

# Epidermal Growth Factor Receptor (EGFR) Targeted Therapies in Non-Small Cell Lung Cancer (NSCLC)

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**Abstract:** The Epidermal Growth Factor Receptor (EGFR) family, including EGFR, HER2, HER3, and HER4, is implicated in the development and progression of cancer, and is expressed in many human epithelial malignancies, including Non-Small Cell Lung Cancer (NSCLC). Several molecules were synthesized to inhibit the extracellular domain of EGFR, such as cetuximab (Erbiximab), the extracellular domain of HER2, such as trastuzumab (Herceptin) or the EGFR tyrosine kinase domain, such as gefitinib (Iressa) and erlotinib (Tarceva). Gefitinib and erlotinib are orally active, selective EGFR tyrosine-kinase inhibitors (EGFR-TKI) that produce objective response rates in about 10% of advanced NSCLC. More recently, erlotinib produced a significant improvement in survival when compared to placebo in pretreated NSCLCs. Among clinical characteristics, although female gender, and adenocarcinoma histology, showed to be significantly associated to TKI sensitivity, never smoking history is probably the most relevant factor. Presence of specific EGFR gene mutations or EGFR gene amplification confer a particularly sensitive phenotype, and patients with activation of the anti-apoptotic protein Akt are more sensitive, when Akt activation is sustained by a EGFR dependent mechanism. Cetuximab is a human-murine chimeric anti-EGFR IgG monoclonal antibody that has demonstrated both *in vitro* and *in vivo* antitumor activity in tumor cell lines expressing EGFR. It has shown impressive activity when combined with radiation by increasing the antitumor effect of radiation therapy. Cetuximab has a synergistic effect with cisplatin and may play a role in reversing resistance to chemotherapy. Cetuximab demonstrated to be active in pretreated NSCLCs, and its activity as first-line therapy in combination with chemotherapy is currently under evaluation. Efforts should be made for the identification of biological mechanism underlying cetuximab sensitivity and emerging data suggest that the drugs is more active in patients with EGFR gene amplification. In NSCLC, trastuzumab produced disappointing results when combined with chemotherapy, but probably patients were not properly selected. Recent findings in gefitinib treated patients support HER2 analysis by fluorescence in situ hybridization as a complementary test for selection of patient candidate for EGFR targeted therapies. Combination of EGFR targeting agents with other biological drugs is under investigation.

**Keywords:** EGFR, Tyrosine Kinase inhibitor, Gefitinib, Non-Small Cell Lung Cancer.

## INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) represents the leading cause of cancer death in the world, and more than 90% of cases are observed in current or former smokers [1]. In patients with NSCLC, genetic abnormalities are often responsible for the tumor survival advantage. Thus, drugs targeting abnormal pathways could result in dramatic tumor regression.

The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor protein found primarily on cells of

epithelial origin [2]. This receptor contains an extracellular ligand-binding domain, a transmembrane region, an intrinsic tyrosine kinase domain, and a carboxyl-terminal tail that contains specific tyrosine-containing sequences, which serve, when phosphorylated, as docking sites for signaling proteins. EGFR is a member of the erbB family, including EGFR (Erb-B1), HER2/neu (Erb-B2), HER3 (Erb-B3) and HER4 (Erb-B4). These receptors represent ideal therapeutic targets because they play a critical role in cancer proliferation and survival [3]. Several mechanisms lead to aberrant receptor activation, including receptor overexpression, gene amplification or mutation. During the last years, several agents have been tested in preclinical and clinical models, especially in NSCLC. These new drugs are monoclonal antibodies that bind to the extracellular domain of the receptor, or small molecules that inhibit the intracellular

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tyrosine kinase domain. In the present review we will present recent data on EGFR targeted therapies in NSCLC patients, focusing on clinical and biological criteria helpful for patient selection.

### Gefitinib and Erlotinib

Gefitinib (Iressa, AstraZeneca, Wilmington, DE) and Erlotinib (Tarceva, OSI Pharmaceutical, Melville, NY), are selective EGFR tyrosine kinase inhibitors (TKIs) showing antitumor activity either as single agent or in combination with chemotherapy and radiation therapy in human tumor xenografts [4-7].

Gefitinib showed promising activity in patients with advanced NSCLC, as well as in other solid cancers, such as head and neck, ovarian and prostate cancer [8-12]. The low toxicity profile and the 10% response rate, along with the prolonged disease stabilization and symptomatic improvement observed in some heavily pretreated NSCLCs, led to further evaluation of the drug especially in lung cancer patients. Because of uncertainties about the optimal biological dose of gefitinib, patients were randomly assigned to receive either 250 or 500 mg daily. The Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL 1 and 2) trials were designed to further investigate efficacy and safety of two different gefitinib doses in patients with pretreated advanced NSCLC [13-14]. The IDEAL 1 was conducted in 210 pretreated NSCLC patients recruited in Europe, Australia, South Africa and Japan [13]. Patients who had previously received one or two chemotherapy regimens, with at least one containing platinum, were randomized to gefitinib 250 mg or 500 mg daily dose. This trial showed an 18% response rate with symptomatic improvement in 40% of patients, without any significant difference between the two dose levels. In the US trial (IDEAL 2), 216 NSCLC patients who had failed two or more prior chemotherapy regimens containing platinum and docetaxel were randomly assigned to gefitinib 250 mg or 500 mg daily. This trial confirmed that gefitinib is active in heavily pretreated NSCLC patients, with a response rate of 11.8% and symptom improvement in 43% of patients in the 250 mg arm [14]. Based on the results of the IDEAL 1 and 2 trials, gefitinib received accelerated approval in May 2003. The approved indication for gefitinib

was as monotherapy for the treatment of patients with locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies. Cufer *et al.* recently presented the results of a randomized phase II trial comparing gefitinib with docetaxel as second-line therapy for patients with advanced NSCLC [15]. In this multicentric trial, 68 patients were assigned to the gefitinib arm, and 73 to the docetaxel arm. Primary end-point was symptom improvement, while secondary end points were quality of life, objective response, disease control rate, overall survival and safety. The study, conducted in patients not selected for any clinical or biological characteristic, showed that gefitinib treated patients had higher symptom and quality of life improvement, lower incidence of hematologic toxicities, and similar outcome in terms of objective response, time to progression and survival than patients receiving docetaxel. A confirmatory phase III trial (INTEREST) comparing gefitinib to docetaxel in unselected, pretreated, NSCLC patients is currently ongoing.

