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REVIEW

Biomarkers for the targeted therapies of non-small cell lung cancer

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Abstract: Targeted therapy includes new biologic agents specifically designed to selectively target molecular pathways responsible for, or that substantially drive, the malignant phenotype of cancer cells. A lot of new biologic agents have been introduced in clinical development for the treatment of advanced non-small cell lung cancer (NSCLC), but unfortunately negative results were more frequent than successes. Two pathways have been deeply studied and have led to the development of corresponding biomarkers for defining the most appropriate therapeutic approach in advanced NSCLC patients. The epidermal growth factor receptor (*EGFR*) pathway is targeted by tyrosine kinase inhibitors, gefitinib and erlotinib, and monoclonal antibody, cetuximab. *EGFR* mutation status, *EGFR* gene copy number determined by fluorescent in situ hybridization, and *EGFR* protein expression determined by immunohistochemistry have been evaluated as potential markers for clinical decision making regarding anti-*EGFR* therapy. Among these, *EGFR* mutation status resulted in the most important predictive/prognostic factor for *EGFR*-tyrosine kinase inhibitor therapy. *EGFR* protein expression seems to be important for cetuximab plus chemotherapy treatment, but further data are needed to define its role in this setting. In the last few years, the anaplastic lymphoma kinase (*ALK*) gene fusion is becoming an important biomarker in defining the specific NSCLC subtype to target with the corresponding inhibitor, crizotinib. To date, considering the *EGFR* activating mutations and the *ALK* gene fusion, generally mutually exclusive, and the availability of the correspondent inhibitors, 20% to 50% of advanced NSCLC could now be treated in Western and Eastern countries, respectively, with a targeted therapy.

Keywords: biomarkers, *EGFR*, *ALK*, NSCLC, TKI, crizotinib

Introduction

Targeted therapy, the key phrase in cancer treatment since the start of the new millennium, refers to using agents specifically designed to selectively target molecular pathways responsible for, or that substantially drive, the malignant phenotype of cancer cells. Targeted agents are designed to be selective in their effects by modulating the activity of proteins essential for the uncontrolled growth, angiogenesis, invasiveness, and metastatic processes of malignant tumors, implying that they should cause fewer side effects on normal cells. On the contrary, most chemotherapeutic agents are relatively nonselective in their activity, although their mechanisms of action work by damaging cells undergoing mitosis, which is usually more common in malignant tumors than in most normal tissues. This has led to an increase in toxicity due to the damage of normal cells too.[1]

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Non-small cell lung cancer (NSCLC) accounts for about 85% of all new lung cancer diagnosis and includes three main histological types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[2] At diagnosis more than 50% of patients have advanced disease for which systemic therapy is the standard of care to be used only in this stage of disease in which the targeted therapy has been investigated, also due to a plateau of effectiveness reached by standard chemotherapy. A lot of new biologic agents have been introduced in clinical investigation for the treatment of advanced NSCLC, but unfortunately negative results have been more frequent than successes. In fact, despite these new biologic agents being developed to block a specific target, when investigated in clinical trials no selection of patients was requested. This has led to a misunderstanding of the real therapeutic power of these drugs. To select patients in order to optimize the effect of targeted therapy, predictive and/or prognostic biomarkers of activity should be identified. The main goal of targeted therapy ought to be the so-called "personalized medicine," meaning the possibility to treat NSCLC with specific biologic characteristics representing the target for a specific inhibitor drug.

The current review will provide an update on the predictive role of the two most clinically relevant molecular biomarkers in NSCLC to date, ie, epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase (*ALK*) gene translocations.

EGFR

EGFR, also known as ErbB-1/HER1, is the first of four members of the ErbB family of cell membrane receptors which are important mediators in cell growth, differentiation, and survival.[3] The *EGFR* binds with a high affinity to several ligands, such as EGF, amphiregulin, and transforming growth factor-α, and it is highly expressed (about 40%–80%) in NSCLC. *EGFR* is the target for two classes of inhibitors: (1) the small-molecule *EGFR*-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, administered orally on a daily basis, which inhibit the *EGFR* activity by competing with adenosine triphosphate for the adenosine triphosphate-binding site localized on the *EGFR* intracellular domain; and (2) the monoclonal antibodies, such as cetuximab, administered intravenously on a weekly basis, directed against the extracellular domain of the *EGFR*, blocking ligand binding and receptor activation.[4]

EGFR mutation status, *EGFR* gene copy number determined by fluorescent in situ hybridization (FISH), and *EGFR* protein expression determined by immunohistochemistry (IHC) have been evaluated as potential markers for clinical decision making regarding anti-*EGFR* therapy.

EGFR mutation status

The identification of its activating somatic mutations in the *EGFR* gene provided the first sight of a clinically relevant NSCLC oncogene.[5–7] These mutations are usually found in exons 18–21 and are either point mutations or in-frame small deletions or insertions. The most common mutations include an in-frame deletion of exon 19 (about 45%–50% of mutations) and the L858R point mutation in exon 21 (about 40%–45% of mutations).[8–10] *EGFR* mutations are more common in patients with adenocarcinoma histology, women, Asians, and never smokers, and are detected in approximately 10%–15% of all NSCLCs in Caucasians and 20%–30% of all NSCLCs in East Asians, with prevalence increasing to 50% or more in never smokers.[8,9] The presence of activating mutations trigger the *EGFR*-signaling pathway in the absence of ligands, and promote *EGFR*-mediated prosurvival and antiapoptotic signals through downstream targets such as phosphatidylinositol-3-kinases/Akt, extracellular signal-regulated kinase/mitogen-activated protein kinase, and signal transducer and activator of transcription.[11,12] However, *EGFR* mutations also alter the tyrosine kinase pocket of the receptor to a degree that enhances the sensitivity to adenosine triphosphate-competitive *EGFR* inhibitors.[13] This is why patients affected by NSCLC harboring an activating *EGFR* mutation are particularly sensitive to EGFR-TKI therapy.

