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Review

Molecular Predictors of EGFR-TKI Sensitivity in Advanced Non–small Cell Lung Cancer

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The epidermal growth factor receptor (EGFR) is overexpressed in the majority of non-small cell lung cancers (NSCLC) and is a major target for new therapies. Specific EGFR tyrosine kinase inhibitors (TKIs) have been developed and used for the treatment of advanced NSCLC. The clinical response, however, varies dramatically among different patient cohorts. Females, East Asians, non-smokers, and patients with adenocarcinoma usually show higher response rates. Meanwhile, a number of biological factors are also associated with EGFR-TKIs responsiveness. In order to better understand the predictive value of these biomarkers and their significance in clinical application we prepared this brief review. Here we mainly focused on *EGFR* somatic mutations, *MET* amplification, *K-ras* mutations, *EGFRvIII* mutation, *EGFR* gene dosage and expression, *HER2* gene dosage and expression, and Akt phosphorylation. We think *EGFR* somatic mutation probably is the most effective molecular predictor for EGFR-TKIs responsiveness and efficacy. Mutation screening test can provide the most direct and valuable guidance for clinicians to make decision on EGFR-TKIs therapy.

Key words: non-small cell lung cancer, EGFR, somatic mutation, tyrosine kinase inhibitor, gene amplification

Introduction

Lung cancer is one of the most common human cancers and the leading cause of cancer death worldwide (1). Lung cancer is generally classified into two histological types, small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC). NSCLC accounts for approximately 85% of the cases and it is further divided into squamous-cell carcinoma (SSC), adenocarcinoma (AC), large cell carcinoma, and others (2). Adenocarcinoma has become the most prevalent subtype of NSCLC in recent decades (3, 4). The treatment of lung cancer is mainly based on the stage of cancer, patients' performance status, comorbidity, etc (5). For patients with early stage disease (stage I or II) surgical resection is considered the primary therapeutic choice. It is worth taking notice, however, that majority of NSCLC cases have reached locally advanced (stage III) or metastatic stage (stage IV) at the time of diagnosis (6), and chemotherapy is usually recommended as the first line therapy.

Chemotherapy is often considered too toxic, particularly for elderly patients and patients with poor performance status. The well-established platinum-based regimen can only bring modest survival benefit by increasing the median survival time about three months in average (7, 8). In recent years more effort has been put onto the development of molecular-targeted drugs.

Epidermal growth factor receptor (EGFR) is overexpressed in the majority of NSCLC and it is an important target in the treatment of NSCLC. EGFR is a member of the family of EGF-related tyrosine kinase receptors. Upon ligands binding, the receptors homo- or hetero-dimerize. Subsequently, it activates receptors' intrinsic tyrosine kinase activity and broad downstream signaling cascades, mainly including Ras-Raf-MAP-kinase pathway, PI3K-Akt pathway, and STAT pathway. All these have strong stimulatory effect on cell proliferation, differentiation, survival, angiogenesis and migration (9-11). EGFR has emerged as a critical tumorigenic factor in the development and progression of NSCLC (12-14). Two specific EGFR tyrosine kinase inhibitors (TKIs), gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva), have been developed and used clinically in the treatment of advanced NSCLC. These two drugs disrupt EGFR signaling by competing with adenosine triphosphate (ATP) for the binding sites at tyrosine kinase domain,

and thus inhibiting the phosphorylation and activation of EGFRs and the downstream signaling network. Both agents can induce dramatic clinical response in patients who fail chemotherapy. Erlotinib and gefitinib have been shown to have survival benefit in Caucasians and Asians respectively when compared to placebo in controlled double-blinded randomized phase III trials (15, 16). However, among unselected NSCLC patients the objective response rate is only about 10% (17, 18). Female patients, nonsmokers, East Asians, and patients with lung adenocarcinoma are noted to have higher response rates (17-19). In addition, many laboratories have found a number of other factors which are associated with EGFR-TKIs sensitivity. In order to better understand and interpret these basic and clinical research knowledge and accelerate the translation of research findings into daily medical practice, we reviewed the literature and carefully evaluated the predictive value of these biomarkers. We hope this brief review could provide useful information for clinicians, patients, and research professionals, help clinicians to select the right subgroup of NSCLC patients for EGFR-TKI therapy with high frequency of success, and to stimulate future research interest and effort in targeted therapy for NSCLC patients.