Erlotinib has been evaluated in a phase II trial conducted in 57 patients pretreated with chemotherapy [16]. This trial showed that erlotinib, at the daily dose of 150 mg produced a response rate of 12.3% that is similar to that reported with gefitinib in larger phase II studies [13-14]. Interestingly, response did not appear to be correlated to the extent of prior exposure to chemotherapy, and survival was significantly associated with the occurrence and degree of skin toxicity ( $p < 0.0001$ ).

More recently, six phase III trials evaluated the impact on survival of gefitinib or erlotinib alone or in combination with chemotherapy in patients with advanced or metastatic NSCLC [17-22]. The INTACT 1 trial randomly assigned 1093 chemo-naïve NSCLC patients to gemcitabine-cisplatin chemotherapy plus placebo or the same chemotherapy regimen in combination with gefitinib at 250 or 500 mg daily dose [17]. In the INTACT 2 study, 1037 untreated NSCLC patients were randomly allocated to carboplatin-paclitaxel chemotherapy plus placebo or plus two different gefitinib doses [18]. Both trials, that had overall survival as main end-point, failed to confirm any survival advantage for patients receiving gefitinib. Similar results were observed in two trials with erlotinib (Table 1). In the TALENT study, 1172

**Table 1. Phase III Trials With Tyrosine Kinase Inhibitors In Non Small Cell Lung Cancer**

Reference	Patient number	Therapy	Overall survival (months)	P value
INTACT 1 <sup>17</sup>	1093	gemcitabine-cisplatin-placebo	10.9	0.45
		gemcitabine-cisplatin-gefitinib	9.9	
INTACT 2 <sup>18</sup>	1037	carboplatin-paclitaxel-placebo	9.9	0.64
		carboplatin-paclitaxel-gefitinib	9.8	
TALENT <sup>19</sup>	1172	gemcitabine-cisplatin-placebo	10.1	N.S.
		gemcitabine-cisplatin-erlotinib	9.8	
TRIBUTE <sup>20</sup>	1059	carboplatin-paclitaxel-placebo	10.6	0.95
		carboplatin-paclitaxel-erlotinib	10.8	
BR 21 <sup>21</sup>	731	placebo	4.7	0.001
		erlotinib	6.7	
ISEL <sup>22</sup>	1692	placebo	5.1	0.09
		gefitinib	5.6	

N.S.: not significant

untreated advanced NSCLC patients received gemcitabine-cisplatin chemotherapy plus placebo or plus erlotinib 150 mg daily dose [19]. In the TRIBUTE study, a total of 1059 chemo-naïve NSCLCs were randomly allocated to carboplatin-paclitaxel plus placebo or erlotinib 150 mg daily dose [20]. These two studies demonstrated that erlotinib combined with standard chemotherapy did not confer a survival advantage over chemotherapy alone in patients with previously untreated, advanced NSCLC.

More recently, two large phase III trials evaluated the efficacy of gefitinib or erlotinib as second or third line therapy [21-22]. The BR21 trial randomly assigned 731 pretreated NSCLC to erlotinib or placebo [21]. This was the first randomized study in lung cancer demonstrating that a TKI prolongs survival after chemotherapy failure. Median survival was 6.7 months in the erlotinib arm, significantly longer than that observed in the placebo arm (4.7 months,  $p=0.001$ , Hazard Ratio:0.73). Based on these data, on November 2004, the U.S. Food and Drug Administration (FDA) has approved erlotinib for the treatment of patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen.

The enthusiasm about TKIs has been partially mitigated by the recent negative results of the ISEL trial, a study comparing gefitinib to placebo in previously treated advanced NSCLC [22]. This study randomized a total of 1692 patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen. Patients were required to be either refractory or intolerant to their most recent prior chemotherapy regimen. Refractory to chemotherapy was defined as patients having either clinical or radiological progressive disease while receiving, or within 90 days of the last dose of their chemotherapy regimen. Although response rate (7.7% versus 1.2%,  $p<0.0001$ ) and time to progression (3.0 versus 2.6 months,  $p=0.0005$ ) significantly favored the gefitinib arm, median survival was not significantly longer (5.6 versus 5.1 months, log rank  $p$  value=0.09, Hazard ratio:0.89). Although difference in dose equivalence and in mechanism of action could be responsible for the different results observed in the BR21 and in the ISEL study, it seems very likely that the greater percentage of patients refractory to chemotherapy included in the ISEL study (90% versus 21%) could have contributed to the different survival result.

More recently, two other important phase III trials have been closed prematurely. The SWOG 0023 compared gefitinib to placebo in patients with stage III NSCLC who had successfully completed chemoradiation and consolidation chemotherapy. This study has been closed based on an interim analysis showing no expected survival benefit for patients receiving gefitinib. The BR19 study is a phase III prospective randomized double-blind, placebo-controlled trial evaluating gefitinib as adjuvant therapy in completely resected NSCLC. On April 22nd, 2005 the National Cancer Institute of Canada Data and Safety Monitoring Committee, based on their review of the SWOG 0023 data, concluded that the lack of efficacy of gefitinib in the SWOG 0023 patient population warranted closure of the BR19 study.

Several hypotheses have been formulated to justify the disappointing results observed with TKIs in combination with chemotherapy [17-20] and the negative results of the most recent study with gefitinib [22]. The most obvious explanation is that patients enrolled onto the trials were not properly selected, and therefore the targeted therapy was not "targeted". Identifying and selecting patients most likely to respond to the therapy is of crucial importance, and represent the best way to improve the chance of achieving a positive result. For instance, in breast cancer, if HER2 overexpression had not been used to select patients for treatment with trastuzumab in combination with chemotherapy in a pivotal phase III study, this trial may have resulted in a negative outcome [23]. In the trials with TKIs and chemotherapy [17-20] no selection was made because, when these trials were designed, no molecular predictor for TKI sensitivity was known. EGFR expression detected by immunohistochemistry seemed not to be associated with TKI sensitivity [24-25], although recent findings suggest that, using a different antibody and scoring system, EGFR expressing patients have better response and longer survival than individuals lacking in EGFR protein [26-27].