At the beginning, gefitinib and erlotinib were clinically investigated within Phase III randomized trials in unselected patients affected by advanced NSCLC in both first-line and second-line therapy, in combination with standard platinum-based chemotherapy[14–17] or administered as single agent.[18–20] Unfortunately, overall results were negative with the exception of the trial in which erlotinib was compared with placebo in previously treated NSCLC patients (BR.21 trial). In this trial, the primary endpoint was met with erlotinib improving overall survival (OS; 6.7 months versus 4.7 months, respectively; hazard ratio [HR] 0.70; $P < 0.001$). Erlotinib also reported a better objective response rate (ORR 8.9% versus >1%, respectively; $P < 0.001$) and progression-free survival (PFS; 2.2 months versus 1.8 months, respectively; HR 0.61; $P < 0.001$).[19] These results led to the marketing of erlotinib for the treatment of unselected previously treated NSCLC patients worldwide. The relative limited activity reported by gefitinib and erlotinib in non-*EGFR* genotyped, or unselected, NSCLC patients was counterbalanced by the significant clinical and radiographic responses registered

through their administration in most patients whose tumors harbor *EGFR*-activating mutations when given as first-line, second-line, or subsequent lines of therapy.[21]

Retrospective analyses from randomized trials reported that some clinical characteristics were associated with high activity of EGFR-TKIs. Thus, two Phase III randomized trials compared gefitinib versus chemotherapy as first-line treatment of advanced NSCLC patients selected for clinical characteristics.[22–24] As reported above, the possibility of detecting an *EGFR* activating mutation is higher in the patients with these clinical characteristics.

The IPASS (Iressa® Pan-Asia Study) study is a randomized Phase III trial comparing gefitinib 250 mg/day to carboplatin plus paclitaxel in 1217 Asian patients. Adenocarcinoma, including bronchioloalveolar carcinoma, and either never smokers or former light smokers were the criteria for patient selection. Gefitinib scored better than chemotherapy in terms of PFS, which was the primary endpoint of the trial (HR for PFS was 0.74; 95% confidence interval [CI]: 0.65–0.85; $P < 0.0001$). However, the median PFS was similar (5.7 months versus 5.8 months for gefitinib and chemotherapy, respectively) due to the crossing shape of the Kaplan–Meier curves, which showed a better outcome with chemotherapy in the first 6 months, but subsequently favored gefitinib. ORR was 43% for the gefitinib arm versus 32.2% for chemotherapy (odds ratio [OR] 1.59; 95% CI: 1.25–2.01; $P = 0.001$), although no differences were reported in OS (HR for death in the gefitinib group was 0.90; 95% CI: 0.79–1.02; $P = 0.109$), with a median OS of 18.8 and 17.4 months, respectively.[22,23] Preplanned retrospective analysis was performed according to molecular markers: *EGFR* mutation status, *EGFR* gene copy number, and *EGFR* protein expression. A total of 437 patients were evaluable for *EGFR* mutation status. Gefitinib scored significantly longer in PFS among the 261 patients with *EGFR* mutation-positive tumors than among those who received carboplatin/paclitaxel (HR for progression or death 0.48; 95% CI: 0.36–0.64; $P < 0.001$), whereas in the subgroup of 176 patients who were *EGFR* mutation-negative, PFS was significantly longer among those who received chemotherapy (HR for progression or death with gefitinib 2.85; 95% CI: 2.05–3.98; $P < 0.001$). When analyzing each activating mutation, a slight difference was reported in the outcomes related to the exon 19 deletion (HR with gefitinib 0.38; 95% CI: 0.26–0.56) and L858R mutation (HR with gefitinib 0.55; 95% CI: 0.35–0.87) subgroups. ORR in *EGFR* mutation-positive patients was 71.2% for gefitinib versus 47.3% for carboplatin/paclitaxel (OR 2.75; 95% CI: 1.65–4.60; $P < 0.001$), and 1.1% versus 23.5% in *EGFR* mutation-negative patients, respectively (OR 0.04; 95% CI: 0.01–0.27; $P = 0.0013$). Surprisingly, a greater ORR was reported for patients harboring the exon 19 mutation (ORR 84.8% for gefitinib and 43.2% for chemotherapy; OR 7.23; 95% CI: 3.19–16.37) and not the L858R mutation (ORR 60.9% and 53.2%, respectively; OR 1.41; 95% CI: 0.65–3.05). No difference in survival in *EGFR* mutation-positive patients (median OS of 21.6 months for gefitinib versus 21.9 months for chemotherapy, respectively; HR 1.00; 95% CI: 0.76–1.33; $P = 0.99$) or *EGFR* mutation-negative subgroup (median OS of 11.2 months for gefitinib versus 12.7 months for chemotherapy; HR 1.18; 95% CI: 0.86–1.63; $P = 0.309$) was reported. The absence of a survival advantage for the gefitinib arm could be explained by the administration of a second-line therapy with about 50% of patients who received the crossover treatment.[22,23]