1. Somatic mutations in *EGFR*

Somatic mutation is the mutation that occurs only in somatic cells, which are in contrast to germ cells. A number of somatic mutations have been identified in the *EGFR* gene in NSCLC. In general these mutations can be classified into three major types: in-frame deletion, insertion, and mis-sense mutation. Most of the mutations are located in the tyrosine kinase coding domain (exons 18-21) of the *EGFR* gene. The amino acids 746~753 encoded by exon 19 and amino acid 858 encoded by exon 21 are two mutation hotspots, which accounts for over 80% of all the detected mutations.

Gefitinib sensitive mutations

A number of retrospective studies have reported that two activating mutations, small in-frame deletion in exon 19 (746~753) and substitution of leucine for arginine at amino acid 858 in exon 21 (L858R), have striking correlation with EGFR-TKI sensitivity (20-28). This discovery has been claimed as the most significant molecular event in lung cancer (29). Both activating mutations are able to enhance kinase activity of EGFR and the activation of its downstream signaling, and play a pivotal role in supporting NSCLC cell survival (20, 30). When specific EGFR-TKIs are applied, the excessive survival signals that cancer cells are "addicted to" are counteracted and dramatic apoptosis occurs (30, 31).

Seven phase II prospective studies (32-38) performed with gefitinib or erlotinib in *EGFR* mutation positive NSCLC patients have also demonstrated over 87% of response and disease control rate, and the duration of progression free survival ranges from 7.7 to 14 months, which is much longer than those reported in the literature by chemotherapy or other targeted therapy in unselected patient population (usually 4~6 months). In addition, the response rates were quite similar regardless race, gender, histology, or smoking history (Table 1). Some of the studies have suggested better quality of life and longer survival occurred in patients treated with gefitinib or erlotinib (26, 27, 39). All these demonstrate that EGFR activating mutations are effective predictor for EGFR-TKIs responsiveness and prognosis. Prospective randomized studies, however, are still needed to compare EGFR-TKIs with chemotherapy in NSLCLC patients with positive *EGFR* mutation to establish the role of EGFR-TKIs as the treatment choice in such patients.

Table 1 Prospective studies of gefitinib/erlotinib in *EGFR* mutation positive NSCLC patients

Author	No. of participating patients with *EGFR* mutations	Ethnicity	EGFR mutation screening method	Overall response and disease control rate	Complete response (%)	Partial response (%)	Stable disease (%)	Median progression-free survival (Months)
Yoshida K et al (35)	21	Japanese	Gene scan & cycleave real-time quantitative PCR technology	91%	3 (14%)	16 (76%)	0	7.7
Sunaga N, et al (32)	21	Japanese	Sequencing	91%	3 (14%)	13 (62%)	3 (14%)	12.9
Inoue A, et al (34)	16	Japanese	Sequencing	88%	0	12 (75%)	2 (13%)	9.7
Asahina H, et al (33)	16	Japanese	Sequencing	81%	2 (13%)	10 (62%)	1 (6%)	8.9
Paz-Ares L, et al (36)	21	Caucasian	Gene scan & TaqMan assay	91%	6 (29%)	13 (62%)	0	>8
van Zandwijk N, et al (37)	13	Caucasian	Sequencing and gene scan	92%	1 (8%)	10 (77%)	1(8%)	14
Sequist LV, et al (38)	31	Asian & others	Sequencing	94%	1 (3%)	16 (52%)	12 (39%)	9.2

Deletion in exon 19 and L858R are usually more common in women, East Asians, light smokers (less than 15 pack-years), and patients with adenocarcinoma (reviewed in (40)). Some studies have reported that exon 19 deletion is superior to L858R in prediction of response rates and survival (26, 39, 41). However, conflict results indicate there is no significant difference observed between these two mutations (33, 34). More studies are required to clarify this issue.