Therefore, identification of molecular predictors for TKI sensitivity is critical for selection of patients that could have a significant benefit in clinical practice (Table 2).

#### Clinical Markers Predicting Sensitivity to TKIs

Retrospective analyses of phase II trials showed that Asian origin, female gender, adenocarcinoma histology and never smoking history were significantly associated with

**Table 2. Clinical And Biological Factors Predictive For Response And Survival To Tyrosine Kinase Inhibitor Therapy**

	Predictive for response	Predictive for survival
<b>CLINICAL</b>	<ul style="list-style-type: none"> <li>▪ Gender</li> <li>▪ histology</li> <li>▪ smoking history</li> </ul>	<ul style="list-style-type: none"> <li>▪ smoking history</li> <li>▪ response to prior therapy</li> <li>▪ performance status</li> <li>▪ histology</li> <li>▪ exposure to prior platinum</li> <li>▪ skin rash</li> </ul>
<b>BIOLOGICAL</b>	<ul style="list-style-type: none"> <li>▪ EGFR gene mutations</li> <li>▪ EGFR high polysomy/amplification</li> <li>▪ HER 2 high polysomy/amplification</li> <li>▪ AKT activation</li> </ul>	<ul style="list-style-type: none"> <li>▪ EGFR gene mutations</li> <li>▪ EGFR high polysomy/amplification</li> </ul>

response to TKIs [13-14, 16, 28]. Recent findings clearly indicate that among clinical characteristics associated with TKI sensitivity smoking history is the most relevant. In the TRIBUTE trial [20], median survival was significantly longer in never smokers treated with erlotinib than in never smoker patients treated with placebo (22.5 versus 10.1 months,  $p=0.01$ ). These results were similar to those observed in the TALENT study, where overall survival was significantly longer in never smokers treated with erlotinib compared to those treated with placebo [29]. In the ISEL trial, a pre-planned subset analysis showed that never smokers treated with gefitinib had a significantly longer survival when compared to never smokers treated with placebo (Hazard Ratio 0.67,  $p=0.01$ ), with no survival difference in smokers irrespective of the treatment [22]. Similar results were observed in the BR21 study, where survival benefit was not confined to objective responders, nor to a single gender or histology, and median survival was significantly longer in never smokers treated with erlotinib compared to never smokers treated with placebo (Hazard Ratio:0.42,  $p=0.0011$ ), without any survival difference in smokers irrespective of the therapy [30].

Two recent prospective phase II studies confirmed the increased sensitivity of never smokers to TKIs [31-32]. Importantly, in both studies, tumor shrinkage was also reported on brain metastases, confirming previous observations suggesting a potential effect of small-molecule TKIs on brain localizations [33-35]. These results suggest that gefitinib could represent a valid alternative to standard chemotherapy in never smoker NSCLC, and prospective phase III trials should compare standard chemotherapy with

TKIs in this subgroup of patients. Although drugs interfering with EGFR are generally well tolerated, skin rash and diarrhea are observed in the majority of treated patients. Development of side effects has been associated with sensitivity to gefitinib [14, 36], erlotinib [37] and cetuximab [38]. Recently, Amador *et al.* observed that polymorphic variations in the intron 1 of the EGFR gene were associated with response to TKIs providing an explanation as to why the development of skin toxicity is associated with a favorable outcome [39]. The association of TKI activity and side effects suggested that TKI dosage should be escalated in all patients to a dosage that causes skin toxicity and diarrhea [37]. Although this hypothesis is fascinating, in our opinion the possibility that this strategy will be successful is scarce. In fact, a recent research has shown that EGFR tyrosine kinases in cell lines with mutated *EGFR* are completely inhibited by lower concentrations of gefitinib than those with wild-type *EGFR* [40]. Moreover, doubling the dose of gefitinib resulted in more diarrhea, more skin toxicity, more grade 3 and 4 toxicity, more dose reductions, and more treatment discontinuations [13-14]. In addition, durable radiographic regressions and symptom improvement have been observed in the absence of rash and diarrhea.

### Biological Markers Predicting Sensitivity to TKIs

#### EGFR Gene Mutations

In May 2004, two groups have shown that mutations in the TK domain of *EGFR* are associated with sensitivity of NSCLC to gefitinib [40-41]. In total, deletions or amino acid substitutions in exons 18, 19, and 21 of *EGFR* were found in

**Table 3. Incidence of EGFR Gene Mutations in NSCLC not Treated with Tyrosine Kinase Inhibitors**

Reference	Total	Population	N Evaluated	Incidence (N/%)	N adenocarcinoma with mutation	Survival p value*
Yang <sup>43</sup>	219	Caucasian African American Other Total	177 41 1 219	25/14.1 1/2.4 0 26/12.0	25	NR
Sasaki <sup>44</sup>	102	Japanese	102	8/7.8	8	0.36
Tokumo <sup>45</sup>	120	Japanese	120	38/32.0	37	NR
Kosaka <sup>46</sup>	277	Japanese	277	111/40.0	110	0.99
Shigematsu <sup>47</sup>	617	Japanese Taiwan US Australia Total	263 93 80 83 519	71/27.0 32/34.0 11/14.0 6/7.0 120/23.0	114	0.5
Sonobe <sup>48</sup>	230	Japanese	230	82/35.6	81	NR
Wu <sup>49</sup>	135	Chinese	135	26/19.3	25	NR
Zhang <sup>50</sup>	56	Chinese	56	9/16.1	4	NR
Zhou <sup>51</sup>	80	Chinese	80	21/26.2	16	NR
Marchetti <sup>52</sup>	860	Caucasian	860	39/4.5	39	NR