In the second randomized Phase III trial (First-SIGNAL study), gefitinib was compared to cisplatin/gemcitabine in 309 patients clinically selected with eligibility criteria similar to those used in the IPASS trial.[24] OS, the primary endpoint of the study, was similar in both groups, with 22.3 months for gefitinib versus 22.9 months for chemotherapy (HR 0.932; 95% CI: 0.716–1.213; $P = 0.604$). About 30% of enrolled patients were analyzed for *EGFR* mutation status with an overall *EGFR* mutation rate of 43.8% (42 out of 96 patients). OS for the gefitinib arm was 27.2 months in the mutation-positive group (n = 26) and 18.4 months in the mutation-negative group (n = 27), while it was similar for the chemotherapy arm with 25.6 months in the mutation-positive group and 21.9 months in the mutation-negative group. However, there was also a high proportion of crossover at disease progression in the chemotherapy arm, with 75% of patients receiving a second-line EGFR-TKI. PFS was 5.8 months in the gefitinib arm versus 6.4 months for the chemotherapy arm (HR 1.198; 95% CI: 0.944–1.520; $P = 0.138$), while the curve crossed over around the median time. In the gefitinib arm, PFS was significantly shorter in the mutation-negative subgroup than in the mutation-positive subgroup with a median time of 2.1 months versus 8.0 months, while there was no difference in the cisplatin/gemcitabine arm (6.3 months versus 6.4 months).[24] ORR was 55.4% for the gefitinib arm and 46% for the cisplatin/gemcitabine group (OR 1.455; 95% CI: 0.929–2.278; $P = 0.101$). In *EGFR* mutation-positive patients, ORR was 84.6% for gefitinib versus 37.5% for chemotherapy (OR 9.167; 95% CI: 2.109–39.847; $P = 0.002$), and 25.9% for gefitinib versus 51.9% for chemotherapy in *EGFR* mutation-negative patients (OR 0.325; 95% CI: 0.103–1.021; $P = 0.051$).

Of interest is the different ORRs obtained with gefitinib in *EGFR* mutation-negative patients between the First-SIGNAL and the IPASS trial (25.9% and 1.1%, respectively). A possible explanation could be that mutation tests were not centralized in the First-SIGNAL study, and this could have determined a higher false negative rate.

Four randomized Phase III trials compared gefitinib[25–27] or erlotinib[28,29] with chemotherapy as first-line treatment of NSCLC harboring *EGFR*-activating somatic mutations. In the WJTOG (West Japan Thoracic Oncology Group) trial, 172 *EGFR* mutation-positive patients were randomized to receive gefitinib or cisplatin plus docetaxel. The median PFS, the primary endpoint, was reached with 9.2 months for the gefitinib arm and 6.3 months the chemotherapy group (HR 0.489; 95% CI: 0.336–0.710; $P < 0.0001$). ORR was significantly higher in the gefitinib arm when compared with chemotherapy (62.1% versus 32.2%, respectively). OS was 30.9 months in the experimental arm, and still not reached in the control arm.[25] In the NEJ002 (North East Japan 002) study, 228 patients were randomized to receive gefitinib or carboplatin/paclitaxel, showing a superiority for gefitinib in terms of PFS, the primary endpoint, with a median time of 10.8 months versus 5.4 months, respectively (HR 0.30; 95% CI: 0.22–0.41; $P < 0.001$). ORR was 73.7% and 30.7%, respectively ($P < 0.001$). No difference was reported in terms of survival, with a median time of 27.7 months in the gefitinib arm and 26.6 months in the chemotherapy group ($P = 0.48$).[26,27]

In the OPTIMAL trial, erlotinib (150 mg daily) was compared with carboplatin/gemcitabine in 154 Chinese patients with advanced NSCLC harboring *EGFR* mutations. The primary endpoint was PFS, which was 13.1 months with erlotinib and 4.6 months with chemotherapy (HR 0.16; 95% CI: 0.10–0.26; $P < 0.0001$). ORR was 83% with erlotinib and 36% with chemotherapy ($P < 0.0001$). The only study ever performed in European countries and investigating an EGFR-TKI in patients with advanced NSCLC harboring an activating *EGFR* mutation is the EURTAC (European Erlotinib Versus Chemotherapy) trial. In this study, erlotinib was compared with a platinum-based doublet in 174 patients. The main endpoint in this trial was also PFS, which was significantly superior in the erlotinib arm (9.7 months) compared with the chemotherapy group (5.2 months; HR 0.37; 95% CI: 0.25–0.54). ORR was 58% and 15%, respectively.[29] In both the OPTIMAL and EURTAC trials, the survival data were not mature.[28,29]

To date, according to the above mentioned results, the European Medicine Agency granted marketing authorization for gefitinib for the treatment of locally advanced or metastatic NSCLC with sensitizing mutations of the *EGFR* gene across all lines of therapy, while erlotinib has not been granted in this setting yet.

In all of these trials, the safety profile, the control of disease-related symptoms, and the global quality of life was better in the group of patients treated with EGFR-TKIs. Skin rash and diarrhea are the most frequent toxicities related to the EGFR-TKI therapy, while less common are nausea, vomiting, anorexia, and transaminase elevations, which usually are mild–moderate and regress after discontinuation of therapy. Among potentially life-threatening events, interstitial lung disease has been reported. However, it was relatively uncommon in patients treated with EGFR-TKIs, as reported by the randomized Phase III trials mentioned above.

Overall, the presence of an *EGFR* mutation resulted in a strong predictor of a better outcome with EGFR-TKIs. Despite this consideration, no survival improvement was reported. A valid explanation is that the percentage of crossover was very high, irremediably influencing the survival results; nevertheless, in all reported studies the median OS for both arms ranged between 18–30 months, results which were never observed in trials addressing advanced NSCLC patients.