EGFR-TKIs resistant mutaions

T790M, D761Y, L747S, and insertion in exon 20 are associated with resistance to EGFR-TKIs (42-47). T790 is located at the key position in ATP binding cleft of EGFR and is considered the gatekeeper residue. The introduction of T790M mutation increases ATP affinity of receptors, which relatively attenuates the binding of EGFR-TKIs (48). T790M is mainly present in relapsed tumors after an initial response and secondary to EGFR-TKIs therapy (42, 43), and it accounts for about half of acquired resistance to gefitinib or elotinib (44). Therefore, T790M has been considered a specific marker for acquired resistance to EGFR-TKIs. L747S, D761Y and insertions in exon 20 also confer modest resistance to EGFR-TKIs. However, they are not as common as T790M among NSCLC patients with acquired resistance to EGFR-TKIs.

2. *MET* amplification

MET is a high affinity tyrosine kinase receptor for hepatocyte growth factor (HGF)/ scatter factor. The binding of HGF results in autophosphorylation of MET at multiple tyrosine residues and activation of many downstream signaling components, which produce profound effect on cellular motility, growth, survival, invasion, and metastasis (49). Alteration of MET pathway contributes to the development and progression of a number of human tumors. Amplification of the *MET* gene has been detected in gastric cancers (10~20%) and esophageal cancers (50, 51). In addition, activating mutations of *MET* are observed in papillary renal carcinoma (52). *MET* amplification has been observed in NSCLC and it is associated with EGFR-TKI resistance (53, 54). Its incidence is about 21% (9 out of 43) among patients with acquired resistance. Among untreated patients it occurs much less frequently (about 3%) (53). *MET* amplification is able to activate ERBB3 (HER3)-dependent PI3K/Akt pathway, and ultimately lead to gefitinib resistance (54). Its occurrence is independent of T790M (53).

3. *K-ras* mutation

Ras is one of the important molecules in the downstream of EGFR signaling pathway. Ras is able to activate serine/theronine kinase Raf, the mitogen-activated protein kinases ERK1 and ERK2, and a number of nuclear proteins to promote cell proliferation. *Ras* genes, especially *K-ras*, have been implicated in the pathogenesis and prognosis of lung cancer (55). Mutated *K-ras* can been observed among 20~30% NSCLC patients. Majority of the mutations (approximately 80~90%) are guanine to thymine transversion in codon 12, which results in constitutive activation of K-ras protein (56, 57). NSCLC patients with *K-ras* mutations are associated with unfavorable prognosis (58-60).

The correlation of *K-ras* mutations with *EGFR* mutations and gefitinib response has been investigated by several groups (61-63). In general, the mutations of *EGFR* and *K-ras* are mutually exclusive. NSCLC patients with *K-ras* mutations have poor sensitivity to EGFR-TKIs (25, 64). Screening *K-ras* mutation among NSCLC patients who are negative for *EGFR* mutations could provide additional information to avoid EGFR-TKIs.

4. Type III epidermal growth factor receptor mutation

Type III deletion mutation (EGFRvIII) is the deletion of exons 2~7, a 801bp fragment of *EGFR* cDNA, which produces a truncated receptor lacking a portion of extracellular ligand binding domain (65). The truncated receptor, however, is oncogenic. It has constitutive kinase activity, which is strong enough to activate downstream signaling cascades and gives cells growth advantage (66, 67). *EGFRvIII* has been identified in a number of human solid tumors, including glioblastoma, breast cancer, ovarian cancer, prostate cancer, and lung caner (66-69). The incidence of *EGFRvIII* in NSCLC varies among studies. Okamoto et al and Garcdia et al have identified 16% (5 of 32) and 39% (30 of 76) of *EGFRvIII* using immunochemistry staining (66, 70). In contrast, low detected rates have been reported using RT-PCR (2.8%~3.2% or undetectable) (71-73). The study performed in transgenic mouse has revealed that *EGFRvIII* mutant cancer cells are relatively resistant to EGFR-TKIs, but sensitive to irreversible EGFR inhibitor (71) and anti-EGFR antibody 806 (74).