\* p value of EGFR mutation positive versus EGFR wild type  
NR= Not reported

13 of 14 tumors sensitive to the drug, but in none of 11 tumors with no response. Lynch and colleagues [40] found mutations in another 2 of 25 primary NSCLCs, and Paez *et al.* [41] found EGFR mutations in 16 of 119 unselected tumors, with a striking predominance of mutations found in 15 of 58 (28%) specimens from Japan as compared to 1 of 61 from the U.S. (2%). These mutations were more frequently observed in never smokers, in female, in adenocarcinoma and in Japanese patients. To ascertain whether EGFR gene mutations were associated with gefitinib or erlotinib sensitivity, Pao *et al.* performed mutation analysis of exon 18-24 of EGFR in tumors from 10 gefitinib- and from 7 erlotinib-sensitive patients [42]. This study showed that EGFR gene mutations were present in 7 out of 10 gefitinib-sensitive and in 5 out of 7 erlotinib-sensitive tumors, confirming that the presence of EGFR gene mutations is associated with sensitivity to TKIs gefitinib and erlotinib. Moreover, it is of note that EGFR gene mutations were present in 7 out of 15 never smoker patients, and only in 4 out of 81 current or former smokers ( $p=0.0001$ ).

Several groups have recently performed EGFR gene mutation analysis in NSCLC patients treated or not treated with TKIs. In unselected series of patients not treated with TKIs (Table 3), the incidence of EGFR gene mutations ranged from 4.5% to 40% [43-52]. All these studies confirmed that these mutations are more frequent in East Asian ethnicity, female, adenocarcinomas and never smokers. The association with smoking status is of particular interest, and a recent study estimated the likelihood of EGFR

**Table 4. Incidence of EGFR Mutations and Cigarette Smoking**

Number of Pack/Year	EGFR Mutations
0	53%
1-5	29%
6-10	50%
11-24	14%
25-40	4%
41-50	8%
51-75	6%
> 75	0

mutation based on cigarette smoking exposure [53] (Table 4). No EGFR mutation has been discovered in non-malignant tissue, indicating that mutations are somatic [47]. In-frame deletion in exon 19 is the most frequent mutation, while missense mutations in exon 18, 20 or 21 are the second most common mutations [47].

In NSCLC patients treated with TKIs (Tables 5-6), EGFR gene mutations were identified in 12%-59% of cases. The difference in EGFR mutation incidence reported [26, 54-62] is due to the different ethnicity of patients and the different methods used for selection. Patients carrying EGFR

**Table 5. EGFR Mutations and Tyrosine Kinase Inhibitor Sensitivity In Published Studies**

Reference	N Evaluated	% EGFR Mutation	Association with RR (p value)	Association with TTP (p value)	Association with OS (p value)
Cappuzzo <sup>26</sup>	89	17.0	<0.001	0.04	0.09
Mitsudomi <sup>54</sup>	59	56.0	<0.0001	NR	0.005
Han <sup>55</sup>	90	18.9	<0.001	<0.001	<0.001
Kim <sup>56</sup>	27	22.2	<0.001	NR	NR

NR: Not Reported

**Table 6. EGFR Mutations and Tyrosine Kinase Inhibitor Sensitivity. Data Presented at the American Society of Clinical Oncology Annual Meeting in 2005**

Reference	N Evaluated	% EGFR Mutation	Association with RR (p value)	Association with TTP (p value)	Association with OS (p value)
Gumerlock <sup>57</sup>	65	18.0	0.026	NR	NR
Takano <sup>58</sup>	66	59.0	<0.001	<0.001	0.001
Cortes-Funes <sup>59</sup>	83	12.0	<0.001	0.002	0.02
Villafior <sup>60</sup>	34	26.4	<0.002	<0.05	Not significant
Tsai <sup>61</sup>	54	53.7	0.020	<0.001	0.029
Rosell <sup>62</sup>	68	25.0	<0.0001	NR	0.009

NR: Not Reported

gene mutations had a significantly higher response rate and time to progression, but the difference in survival was not significant in all studies (Tables 5-6). Although the majority of studies included patients enrolled onto the expanded access program of gefitinib, some of these series were enriched with responding patients, and biological analyses were retrospective. Therefore, prospective studies should validate these results. Moreover, EGFR mutation analyses conducted among the participating to large phase II-III trials with TKIs (Table 7) did not show any association of EGFR mutations with survival [20, 27, 29, 63]. A total of 228 patients enrolled onto the TRIBUTE trial [20] were evaluated for EGFR gene mutations. These mutations were found in 13% of cases, and, importantly, response rate and time to progression favored the EGFR mutated patients treated with chemotherapy plus erlotinib (response rate 53% versus 18%,  $p=0.12$ ; time to progression 12.5 versus 6.6 months,  $p=0.092$ ). Nevertheless, survival analysis showed that the outcome of patients carrying EGFR mutations was better than patients with wild type EGFR, irrespective of erlotinib therapy, suggesting that EGFR mutations are positive prognostic factors. Among the participating to the TALENT trial, 233 patients were evaluated for EGFR gene mutations [29]. These mutations were found in 9.8% of cases, and predicted for improved overall survival and time to progression compared to EGFR wild-type irrespective of the treatment, suggesting again a potential prognostic value of EGFR gene mutations. Similar results were recently reported by Lynch *et al.* in a retrospective analysis of patients enrolled in the IDEAL and INTACT trials [63]. In the IDEAL trials, EGFR gene mutation were detected in 18% of cases, more frequently in woman (57%) and adenocarcinomas (86%). Response rate was 46% in patients with EGFR gene mutation, significantly higher than in patients with wild type EGFR (10%,  $p=0.005$ ). Time to progression significantly favored patients with mutations, but overall survival was not significantly improved. In the INTACT trials, EGFR gene mutations were identified in 10.2% of evaluable specimens. In the cohort with EGFR gene mutations, response rate was higher in patients treated with chemotherapy plus gefitinib than in patients treated with chemotherapy alone (72% versus 40%). Again, time to progression and survival were longer in the cohort with EGFR gene mutations irrespective of the therapy, confirming a potential positive prognostic value of mutations [63].