The role of *EGFR* mutation status has also been investigated for cetuximab therapy. A randomized Phase III trial, BMS099, compared carboplatin/taxanes (paclitaxel or docetaxel) with or without cetuximab in 676 chemotherapy-naïve patients with advanced NSCLC, without restrictions based on histology or *EGFR* expression. The primary endpoint was median PFS, assessed by independent radiologic review committee, which was 4.4 months for cetuximab plus chemotherapy and 4.24 months for chemotherapy alone (HR 0.902; 95% CI: 0.761–1.069; $P = 0.236$). Median OS was 9.69 months versus 8.38 months, respectively (HR 0.890; 95% CI: 0.754–1.051; $P = 0.169$). ORR by independent radiologic review committee was 25.7% for cetuximab plus chemotherapy versus 17.2% for chemotherapy alone ($P = 0.007$).[30] Retrospective analyses evaluated the role of *EGFR* mutations. A total of 166 patients were evaluable for the *EGFR* mutational status and 17 (10.2%) resulted positive. Cetuximab plus chemotherapy did not significantly affect any outcome. PFS in patients with *EGFR* wild-type was 5.1 months in the cetuximab-based arm versus 4.6 months in the chemotherapy alone group (HR 0.95; 95% CI: 0.66–1.35; $P = 0.76$). In patients *EGFR* mutation-positive, PFS was 6.1 months versus 6.4 months, respectively (HR 1.17; 95% CI: 0.36–3.77; $P = 0.79$). Median OS in patients with *EGFR* wild-type was the same

in both groups (9.8 months; HR 0.91; 95% CI: 0.64–1.29; $P = 0.61$), while in *EGFR* mutation-positive patients it was 17.6 months in the cetuximab-based arm versus 20 months in the chemotherapy alone group (HR 1.62; 95% CI: 0.54–4.88; $P = 0.38$). ORR tended to be higher in *EGFR* mutation-positive patients receiving cetuximab plus chemotherapy (50% versus 11.1%; $P = 0.13$); a similar pattern was found in the *EGFR* wild-type group (32.4% versus 21.8%).[31] A randomized Phase III study (FLEX trial) enrolled 1125 patients affected by *EGFR*-expressing NSCLC to receive, as first-line therapy, cisplatin/vinorelbine with or without cetuximab. OS was the primary endpoint, which was reached with cetuximab, improving median OS significantly compared with chemotherapy alone (11.3 months versus 10.1 months, respectively; HR 0.871; 95% CI: 0.762–0.996; $P = 0.044$). The median PFS was 4.8 months in both groups (HR 0.943; 95% CI: 0.825–1.077; $P = 0.39$). ORR was 36% in the cetuximab plus chemotherapy group and 29% in the chemotherapy arm. The main cetuximab-related toxicity was acne-like rash which was grade 3 in 10% of cases.[32] Also for this trial, retrospective analyses were performed according to several biomarkers. *EGFR* kinase domain mutation status was assessable in 436 (39% of all cases) patients. Activating *EGFR* mutations were identified as indicators of good prognosis in both groups of treatment. In fact, *EGFR* mutation-positive and *EGFR* mutation-negative patients treated with chemotherapy plus cetuximab reported a median OS of 17.5 months and 8.5 months, respectively (HR 0.52; 95% CI: 0.32–0.84; $P = 0.0063$), while median OS in the chemotherapy group was 23.8 months and 10 months, respectively (HR 0.35; 95% CI: 0.21–0.59, $P < 0.0001$). PFS in the *EGFR* mutation-positive and the *EGFR* mutation-negative patients treated with chemotherapy plus cetuximab was 5.4 months and 4.2 months (HR 0.78; 95% CI: 0.51–1.18; $P = 0.24$), while in the chemotherapy arm it was 5.6 months and 4.9 months, respectively (HR 0.92; 95% CI: 0.61–1.38; $P = 0.68$). ORR in the *EGFR* mutation-positive and the *EGFR* mutation-negative patients treated with chemotherapy plus cetuximab was 46.4% and 31.7% (OR 1.87; 95% CI: 0.83–4.17; $P = 0.13$), while in the chemotherapy arm it was 38.9% and 28.5%, respectively (OR 1.60; 95% CI: 0.76–3.35; $P = 0.21$).[33]

Looking at these results, contrasting with what was reported for EGFR-TKI therapy, *EGFR* mutation status was not predictive for the efficacy of chemotherapy plus cetuximab, but it was predictive of an overall better prognosis. This finding is consistent with the role of being a prognostic factor in NSCLC.

EGFR gene copy number

Several retrospective analyses evaluated the clinical results of *EGFR* inhibitors using the predictive markers *EGFR* gene copy number detected by FISH. A retrospective analysis demonstrated that *EGFR* FISH-positive patients when treated with gefitinib had a significantly longer time to progression and survival than patients *EGFR* FISH-negative.[34] For the trial comparing carboplatin/paclitaxel with or without erlotinib (TRIBUTE trial), the results of *EGFR* FISH were available for 245 of the 1059 patients who participated in the study. In FISH-positive patients, time to progression was significantly longer (HR 0.59; 95% CI: 0.35–0.99; $P = 0.043$) for patients on erlotinib, but there was no difference in OS, nor was there any difference in time to progression or OS for the FISH-negative patients.[35] In the BR.21 trial, *EGFR* FISH-positive patients treated with erlotinib reported an ORR significantly higher ($P = 0.02$) when compared with FISH-negative patients (21.4% versus 4.8%, respectively). The survival benefit for erlotinib compared with placebo was significant in FISH-positive patients (HR 0.43; 95% CI: 0.23–0.78; $P = 0.004$), but not in FISH-negative patients (HR 0.80; 95% CI: 0.49–1.29; $P = 0.35$).[36] In the ISEL (Iressa Survival Evaluation in Lung Cancer) trial comparing gefitinib versus placebo in unselected previously treated NSCLC patients, a total of 114 patients (30.8%) were *EGFR* FISH-positive and achieved a significantly better OS with gefitinib compared with placebo than *EGFR* FISH-negative patients ($P = 0.045$). Median OS among *EGFR* FISH-positive patients was 8.3 months for gefitinib and 4.5 months for placebo. No apparent difference in OS between gefitinib and placebo was observed in *EGFR* FISH-negative patients (HR 1.16; 95% CI: 0.81–1.64; $P = 0.417$). *EGFR* FISH-positive patients achieved better ORR and time to failure than *EGFR* FISH-negative patients.[37] However, in both studies, *EGFR* FISH status was not an independent predictive factor in the multivariate analysis. This may in part be due to the lack of sufficient tumor samples for the majority of patients in these trials. In the INTEREST (Iressa NSCLC Trial Evaluating Response and Survival against Taxotere®) trial comparing gefitinib and docetaxel in unselected previously treated NSCLC patients, 374 out of 1466 randomized patients were assessable for *EGFR* FISH status. A total of 174 (47%) assessable patients were *EGFR* FISH-positive. OS was superior for gefitinib versus docetaxel in *EGFR* FISH-positive patients (HR 1.09; 95% CI: 0.78–1.51; $P = 0.62$). OS outcomes in *EGFR* FISH-negative patients were also similar for both treatments (HR 0.93; 95% CI: 0.68–1.26; $P = 0.64$). Gefitinib was similar to docetaxel in terms of PFS in *EGFR* FISH-positive (HR 0.84; 95% CI: 0.59–1.19; $P = 0.33$) and