5. *EGFR* gene dosage

Gene dosage is the number of copies of a gene present in a cell or nucleus. An increase in gene dosage means the gene is amplified. Gene amplification is a molecular mechanism responsible for oncogene overexpression. By production of multiple copies of a particular gene or genes, the phenotype that the gene confers is amplified in the cell. High copies of *EGFR* (amplification or high polysomy) have been detected in approximately 30% of NSCLC patients using fluo-

rescence in situ hybridization (FISH), and it is usually associated with poor clinical prognosis (75). High copies of *EGFR* probably is an effective predictor for better treatment response to EGFR-TKIs (Table 2)(22, 23, 76, 77). Patients who have increased copies of *EGFR* gene show significant survival benefit from EGFR-TKIs treatment in both Phase II (23, 78) and Phase III clinical trials (Iressa Survival Evaluation in Lung cancer and BR.21) (79, 80) (Table 2).

High *EGFR* copy number is frequently correlated with *EGFR* somatic mutations(22, 27, 31, 81). This casts doubt about the independent predictive value. Additional preclinical and clinical studies with large sample size are paramount to resolving this issue. Since the mutation rate of *EGFR* is much lower among Caucasians (~10%) comparing with Asians (30~50%) and a substantial portion of patients without *EGFR* mutations still benefit from EGFR-TKIs treatment, increased *EGFR* gene copy number could play its unique role in predicting EGFR-TKIs susceptibility. Japanese patients with *EGFR* gene amplification, however, do not benefit from gefitinib treatment (72).

Table 2 Detected *EGFR* copy number using FISH and EGFR-TKI treatment response in NSCLC

Study subjects	Scoring criteria		Result	Conclusion
81 (Southwest Oncology Group study 0126)	FISH negative	with no or low genomic gain (≤4copies in 40% cells)	68%	EGFR copy number is associated with improved survival after gefitinib therapy (78)
	FISH Positive	high level of polysomy (≥4copies in 40% cells)	32%	
		Gene amplification (EGFR/chr7≥2, or≥15 copies per cell in ≥10% cells)		
102	Disomy	≤2 copies in >90% of cells	35%	Gene amplification and high polysomy has higher response rate and better survival (23)
	Low trisomy	≤2 copies in ≥40% of cells, 3 copies in 10%–40% of the cells, ≥4 copies in <10% of cells	17%	
	High trisomy	≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells	2%	
	Low polysomy	≥4 copies in 10%–40% of cells	14%	
	High polysomy	≥4 copies in ≥40% of cells	20.0%	
	Gene amplification	EGFR/chr7≥2, or≥15 copies per cell in ≥10% cells	13%	
370 Phase III Iressa Survival Evaluation in Lung Cancer	Disomy	≤2 copies in >90% of cells	69%	*EGFR* gene copy number is a predictor for survival benefit from gefitinib (80).
	Low trisomy	≤2 copies in ≥40% of cells, 3 copies in 10%–40% of the cells, ≥4 copies in <10% of cells	16%	
	High trisomy	≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells	24%	
	Low polysomy	≥4 copies in 10%–40% of cells	27%	
	High polysomy	≥4 copies in ≥40% of cells	17%	
	Gene amplification	EGFR/chr7≥2, or≥15 copies per cell in ≥10% cells	14%	
125 Phase III clinical trial BR.21 study	Disomy	≤2 copies in >90% of cells	10%	High copies of *EGFR* was associated with survival benefit from Erlotinib (79).
	Low trisomy	≤2 copies in ≥40% of cells, 3 copies in 10%–40% of the cells, ≥4 copies in <10% of cells	18%	
	High trisomy	≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells	2%	
	Low polysomy	≥4 copies in 10%–39% of cells	24%	
	High polysomy	≥4 copies in ≥40% of cells	34%	
	Gene amplification	EGFR/chr7≥2, or≥15 copies per cell in ≥10% cells	11%	
183 Pooled study subjects from Italy and SWOG study 0126	FISH negative	no or low genomic gain (≤4copies in 40% cells)	68%	EGFR gene copy number is an independent predictive biomarker for survival (77)
	FISH Positive	Gene amplification (EGFR/chr7 ≥2, or ≥15 copies per cell in ≥10% cells)	32%	