Among the participating to the BR21 trial, 177 patients were successfully analyzed for EGFR gene mutation [27]. Mutations were detected in 23% of cases, and were

significantly associated with adenocarcinoma histology ( $p=0.05$ ), and Asian ethnicity ( $p=0.03$ ), with no association with gender or smoking history. Among patients treated with erlotinib, although response rate was doubled in individuals with EGFR gene mutation when compared to those with EGFR wild type (15.8% versus 7.4%), the difference was not statistically significant ( $p=0.37$ ). Moreover, no survival difference was observed in EGFR mutated patients treated with erlotinib when compared to EGFR mutated patients receiving placebo ( $p=0.13$ ).

In large studies (Table 7), EGFR gene mutations resulted not to be associated with survival probably because the benefit is not confined to responding patients, and also patients with prolonged disease stabilization have a significant survival improvement. So far, EGFR gene mutations have been detected in few patients with stable disease. In our experience, among the 21 patients with stable disease, only 1 had EGFR mutation [26]. In the BR-21 study from NCI Canada, it has been shown that when considering only patients with stable disease or progressive disease, erlotinib still produced a significant survival advantage compared to placebo [21]. Thus, stable disease contributes to the overall survival benefit derived from EGFR-TKI treatment of NSCLC.

#### EGFR Gene Copy Number

Although initial reports, conducted in small cohorts of patients selected on the basis of response to TKIs showed that EGFR gene mutations were present in almost the totality of responders [40-42], recent studies demonstrated that there is a significant fraction of patients with EGFR mutations refractory to the therapy [26-27, 55, 57], probably because not all EGFR gene mutations have the same value as predictive factor for TKI sensitivity. Moreover, response to TKI is not confined to patients with mutations, and also some individuals with EGFR gene wild-type experienced dramatic response. These findings suggest that other mechanisms are involved in TKI sensitivity, and recent findings indicate that increased copy number of the EGFR gene is critical for TKI sensitivity [26,64]. Amann *et al.* investigated whether alterations in copy number or coding sequence correlated with gefitinib sensitivity in NSCLC [64]. They found an amplified EGFR gene locus and an in-frame deletion in the EGFR kinase domain in the tumors of 3 patients who had experienced dramatic responses to gefitinib. Moreover, in examining NSCLC cell lines, they found that the presence of EGFR coding sequence alterations

**Table 7. Association of EGFR Gene Mutations with Survival In Large Phase II and III Trials**

Study	N Evaluated	EGFR mutation (%)	Association with survival
IDEAL 1&2 <sup>63</sup>	78	18.0	Not Significant
INTACT 1&2 <sup>63</sup>	312	10.2	Not Significant
TRIBUTE <sup>20</sup>	228	13.0	Not Significant
TALENT <sup>29</sup>	233	9.8	Not Significant
BR 21 <sup>27</sup>	177	23.0	Not Significant

was associated with increased gene copy number, and both phenomena were associated with enhanced sensitivity to TKIs. By using a commercial FISH probe for EGFR (Vysis/Abbot, USA), we studied the number of EGFR gene copies in 102 NSCLC patients, who were treated with gefitinib (250 mg daily) as a single agent treatment [26]. The study demonstrated a significant association between increased EGFR gene copy numbers and response to gefitinib, time to progression and survival. In particular, patients with EGFR gene high polysomy or amplification, defined as EGFR FISH+, had a significantly higher response rate (36% versus 3%,  $p < 0.001$ ), longer median time to progression (9.0 versus 2.5 months,  $p < 0.001$ ), longer median survival and higher 1-year survival rate (18.7 versus 7.0 months and 57% versus 33%, respectively,  $p = 0.03$ ) than patients with low or no EGFR gene gain, defined as EGFR FISH – . FISH+ patients were more likely to be female ( $p = 0.04$ ) and never smokers ( $p = 0.001$ ), and therefore, significantly associated with clinical characteristics known to be related to TKI sensitivity. A very interesting finding was the evidence of a significant association between EGFR mutations and EGFR gene gain ( $p = 0.01$ ), a phenomena probably important for the sensitivity to the drug. In fact, among patients with EGFR mutations responding to the therapy, 7 out of 8 were also FISH positive, and among the 6 non-responding patients with mutations, 4 presented a disomic pattern, suggesting that EGFR mutations are critical for gefitinib sensitivity only in a context of gene gain.

Using the same methods and criteria adopted in our study [26], Tsao *et al.* evaluated by FISH 125 specimens among the participating to the BR21 trial [27]. EGFR gene gain was reported in 45% of cases. In the cohort of patients treated with erlotinib, EGFR FISH positive patients had a significantly higher response rate (20% versus 2.4%,  $p = 0.03$ ), and a significantly longer survival than patients with no or low EGFR gene gain (Hazard Ratio:0.44,  $p = 0.008$ ).

Same results were observed by Hirsch *et al.*, analyzing 81 specimens from participating to the SWOG S0126 trial [65]. In the SWOG S0126 trial, patients with bronchioloalveolar carcinoma received gefitinib at the daily dose of 500 mg. Patients with increased EGFR gene copy number had a significant improvement in survival compared to individuals with no EGFR genomic gain (Hazard Ratio=2.02,  $p = 0.042$ ).

Nevertheless, two groups presented different results [58, 63]. In the retrospective analysis of IDEAL and INTACT trials, increased EGFR gene copy number was not significantly associated with response, time to progression or survival, but the number of patients evaluated was too small for any definitive conclusion [63]. Takano analyzed 66 surgical specimens from patients treated with gefitinib [58]. This study showed that patients with increased EGFR gene copy number had a significantly higher response rate (72% versus 38%,  $p = 0.005$ ), longer time to progression (9.4 versus 2.6 months,  $p = 0.038$ ), with no difference in survival (16.4 versus 15.7 months,  $p = 0.33$ ). The different results observed in these two studies [58, 63] are probably due to the low number of patients, and, more importantly, to the different methods used for EGFR gene evaluation. Both trials used quantitative real time PCR, that is not probably the best method. In our previous experience, using quantitative real time PCR, no association with clinical characteristics nor with response or survival was observed in the same cohort of patients in whom FISH analysis demonstrated to identify gefitinib sensitive population [26] (Table 8).