EGFR FISH-negative patients (HR 1.30; 95% CI: 0.93–1.83; $P = 0.12$). ORR was higher in *EGFR* FISH-positive patients receiving gefitinib compared with those receiving docetaxel (13.0% versus 7.4%; $P = 0.04$).[38]

A prospective Phase II study (ONCOBELL trial), selected patients who were *EGFR* FISH-positive and were phospho-Akt positive or never smokers. Of the 183 patients who were evaluated, 42 patients were enrolled in the trial and treated with gefitinib. ORR observed was 47.6% (68% in *EGFR* FISH-positive), whereas no responses were observed in never smokers who were also negative for *EGFR* FISH and mutation. The median time to progression was 6.4 months and the 1-year survival rate was 64.3%. *EGFR* mutations were detected in 24 patients (66.8%) and ORR observed in those patients was 62.5%.[39] A randomized Phase II trial performed in elderly patients (≥70 years) compared gefitinib and vinorelbine as first-line therapy. The exploratory endpoint included the association of *EGFR* gene copy number with gefitinib and vinorelbine activity. Of the 196 patients who underwent random assignment, 191 patients provided a tumor sample and, of these, 158 were assessable for *EGFR* gene copy number detection. Surprisingly, in the 54 *EGFR* FISH-positive patients, those treated with vinorelbine achieved better PFS and survival outcomes than patients treated with gefitinib: HR was 3.13 for gefitinib (95% CI: 1.45–6.76) versus 2.88 for vinorelbine (95% CI: 1.21–6.83). Furthermore, patients who were FISH-positive and who were treated with gefitinib had a nonsignificant trend toward poorer PFS and OS outcomes compared with patients who were FISH-negative and who were treated with gefitinib: HR for FISH-positive versus FISH-negative patients who were treated with gefitinib was 1.31 (95% CI: 0.77–2.22) for PFS and 1.61 (95% CI: 0.87–3.01) for OS. On the other hand, patients who were FISH-positive and who were treated with vinorelbine had a nonsignificant trend toward improved PFS and OS outcomes than patients who were FISH-negative and who were treated with vinorelbine: HR for FISH-positive versus FISH-negative patients who were treated with vinorelbine was 0.77 (95% CI: 0.43–1.39) for PFS and 0.52 (95% CI: 0.25–1.10) for OS.[40]

In the IPASS trial, the *EGFR* gene copy number was evaluable in 406 patients. In patients *EGFR* FISH-positive (n = 249), PFS was significantly longer with gefitinib versus carboplatin/paclitaxel (HR 0.66; 95% CI: 0.50–0.88; $P = 0.005$). ORR favored gefitinib (58.9% versus 44.8%; OR 1.79; 95% CI: 1.08–2.96; $P = 0.024$). In patients *EGFR* FISH-negative (n = 157), PFS was longer (HR 1.24; 95% CI: 0.87–1.76; $P = 0.237$) and ORR was higher (26.3% versus 22.2%; OR 0.80; 95% CI: 0.38–1.68; $P = 0.558$) with carboplatin/paclitaxel versus gefitinib. However, 190 (78%) *EGFR* FISH-positive patients also harbored *EGFR* mutations. Of the 153 *EGFR* FISH-negative patients, only 51 (33%) were also *EGFR* mutation-positive. PFS was significantly shorter with gefitinib versus carboplatin/paclitaxel in *EGFR* FISH-positive patients in the absence of a coexisting *EGFR* mutation (n = 55; HR 3.85; 95% CI: 2.09–7.09), although patients with *EGFR* mutation achieved significantly longer PFS with gefitinib versus carboplatin plus paclitaxel, irrespective of whether they were *EGFR* FISH-positive (HR 0.48; 95% CI: 0.34–0.67; n = 190) or *EGFR* FISH-negative (HR 0.51; 95% CI: 0.25–1.04; n = 51). No survival difference for gefitinib versus chemotherapy in *EGFR* FISH-positive (HR 1.03; 95% CI: 0.78–1.37; $P = 0.816$) or *EGFR* FISH-negative patients (HR 1.30; 95% CI: 0.92–1.85; $P = 0.137$) was reported.[23]

A systematic review and meta-analysis, including 22 trials, assessed the *EGFR* gene copy number as a potential marker of OS for patients affected by NSCLC and treated with EGFR-TKIs. The *EGFR* FISH-positive status was associated with increased OS (HR 0.77; 95% CI: 0.66–0.89; $P = 0.001$), PFS (HR 0.60; 95% CI: 0.46–0.79; $P < 0.001$), and time to progression (HR 0.50; 95% CI: 0.28–0.91; $P = 0.02$). Of interest was that among predominantly white populations *EGFR* FISH-positive status was strongly associated with improved OS (HR 0.70; 95% CI: 0.59–0.82; $P < 0.001$), whereas it did not influence OS in East Asians (HR 1.11; 95% CI: 0.82–1.50; $P = 0.50$). This difference was statistically significant ($P = 0.02$).[41]

Overall, these contrasting results suggest that the predictive value of *EGFR* gene copy number for outcomes benefit with gefitinib was probably driven by the overlap of the concurrent high incidence of *EGFR* mutation-positive status in this subgroup.