Table 3 EGFR protein expression and EGFR-TKI treatment response

Sample size	Scoring criteria		Results	Conclusion
325 (Phase III clinical trial BR.21 study)	Negative	<10% cells positive for membranous staining	43%	EGFR expression is associated with erlotinib treatment response(79)
	Positive	≥10% of tumor cells positive for membranous staining	57%	
100	Negative	0~99	40%	EGFR protein status is associated with gefitinib treatment response (23)
		100~199		
	Positive	200~299	58%	
		300~400		
200 (Pooled study subjects from Italy and SWOG study 0126)	Negative	0~99	39%	EGFR protein status is associated with treatment response (77)
		100~199		
	Positive	200~299	61%	
		300~400		

379 (Phase III Iressa Survival Evaluation in Lung Cancer)	Negative	0~99	30%	EGFR protein status is associated with treatment response (80)
		100~199		
	Positive	200~299	70%	
		300~400		
50	0/1+	Negative to faint immunoreactive cells	54%	EGFR protein is not a significant predictive factor for response to gefitinib (88)
	2+/3+	Medium to strong immunoreactive cells	46%	

*Percentage of positive tumor cells per slides ×dominant intensity pattern of staining

6. EGFR protein expression

Overexpression of EGFR protein is very common in NSCLC patients (40-80%) (13, 14), and it is associated with aggressive clinical behaviors and poor prognosis (82-87). The relationship between EGFR protein level and EGFR-TKIs sensitivity has been studied intensively. Both positive (23, 77, 79, 80) and negative correlation (88, 89) have been reported (Table 3). The conflict observations partially could be attributed to the methodology (immunohistochemistry staining, IHC) applied for EGFR protein quantification because different laboratories use different antibodies, different scoring systems, and different protocols. EGFR protein is often associated with *EGFR* gene copy number (23, 75, 90, 91). Hirsch et al have recently suggested that patients with FISH and IHC double positive (approximately 23%) probably can benefit more from EGFR-TKIs (77).

7. *HER2* expression and gene dosage

HER2 is another member of erbB transmembrane receptor family. It has intrinsic kinase activity. HER2 is known to be a preferred coreceptor for EGFR in the process of EGFR heterodimerization. Increased expression of HER2 is associated with inferior survival in NSCLC patients, and high EGFR and HER2 coexpression has additive impact on unfavorable prognosis (92). Overexpression of HER2 protein is not associated with gefitinib response and survival (76, 93). Neither is *HER2* copy number (78). However, *HER2* amplification could predict gefitinib sensitivity and survival among NSCLC patients with increased *EGFR* copy number (76, 94).

8. Akt phosphorylation

The phosphatidylinositol 3′-kinases (PI3K)/Akt pathway is one of the important downstream signal transduction pathways of EGFR. It plays critical role in regulating cell survival and apoptosis. Akt activation is able to protects cells from apoptosis by inactivating pro apoptotic proteins (95, 96). Increased PI3K/Akt activity has been observed in NSCLC. Positive p-Akt expression is associated with better gefitinib responsiveness and prognosis (77, 97, 98). Conflicting result have also indicated that p-Akt is not associated with EGFR-TKI efficacy (99).