All together these data clearly show that FISH methodology offers a good prediction of sensitivity to EGFR inhibitors in NSCLC. Because of the association with mutations and because EGFR mutations are unusual in patients with stable disease, FISH analysis seems to be an ideal test to select patients candidate to TKI therapy. In fact, EGFR FISH test allow us to select not only patients with the higher possibility to have a benefit but also patients with intermediate benefit, such as those experiencing stable disease. Moreover FISH analysis presents other advantages over mutation analysis. FISH technique is easily applicable in any hospital, EGFR probe is commercially available and FISH can be routinely applied to stored paraffin embedded material.

#### K-ras Mutations and Phosho-Akt

Mutations in K-ras, a well known downstream signaling molecule in the EGFR signaling pathway, have been described in approximately 30% of lung adenocarcinomas [66, Fig. (1)]. K-ras mutations occur in smokers, and are significantly associated with TKI resistance [67]. Importantly, EGFR and K-ras mutations are mutually exclusive, suggesting a different pathogenic mechanism in smokers and

**Table 8. Association of EGFR Gene Copy Number with Survival In NSCLC Treated with Tyrosine Kinase Inhibitors**

Study	N Evaluated	Method	EGFR Gene Gain (%)	Association with survival (p value)
Cappuzzo <sup>26</sup>	102	FISH	33.0	0.03
Hirsch <sup>66</sup>	81	FISH	34.0	0.042
Tsao <sup>27</sup>	125	FISH	45.0	0.008
IDEAL <sup>63</sup>	78	RT-PCR	8.0	Not Significant
INTACT <sup>63</sup>	312	RT-PCR	7.0	Not Significant
Takano <sup>58</sup>	66	RT-PCR	44.0	0.33
Cappuzzo <sup>26</sup>	63	RT-PCR	36.5	Not Significant

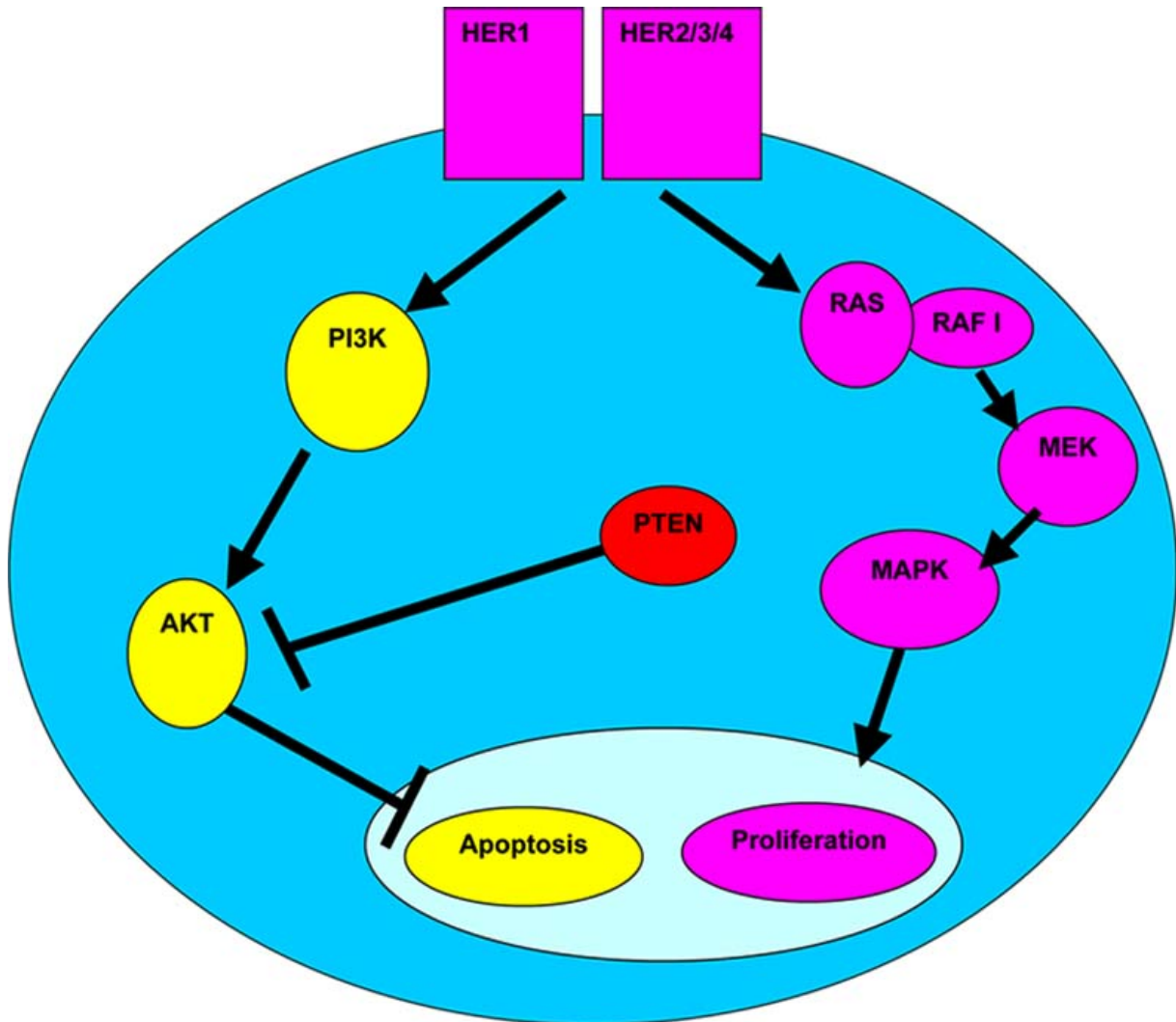


Fig. (1).

in never smoker patients [47]. Recently, Eberhard *et al.* showed that both time to progression and survival were significantly worse in patients with K-ras mutations when they were treated with erlotinib plus chemotherapy compared with those who received chemotherapy alone [68]. These findings suggest that K-ras mutations represent a potential mechanism for primary resistance to TKIs.

Phosphorylation status (a measure of activation) of the two major downstream signaling pathways, namely the phosphatidylinositol 3-kinase/Akt (Akt) and RAS/RAF/mitogen activated protein kinase (MAPK) cascades, has also undergone initial investigation as a potential molecular marker of tumor responsiveness to gefitinib.