Concerning the possible relationship between cetuximab and *EGFR* copy number, retrospective analysis from the two main randomized Phase III trials (BMS009 and FLEX) reported the following results. In the BMS099 trial, 54 of 104 evaluable patients (51.9%) were *EGFR* FISH-positive. The addition of cetuximab to carboplatin/taxane did not significantly affect PFS in the FISH-positive group (5.4 months in both arms; HR 1.54; 95% CI: 0.81–2.93; $P = 0.18$) or in the FISH-negative group (4.3 months versus 3.8 months; HR 0.65; 95% CI: 0.35–1.18; $P = 0.15$). In the cetuximab plus chemotherapy group, no PFS difference was evident between patients in the FISH-positive and FISH-negative groups (HR 0.99; $P = 0.97$). Patients with FISH-positive tumors treated with chemotherapy had significantly longer PFS than those with FISH-negative tumors (HR 1.41; $P = 0.007$).

Patients with *EGFR* FISH-positive tumors had significantly shorter OS with cetuximab plus chemotherapy than with chemotherapy alone (8.6 months versus 12.5 months; HR 1.92; 95% CI: 1.05–3.54; $P = 0.03$), whereas OS did not differ by treatment in patients with FISH-negative tumors (7.4 months in both groups; HR 0.84; 95% CI: 0.47–1.52; $P = 0.57$). Patients with FISH-positive tumors had longer OS than those with FISH-negative tumors when treated with chemotherapy alone (HR 0.48; $P = 0.017$), but not when treated with cetuximab plus chemotherapy (HR 1.07; $P = 0.81$). In the *EGFR* FISH evaluable population overall, ORR was higher with cetuximab plus chemotherapy than with chemotherapy alone (34.0% versus 24.0%), as in patients with FISH-positive (37.0% versus 22.2%; $P = 0.37$) and FISH-negative (30.8% versus 16.7%; $P = 0.33$) tumors.[31] In the FLEX trial, tumor *EGFR* gene copy number was analyzed in 330 of 1125 (29%) patients. In the *EGFR* FISH-positive patients, the addition of cetuximab to cisplatin plus vinorelbine did not improve any outcomes, with median OS of 11.6 months and 9.9 months (HR 0.85; 95% CI: 0.56–1.29; $P = 0.44$), median PFS of 4.2 months and 4.4 months (HR 0.80; 95% CI: 0.51–1.25; $P = 0.33$), and ORR of 36.7% and 26.4%, respectively, (OR 1.62; 95% CI: 0.70–3.76; $P = 0.26$) for cetuximab plus chemotherapy and chemotherapy alone. No differences in any outcomes were reported in the *EGFR* FISH-negative patients, with a median OS of 10.6 months and 10 months (HR 0.91; 95% CI: 0.65–1.26; $P = 0.56$), median PFS of 4.2 months and 5.2 months (HR 1.05; 95% CI: 0.75–1.47; $P = 0.77$), and ORR of 32.9% and 34.7%, respectively (OR 0.92; 95% CI: 0.49–1.72; $P = 0.80$) for cetuximab plus chemotherapy and chemotherapy alone.[33] In both these large randomized trials, increased *EGFR* gene copy number was not a predictive or prognostic biomarker for cetuximab activity.

Overall, when considering *EGFR* gene copy number as a potential marker of *EGFR* inhibitor therapy, it is important to take into account that its assessment in clinical trials varies greatly. This may be due to different patient populations, different study designs, and variability in the methods used by different laboratories for reading and interpreting FISH. Thus, adequate assay reproducibility is mandatory, and therefore guidelines have been produced to provide information on the inclusion/exclusion criteria, reading and scoring of slides, assessment of signal clusters, and assessment of borderline cases.[42]

EGFR protein expression

EGFR protein expression assessed by IHC was the first biological marker to be retrospectively explored in cohorts of NSCLC patients treated with EGFR-TKIs. In the BR.21 study, tumor samples were available for 325 out of 731 patients treated on the trial. *EGFR* IHC-positive patients treated with erlotinib had a significant OS improvement when compared with *EGFR* IHC-positive patients who received placebo (HR 0.68; 95% CI: 0.49–0.95; $P = 0.02$). No difference was observed among patients who were *EGFR* IHC-negative. However, *EGFR* IHC was not an independent predictor for OS in the multivariate analysis, with a negative interaction test between *EGFR* expression and treatment effect.[43] On the other hand, in the ISEL trial, *EGFR* IHC-positive patients achieved significantly better OS with gefitinib versus placebo than *EGFR* IHC-negative patients (interaction test, $P = 0.049$). The OS benefit for *EGFR* IHC-positive patients treated with gefitinib was slightly higher than the overall study population (HR 0.77; 95% CI: 0.56–1.08; $P = 0.126$), with no evidence of an OS benefit among patients with *EGFR* protein-negative tumors who received gefitinib (HR 1.57; 95% CI: 0.86–2.87; $P = 0.140$).[37]