Gene expression signature and mass spectrometry

Gene expression signature and mass spectrometry are fast growing area in cancer research. Although both biotechnologies are costly, they are robust for new biomarkers discovery. For patients who are negative for EGFR mutations and/or other markers, gene expression and mass spectrometry analysis probably could introduce new insight into clinical practice to assure better clinical outcomes. By comparing the gene expression patterns of gefitinib sensitive and gefitinib resistant lung cancer, Balko and Coldren *et al* have found several novel markers associated with gefitinib sensitivity (100, 101). In addition, they have generated a multivariate model, which is supposed to provide more accurate prediction for EGFR-TKI sensitivity than single biomarkers or clinical characteristics (100).

Mass spectrometry is currently the most powerful analytic proteomic tool. Using mass spectrometry Taguchi *et al* have performed a multicohort cross-institutional study to investigate serum predictive biomarkers for clinical outcome after EGFR-TKIs treatment. They have identified eight distinct peaks and developed an algorithm, which could be used for patients selection and to predict prognosis after EGFR-TKI treatment (102). However, there are some concerns regarding the predictive value because the identities of the eight discriminatory peaks remain unknown and there are no other validation tests performed beyond their laboratory.

Discussion

Identifying a panel of predictive markers is important for selection of advanced NSCLC patients for EGFR-TKI therapy. Although several important demographic and clinical factors are associated with treatment response, *EGFR* somatic mutations are still the most effective predictor for EGFR-TKI sensitivity. *EGFR* mutation screening could be number one test to provide the most direct and valuable information to help clinicians to make treatment decision. Among NSCLC patients with EGFR-TKI susceptible mutations 70% of objective response rate or higher can be expected with progression-free survival of at least 7.7 months upon gefitinib/erlotinib treatment. Moreover, mutation analysis can also provide insight into resistance mechanisms to EGFR-TKIs by NSCLC cells.

The question, however, is who should have *EGFR* mutation screening test. We recommend all advanced NSCLC patients to consider mutation test before EGFR-TKIs treatment. For female patients with favorable clinical factors such as adenocarcinoma and/or low exposure to smoking, mutation test might not be necessary if the patients object to the test or the test is not available. Male patients with squamous-cell carcinoma or heavy smoking history and failing standard chemotherapy had little possibility responding to EGFR-TKI. It is prudent to test *EGFR* mutation before starting EGFR-TKI treatment.

Regarding the specimen and the method used for mutation analysis, we do not think the answer is universal, and the choices are multiple. By now direct sequencing is the most commonly used method for *EGFR* mutation screening although the sensitivity is often concerned, especially for heterogeneous specimens, such as pleural effusion drainage, blood or plasma. In addition, a number of genotyping methods with high sensitivity have been developed for *EGFR* mutation screening, such as single-strand conformation polymorphism (SSCP), scorpion allele specific PCR, mutation enriched PCR, and peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp. Most of them are able to detect even one *EGFR* mutant tumor cell with the presence of up to 1000-2000 normal cells(103-106). However, these sensitive methods have only been tested in small number of patients, and they are available in limited numbers of research laboratories. These methods are also needed to be standardized and validated. Therefore, under current situation direct sequencing probably is a mature method which could be used in health institutions for routine clinical mutation screening. For the commonly known mutations, such as deletion in exon 19, L858R, and T790M, gene scan, Scorpion allele specific PCR, and TaqMan genotyping assay are applicable. These methods are highly sensitive and easy to handle.

Among *EGFR* mutation negative patients, other predictive markers, such as *EGFR* copy number detected by FISH or *K-ras* mutation could provide important information in deciding the use of EGFR-TKIs for NSCLC patients.

Conclusions

EGFR mutation is the most effective molecular predictor of sensitivity in patients with advanced NSCLC to EGFR-TKIs treatment. Almost 75% of patient with *EGFR* mutations will have objective response to either gefitinib or erlotinib. Other molecular markers or methods, such as EGFR gene copy numbers, *K-ras* mutation, gene expression signature or serum protein profiles by mass spectroscopy may add additional value but require further studies.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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