance of 18%, whereas no EGFR mutations were found. The mechanism by which metastases arise with different profiles from the primary tumor is still unclear, but the possibility of heterogeneous tumor populations, genetic drift, or clonal selection of tumor clones, may exist. Ultimately, these results advocate molecular testing for metastatic lesions in addition to, or in lieu of the primary tumors, in view of the fact that the main aim of advanced NSCLC treatment is to attack the metastatic cells.

Biological samples suitable for molecular characterization
Another important point to consider in the molecular characterization of NSCLC patients is to provide sufficient sampling materials that are not always available for inoperable stage IIIB and IV tumors. Although frozen specimens are the preferred source for EGFR and KRAS analysis, mutation testing is regularly carried out on Formalin-Fixed, Paraffin-Embedded (FFPE) specimens obtained from surgery for resectable tumors, and from biopsy for advanced tumors.

About one-third of primary NSCLC diagnoses are performed on cytological samples, and usually no other biopsy materials are available for molecular analyses. Effort has, therefore, been focused on detecting EGFR mutations in cytological samples. Results from several studies have shown that, after decontamination of cytological slides, extracted DNA is of sufficient quality for analysis. Transesophageal ultrasound-guided fine needle aspiration (EUS-FNA) has proven to be a useful method for NSCLC staging and diagnosis. Recently, in our laboratory, we have successfully used this methodology to obtain fresh lymph node material suitable for DNA extraction and EGFR analysis (P Ulivi et al., unpublished data, 2009). However, the macro-

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selection of tumor cells from fresh EUS-FNA samples cannot be performed, and so the lack of a mutation could indicate either a real absence or an insufficient number of cells in the starting material.

A non-invasive approach able to overcome the scarcity of tumor material is the analysis of DNA extracted from plasma/serum or from circulating tumor cells (CTC). It has recently been demonstrated that free-tumor derived DNA levels in plasma or serum are significantly higher in lung cancer patients compared to healthy donors.102,103 This could be explained by the presence of necrotic cells sloughed from primary tumor or circulating tumor cells, which possess the same genetic lesions.

Kimura et al. were the first group to report on the detection of EGFR mutations in tumor.104 In the 42 patients analyzed, EGFR mutations were detected in 8 tumor samples and in 7 serum samples (one of the serum positive cases was not mutated in the corresponding tumor), demonstrating a high concordance between tumor and serum.105 Subsequent studies have attempted to confirm these results in larger case-series.106-112 Indeed, using a range of different methodologies, serum/plasma EGFR mutations have been reported in over 70% of patients in which the tumor tissue showed the same mutation (Table 2).

However, in some of these studies, EGFR mutations were found in the plasma but not in the corresponding tumor tissue. In the study of Bay et al.,113 consisting of 77 patients with primary tumor EGFR mutations, 63 reported identical alterations in the matched plasma. Moreover, 7% of patients with plasma mutations had no detectable alterations in the corresponding primary tumors and, similarly, 6% of patients with tumor mutations had no detectable EGFR alterations in the corresponding plasma. The authors tried to explain this apparent inconsistency in terms of the heterogeneity of genetic tumor abnormalities, in which tumoral cells may or may not carry the mutation. The lower tumor cell content in some of the samples may also contribute to the lack of detectable mutations in some tumor tissues in which the corresponding plasma was mutated. The only study reporting a low plasma EGFR mutation frequency is that of Maheswaran et al.,4 with a sensitivity in plasma and CTC of 34% and 94%, respectively. Plasma DNA analysis has also been used to monitor patients during gefitinib treatment, for example to characterize secondary mutations, such as the T790M alteration.107 Nevertheless, the scarcity of materials obtained from the primary tumor tissue of advanced-stage lung cancer patients and from biopsy or cytological samples, highlights the potential clinical importance of plasma/serum as a surrogate tissue for genetic analysis.

Conclusions

To date, specific EGFR mutations are the only alterations strongly correlated with tumor response to EGFR-TKIs. Clearly, more studies are necessary to investigate the potential role of other promising predictive markers, such as miRNA. The scarcity of tumor samples and the poor correlation between primary and metastatic lesions represent a major problem for the molecular characterization of patients to decide the best therapeutic strategy. In view of the fact that the main goal of advanced NSCLC therapy is to treat the metastasis, analysis should be focused on the metastatic lesions. Moreover, improvements in the analysis of biological fluids such as plasma or serum could represent an important strategy to overcome these problems.

References

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Table 2. Correlation between EGFR mutation status in paired plasma and tumor samples.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>N. EGFR-mutated tumors</th>
<th>Biological material</th>
<th>Methodology</th>
<th>Mutations in paired samples (%)</th>
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<tr>
<td>Kimura, 2007</td>
<td>8</td>
<td>Serum</td>
<td>SARMS</td>
<td>6/8 (75%)</td>
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<tr>
<td>Maheswaran, 2008</td>
<td>18</td>
<td>Plasma</td>
<td>CTC</td>
<td>7/18 (39%)</td>
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<tr>
<td>Kuang, 2009</td>
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<td>Plasma</td>
<td>SARMS</td>
<td>11/12 (92%)</td>
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<tr>
<td>He, 2009</td>
<td>30</td>
<td>Plasma</td>
<td>SARMS and WAVE/Surveyor</td>
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<td>Bai, 2009</td>
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<td>Plasma</td>
<td>Mutant-enriched PCR</td>
<td>17/18 (94.4%)</td>
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<tr>
<td>Mack, 2009</td>
<td>7</td>
<td>Plasma</td>
<td>SARMS</td>
<td>63/77 (82%)</td>
</tr>
</tbody>
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CTC: circulating Tumor cells; SARMS: scorpion amplification refractory mutation system; DHPLC: denaturing high performance liquid chromatography.


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