In the IPASS trial, 365 patients were evaluable for the *EGFR* protein expression status. PFS was significantly longer for gefitinib versus carboplatin/paclitaxel in patients with *EGFR* IHC-positive tumors (n = 266; HR 0.73; 95% CI: 0.55–0.96; $P = 0.024$), while no difference was reported in patients with *EGFR* IHC-negative tumors (n = 99; HR 0.97; 95% CI: 0.64–1.48; $P = 0.893$). ORRs were similar between the gefitinib and carboplatin/paclitaxel groups for patients with either *EGFR* IHC-positive (51.5% versus 41.8%; OR 1.49; 95% CI: 0.92–2.42; $P = 0.109$) or *EGFR* IHC-negative (34.0% versus 26.1%; OR 1.44; 95% CI: 0.60–3.47; $P = 0.415$) tumors. No difference in OS between gefitinib and chemotherapy in patients with *EGFR* IHC-positive (HR 1.05; 95% CI: 0.80–1.37; $P = 0.731$) or *EGFR* IHC-negative (HR 1.09; 95% CI: 0.70–1.70; $P = 0.692$) tumors was reported.[23]

Although conflicting, these data are provocative for positive *EGFR* protein expression as a predictive molecular factor. However, these data are extrapolated retrospectively and are based on relatively small numbers of patients who had available tumor tissue. Further investigations should better clarify the role of *EGFR* protein expression as a biomarker for EGFR-TKI therapy and the relationship with the concurrent presence of *EGFR* activating mutations.

The predictive role of *EGFR* protein expression seems to be clearer when administering cetuximab therapy. In the BMS099 trial, 17 (11.5%) out of 148 patients were *EGFR* IHC-negative. PFS results were comparable in the *EGFR* IHC-positive subgroup with a median of 4.6 months for cetuximab plus carboplatin/taxane versus 4.5 months for chemotherapy alone (HR 1.15; 95% CI: 0.78–1.68; $P = 0.48$) and in the

subset with *EGFR* IHC-negative tumors (4.1 months versus 6.4 months, respectively; HR 1.17; 95% CI: 0.37–3.72; $P = 0.79$). Adding cetuximab to chemotherapy did not significantly affect OS in patients with *EGFR* IHC-positive tumors, with a median OS of 8.3 months for the cetuximab-based group and 9.7 months for chemotherapy alone arm (HR 1.02; 95% CI: 0.71–1.48; $P = 0.91$) or in the small subset of patients with *EGFR* IHC-negative tumors (median OS, 11.2 months versus 17.6 months, respectively; HR 1.86; 95% CI: 0.57–6.11; $P = 0.30$). No significant ORR differences between cetuximab plus chemotherapy and chemotherapy alone arms were observed either in patients with *EGFR* IHC-positive tumors (31.8% versus 21.5%; $P = 0.24$) or with *EGFR* IHC-negative tumors (18.2% versus 33.3%, respectively; $P = 0.58$).[31] Very interesting are the results reported by the FLEX trial, in which patients tumor *EGFR* expression data were used to generate an IHC score on a continuous scale of 0–300 with a defined threshold of 200. Treatment outcome was analyzed in patients with low (IHC score < 200) and high (IHC score ≥ 200) tumor *EGFR* expression. Tumor *EGFR* IHC data were available for 1121 of 1125 (99.6%) patients randomized in this trial. High *EGFR* expression was scored for 345 (31%) evaluable patients and low for 776 (69%) patients. For patients in the high *EGFR* expression group, OS was longer in the cetuximab plus cisplatin plus vinorelbine group than in the chemotherapy alone arm, with a median OS of 12.0 months versus 9.6 months, respectively (HR 0.73; 95% CI: 0.58–0.93; $P = 0.011$). No OS benefit for patients in the low *EGFR* expression group was reported with a median OS of 9.8 months versus 10.3 months, respectively (HR 0.99; 95% CI: 0.84–1.16; $P = 0.88$). A treatment interaction test assessing the difference in the HR for OS between the *EGFR* expression groups suggest a predictive value for *EGFR* expression ($P = 0.044$). These results suggest that high *EGFR* expression is a tumor biomarker that can predict OS benefit from the addition of cetuximab to first-line chemotherapy in patients with advanced NSCLC.[44]

Overall, further trials are needed to evaluate the assessment of *EGFR* expression to select advanced NSCLC patients who could benefit from cetuximab plus chemotherapy first-line therapy offering a personalized treatment approach in this setting.

ALK

In the last few years, a new and promising target has emerged for the treatment of advanced NSCLC patients. *ALK* is a transmembrane receptor tyrosine kinase in the insulin receptor superfamily. About 2%–7% of patients with NSCLC have tumors with an inversion in the short arm of chromosome 2 (inv [2][p21p23]) which results in the fusion of exons 1–13 of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene with exons 20–29 of the *ALK* gene, leading to the production of an *EML4-ALK* fusion tyrosine kinase which is involved in cell proliferation, differentiation, and antiapoptosis.[45] *EML4-ALK* also contains the hydrophobic protein domain of *EML4*, which is critical for dimerization of *EML4-ALK* and the resulting aberrant constitutive activity.[46] There are also other less frequent fusion partners of *ALK*, such as TFG and KIF5B, which mediate ligand-independent dimerization, and therefore constitutive activity of the *ALK* tyrosine kinase domain.[47,48] In cell line and mouse models, *EML4-ALK* is highly oncogenic, activates the phosphatidylinositol-3-kinases-Akt and mitogen-activated protein kinase-extracellular signal-regulated kinase pathways, and induces lung tumors.[45] Clinical patients' characteristics, including never or light smoking history, young age, and adenocarcinoma histology with signet ring cells seem to be associated with NSCLC tumors with a higher probability to detect *ALK* translocations.[49–53] In never or light smokers with NSCLC, the prevalence of *ALK* translocations may be as high as 20%–30%.[52] *ALK* translocations are usually mutually exclusive with *EGFR* or *KRAS* mutations and predict for a poor response to EGFR-TKIs, less responsiveness to platinum-based chemotherapy, and a lower OS in patients with advanced NSCLC.[52,54] The evidence of *EML4-ALK* in lung tumors has been detected by FISH, which appears to be the most clinically applicable test.[47,51,52,54]