In a recently published study, we demonstrated that P-Akt assessment by IHC is useful for selection of patients candidate to TKIs [69]. In this study, conducted in 103 NSCLC, we observed that P-Akt positive patients had a significantly higher response rate, disease control rate, and time to progression than P-Akt negative. Moreover, P-Akt positive status resulted significantly associated with being female ( $p < 0.001$ ), never-smoking history ( $p = 0.004$ ), and

with bronchioloalveolar carcinoma histology ( $p = 0.034$ ). In a more recent paper Han *et al.* also found that activation of Akt predicted gefitinib sensitivity, while expression of p-MAPK was associated with poor response [70]. Consistent with these findings is a preclinical report that anti-apoptotic (Akt and STAT), but not proliferative (Erk/MAPK), pathways are selectively activated by gefitinib-sensitising EGFR mutations [71-72], and a recent report demonstrating that Akt, but not MAPK is constitutively activated in gefitinib sensitive cell line H3255, and treatment with gefitinib completely inhibited the Akt [73]. These results suggest that gefitinib responsiveness in NSCLC cells expressing EGFR mutations may result, in large part, from its effective inhibition of essential anti-apoptotic signals transduced by the mutant receptor [71-72]. The general conclusion seems to be that high sensitivity to gefitinib in NSCLC is closely correlated with dependence on Akt activation in response to EGFR signaling. In a recently completed study we analyzed by IHC the P-Akt status of 102 tumors previously evaluated for EGFR status [26]. Irrespectively of the method of EGFR assessment, when the EGFR status was positive, presence of Akt phosphorylation

was significantly related to better response, disease control rate, time to progression and survival. Importantly, better outcome was observed not only when the group EGFR+/P-Akt+ was compared to patients negative for EGFR and/or P-Akt, but also when this group was compared to patients positive for EGFR and negative for P-Akt. These findings support the hypothesis that when the gefitinib target is present but the anti-apoptotic pathway is not activated, the patient is not sensitive to the inhibitory effects of gefitinib, as suggested by our previous study [69] and demonstrated in preclinical models [73-74]. As expected, the double positive EGFR/P-Akt group had a significantly better outcome also when compared to the group negative for EGFR and positive for P-Akt, confirming preclinical data indicating that aberrant and not EGFR dependent Akt activation may lead to gefitinib resistance [74-75].

In conclusion, these data indicate that P-Akt status assessed by IHC is a complementary test useful for the identification of EGFR positive patients with the highest possibility to have a benefit from TKI therapy.

### Monoclonal Antibodies

The two most widely tested monoclonal antibodies in NSCLC are cetuximab (Erbix), that binds to the extracellular domain of EGFR, and trastuzumab (Herceptin), that binds to HER2.

### Cetuximab

Cetuximab (C225, Erbitux, Imclone Systems Inc, New York, NY) is a human-murine chimeric anti-EGFR IgG monoclonal antibody that has demonstrated both *in vitro* and *in vivo* antitumor activity in tumor cell lines expressing the EGFR. It has shown impressive activity when combined with radiation by increasing the antitumor effect of radiotherapy [76]. Cetuximab has a synergistic effect with cisplatin and may play a role in reversing resistance to chemotherapy. In all so far published trials, cetuximab has been administered at the weekly doses of 250 mg/m<sup>2</sup> after the first loading dose of 400 mg/m<sup>2</sup>. Kim *et al.* investigated the combination of cetuximab and docetaxel as second-line treatment in chemotherapy refractory/resistant patients with advanced NSCLC [77]. In this study, conducted in 47 EGFR immunohistochemistry (IHC) positive NSCLCs, response rate was 27.6%, suggesting that this combination is active in this unfavourable group of patients. More recently, Lilenbaum *et al.* evaluated the activity of cetuximab as single agent in 60 EGFR IHC positive patients with recurrent NSCLC [78]. The majority of patients (58%) had received at least two prior chemotherapy regimens, and 13 patients were never smokers. In this study, although response rate was only 4.5%, median survival and 1-year survival were encouraging (8.1 months and 41%, respectively). Grade 3 and 4 side effects consisted of dyspnea (15.2%) and fatigue (13.6%), and only 1% of patients discontinued the trial due to toxicity. These data suggest that cetuximab is reasonably well tolerated and active in pretreated NSCLCs. Identification of biological predictor of cetuximab sensitivity is crucial for a better patient selection. It is unknown whether mutations in EGFR predict for sensitivity to cetuximab. In the Lilenbaum study, the two responding patients did not

harbour EGFR gene mutation, while 3 patients with stable or progressing disease had EGFR mutation, suggesting that mutation analysis will not likely predict for sensitivity to cetuximab and other biomarkers should be investigated, such as EGFR gene gain, as recently described in colon cancer [79].

Other three trials evaluated the combination of cetuximab with chemotherapy as first line treatment of advanced NSCLC. Rosell *et al.* conducted a randomized phase II study comparing cetuximab plus cisplatin plus vinorelbine versus cisplatin plus vinorelbine alone as first-line treatment in 86 EGFR IHC positive NSCLCs [81]. This study suggested that cetuximab can be safely combined with chemotherapy with evidence suggesting enhancement of activity. A large phase III trial comparing cisplatin-vinorelbine-cetuximab combination versus chemotherapy alone as first-line therapy is ongoing. A phase I/II trial investigated the association of cetuximab with carboplatin-gemcitabine in 35, stage IV, EGFR IHC positive NSCLC patients [81]. Response rate was observed in 22.8% of patients, and median survival was 9.2 months. Another phase I/II study evaluated the association of cetuximab with carboplatin and paclitaxel [82] in 30 EGFR IHC positive, stage IV NSCLCs. In this study, response rate was 29% and median survival was 15.7 months, supporting further evaluation of this regimen.

All these data indicate that cetuximab is a promising agent in NSCLC. Further prospective studies should define the best way to combine this agent with other new drugs in patients selected on the basis of the presence of biological predictors of drug sensitivity.