Crizotinib (PF0234-1066) is an oral adenosine triphosphate-competitive selective inhibitor of the *ALK* and *MET* tyrosine kinases that inhibits tyrosine phosphorylation of activated *ALK*.[55] In a Phase I–II study, the escalated doses of crizotinib were from 50 mg once daily to 300 mg twice daily, using a standard dose-escalation design. Dose-limiting fatigue in the cohort receiving 300 mg twice daily led to the establishment of a regimen of 250 mg twice daily as the maximum tolerated dose. The expanded cohort with FISH-positive results for *ALK* rearrangement received 250 mg twice daily as long as they did not have progressive disease or intolerable side effects. A total of 82 patients with advanced *ALK*-positive NSCLC received crizotinib with the mean duration of treatment of 6.4 months and an ORR of 57%, while in 33% of patients a stable disease was detected. The estimated probability of 6-month PFS was 72%, with no median PFS for the study reached. In terms of safety, grade 1 nausea and diarrhea were the adverse events most commonly reported, and grade 3 or

Table 1 Frequency of main genetic abnormalities in non-small cell lung cancer[62–65]

Gene	Event type	Adenocarcinoma (%)	Squamous cell carcinoma (%)
EGFR	Mutation	5–15	<5
EML4-ALK	Fusion	5–15	1
KRAS	Mutation	15–38	<5
FGFR1	Amplification	1	20–25
FGFR2	Mutation	18	5
PIK3CA	Mutation	2–8	9
PTEN	Mutation/deletion	2	18
CDKN2A	Deletion/mutation	15	45
PDGFRA	Amplification/mutation	4–9	9
BRAF	Mutation	<5	3
DDR2	Mutation	1	4

4 transaminase elevation was the most commonly detected serious adverse event. To date, median OS was not reached.[56] Additional data from an expanded cohort of 116 patients with a median follow-up of 11 months were reported. ORR was 61%, including two complete and 69 partial responses, and clinical benefit rate was 88%. ORRs were reached rapidly with a median of 8 weeks to response. Preliminary median response duration estimate was 48 weeks. Preliminary median PFS was 10 months. The benefit was consistent across the line of therapy, sex, age, and general fitness.[57] These results led to ongoing trials which are investigating crizotinib in first-line and second-line advanced *ALK*-positive NSCLC patients. Preliminary data of a large Phase II study which recently closed its accrual have been presented. The data are related to the first 136 enrolled patients who were evaluable for safety and 76 for activity. The majority of them had received at least two prior systemic therapy regimens (93%). At the time of this analysis, patients had received a median of 9 weeks of crizotinib treatment and 88% remained on therapy. A waterfall plot of tumor measurements in evaluable patients showed target lesion shrinkage in approximately 90% of patients (41 patients had ≥30% shrinkage).[58]

The final results of the ongoing trials investigating crizotinib in patients with advanced NSCLC with *ALK* rearrangements give the real impact in the clinical practice of this biomarker and its inhibitor.

Conclusion

To date, two biomarkers are available for the personalized medicine in the treatment of advanced NSCLC patients. The *EGFR* activating mutation has already entered the clinical practice, while the *ALK* gene fusion is about to. This implies that, whenever possible, an adequate tumor sample tissue for molecular characterization has to be obtained at the moment of the initial diagnosis to start treatment off with the most appropriate therapeutic strategy. The clinical characteristics strongly related with the presence of these biomarkers should guide the selection of patients who must be investigated for their detection. An important issue is that evidence shows that ORR in *EGFR*-mutated or *ALK*-positive patients was not 100% and, unfortunately, patients who initially benefited from the specific inhibitors experienced – at a certain point of their illness – a progression of their disease. Possible explanations could be the presence of a primary resistance due to the contemporary activation of other pathways which bypass the *EGFR* pathway, or of an acquired resistance due to the occurrence, during EGFR-TKI therapy, of an additional *EGFR* gene mutation. The most studied mutation occurs in exon 20, with the substitution of a threonine for methionine at position 790 (T790M), which is supposed to change the conformation of the receptor and block the binding of gefinitib or erlotinib to the active site, creating resistance to these EGFR-TKIs.[59] In contrast to the reversible TKIs like gefitinib and erlotinib, the second generation *EGFR* inhibitors, the irreversible TKIs, among which afatinib (BIBW2992) is in advanced phase of clinical development in NSCLC patients, seem to effectively inhibit *EGFR* T790M and block the growth of NSCLC cell lines harboring T790M mutations.[60]

Preliminary studies revealed that also in the case of *ALK* rearrangements, the onset of secondary acquired mutations in the kinase domain of *EML4-ALK* confer resistance to the specific inhibitor crizotinib.[61] The interesting retrospective results from the FLEX trial seem to open a new therapeutic option for patients with *EGFR* IHC-positive tumors (score ≥ 200). If these data are further confirmed, a new therapeutic personalized approach will be available in the clinical practice. Further biomarkers are under investigation for the targeted therapy of both adenocarcinoma and squamous cell carcinoma of the lung, with several trials ongoing and relative results still pending (Table 1).[62–65]

To date, considering the *EGFR* activating mutations and the *EML4-ALK* gene fusion, which is generally mutually exclusive, and the availability of the correspondent EGFR-TKIs and crizotinib, about 20% to 50% of advanced NSCLC could now be treated in Western and Eastern countries, respectively, with a really targeted therapy.

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