### Trastuzumab

HER2 protein is overexpressed in 25% to 30% of breast cancers, more typically as a result of gene amplification [83-84]. This overexpression is a marker of a more aggressive disease phenotype, with lower rates of estrogen receptor expression, higher rate of recurrence after surgery, and worse prognosis [85-87]. With immunohistochemistry (IHC), about 25% of NSCLC demonstrated 2+ HER2 expression, but only 6% to 8% of such tumors have 3+ overexpression. Adenocarcinomas frequently overexpress HER2, while squamous-cell carcinomas have a low frequency of HER2 expression. With FISH analysis, less than 10% of NSCLC have true HER2 gene amplification, while the majority of lung cancers that overexpress the HER2 protein have extra-copies of the HER2 gene because of polysomy of chromosome 17 [88].

Trastuzumab (Herceptin, Genentech, Inc, South San Francisco, CA) is a humanized monoclonal antibody that binds to HER2, originally developed for use against breast cancer because of the high levels of HER2 expression in breast tumors. While in breast cancer FISH analysis better correlate with trastuzumab sensitivity, in lung cancer which analysis (IHC or FISH) correlates best with clinical outcome is unknown. In preclinical studies, trastuzumab was found to have additive and synergistic effects with some chemotherapeutic agents [89]. A recent phase III trial comparing chemotherapy to chemotherapy plus trastuzumab in breast cancer patients overexpressing HER2 demonstrated that the addition of trastuzumab to chemotherapy was

associated with improvement in time to progression and overall survival [90].

Four trials evaluated the role of trastuzumab in advanced NSCLC. The Eastern Cooperative Oncology Group (ECOG) conducted a phase II trial evaluating the carboplatin-paclitaxel-trastuzumab combination in 56 HER2 positive advanced NSCLC patients. In this trial, HER2 was determined by IHC, and also 1+ HER2 positive patients were considered eligible [91]. The results indicate that this combination is feasible, with toxicity profile no worse than cytotoxic therapy alone. Krug *et al.* conducted a randomized phase II study evaluating efficacy and toxicity of trastuzumab combined with weekly docetaxel or weekly paclitaxel in 44 untreated advanced NSCLC patients. In this trial, HER2 was determined by IHC, and only 10 patients were HER2 2+ or 3+. Overall response rate was 26%; in HER2 overexpressing patients response rate was 20%, whereas it was 28% in HER2 non overexpressing patients [92]. Another phase II trial evaluated efficacy and tolerability of a gemcitabine-cisplatin-trastuzumab combination in 76 untreated advanced NSCLC patients overexpressing HER2. HER2 was determined by IHC and in serum by ELISA. Although only 13 patients were ELISA positive (6 of whom had at least 1+ at IHC), preliminary results were encouraging, with 50% of partial responses and 42% of stable diseases [93]. Gatzemeier *et al.* presented the results of a two-arm, randomized, multicenter, phase II trial, in which patients with HER2 positive NSCLC were allocated to treatment with either gemcitabine-cisplatin-trastuzumab, or gemcitabine-cisplatin alone. Although the trial failed to demonstrate any advantage for both primary (response rate) and secondary end-points (time to progression and overall survival), the low number of patients with strongly HER2 positive disease (3+ by IHC) or HER2 gene amplification preclude any firm conclusion [94]. In fact, of seven patients who had strongly HER2 positive disease as demonstrated by FISH analysis, five seemed to have a relatively long duration of stable disease or response.

A possible reason for the failure of studies combining chemotherapy with trastuzumab could be an inadequate patient selection. In all phase II trials, IHC was the method used for patient selection, but probably IHC is not optimal for HER2 analysis [25]. Preclinical data indicate that, although gefitinib selectively inhibits EGFR activity, tumors with HER2 overexpression are particularly sensitive to it [95]. Recently, we evaluated by FISH the HER2 status of a cohort of 102 NSCLC patients treated with gefitinib and previously evaluated for EGFR status by FISH, IHC and DNA sequencing [96]. In this study we observed that the group of patients with HER2 high polysomy or gene amplification (HER2 FISH+), had a significantly higher response, and a significantly longer time to progression than patients with low or no genomic gain, defined as HER2 FISH-. Importantly, independently of the method used for EGFR assessment, EGFR+ patients who also had increased copy numbers of the HER2 gene had better response rate, disease control rate, time to progression and survival. The outcome of double positive patients was significantly better than the EGFR- and/or HER2- patients, but most importantly, significant differences were also observed when double positive patients were compared with EGFR+/HER2-

patients. In particular, when EGFR was evaluated for the presence of mutations, among the 7 patients EGFR mutation+/HER2-, only one patient had major response, and this group had a similar worse outcome of EGFR mutation negative groups. These findings suggest that high copy numbers of the HER2 gene may impact its dimerization with EGFR, increasing gefitinib sensitivity. Conversely, in absence of the gefitinib target EGFR, HER2 alone is not able to drive gefitinib sensitivity. In fact, the outcome of patients EGFR-/HER2+ was not different from the outcome of patients negative for both receptors, and this double negative group of patients had the worst outcome for all the clinical endpoints. Moreover, in this study we observed the association of HER2 gene amplification with EGFR gene amplification and mutations in the tyrosine kinase domain of the EGFR gene. A recently published study confirmed our findings [64], and it is relevant that these three phenomena are associated with never smoking history [26, 40-42, 96], reinforcing the hypothesis that NSCLC arising in smokers and in never smokers are different diseases and, in never smokers, an unidentified carcinogen probably induces mutations and gene amplification [97]. These results support the use of HER2 FISH analysis as a complementary test to EGFR assay for selection of patients candidate to TKIs therapy and therapeutic strategies against both EGFR and HER2 deserve further evaluation in lung cancer.

## CONCLUSION

Novel targeted therapies are giving a new hope for patients with cancer, also in patients with NSCLC, a disease scarcely sensible to standard treatments. TKIs produced impressive and durable responses in a small fraction of patients. Moreover, the negative results observed in phase III trials, and the limited activity in other cancer types, clearly indicate that the best way to develop targeted therapies should be based on patient selection. We are moving to a new era in which cancer therapy should not be given to all patients irrespectively of their characteristics, but given only to individuals presenting the molecular target of the therapy and in whom this target is crucial for cancer cell survival.

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