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REVIEW

Genetic profiling and epidermal growth factor receptor-directed therapy in nonsmall cell lung cancer

J. Cadranel*, G. Zalcman# and L. Sequist¶

ABSTRACT: The principle of preferentially selecting patients most likely to benefit from therapy according to their genetic profile has led to substantial clinical benefit in some tumour types, and has potential to considerably refine treatment in advanced nonsmall cell lung cancer (NSCLC). Effective, reliable use of molecular biomarkers to inform clinical practice requires the standardisation of testing methods and careful assessment of biomarkers' predictive and prognostic value.

Although a number of studies have shown that patients with activating mutations in exons 18–21 of the epidermal growth factor receptor (EGFR) gene respond particularly well to gefitinib and erlotinib, a prospective, randomised study was needed to differentiate between the prognostic and predictive value of *EGFR* mutations. From one such study, it appeared that mutational testing should become standard at diagnosis, at least for adenocarcinoma patients with a never or low smoking history, as clinical predictors are insufficient to optimise treatment.

However, outstanding questions remain: what are the treatment options for patients with tumours resistant to erlotinib/gefitinib? What conclusions about treatment can we draw from *EGFR* copy number or *KRAS* mutation status? What role should anti-EGFR antibodies play in NSCLC treatment, and in which patients?

This review considers current evidence linking biomarker profile to efficacy of EGFR-targeted therapy in NSCLC, and clinical implications of recent findings.

KEYWORDS: Biomarker, epidermal growth factor receptor, epidermal growth factor receptor inhibitors, genetic profiling, mutational testing, nonsmall cell lung cancer

AFFILIATIONS
*Hôpital Tenon, Assistance Publique-Hôpitaux de Paris and Faculté de Médecine Pierre et Marie Curie, Université Paris VI, Paris, and
#Service de Pneumologie, Université de Caen-Basse-Normandie, CHU de Caen, Caen, France.
¶Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA, USA.

CORRESPONDENCE
J. Cadranel
Service de Pneumologie
Hôpital Tenon
4 rue de la Chine
75970
Paris
France
E-mail: jacques.cadranel@tnn.aphp.fr

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ESTABLISHING THE PRINCIPLE OF MUTATION TESTING: LESSONS FROM OTHER TUMOUR TYPES

Therapies tailored to specific genetic lesions and diagnostic tests that assay for their respective molecular targets are now an established part of clinical practice across various tumour types, including chronic myeloid leukaemia [1], gastrointestinal stromal tumours and epithelial tumours, such as breast and colon cancer [2].

Clinically relevant improvements in survival have been attained by administering targeted therapy to the appropriate patient population: for example, the addition of trastuzumab to standard first-line chemotherapy in patients with human epidermal growth factor receptor-positive (HER2+) metastatic breast cancer [3]. A *HER2* amplification diagnostic test is now required in breast cancer before patients are treated with trastuzumab [2]. Clinical practice in colon cancer also reflects the need for mutational testing to identify patients most likely to benefit from cetuximab: patients whose tumours lack a *KRAS* mutation (also called wild-type) show significantly increased overall survival (OS) (median 9.5 *versus* 4.8 months) with cetuximab, whereas those with *KRAS* mutations do not benefit from therapy [4].

These successful examples validate the concept of understanding the genetic profile of patients most likely to benefit from a targeted agent and preferentially selecting those patients for therapy. However, the use of molecular biomarkers to optimise clinical outcomes requires careful

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assessment of their role in terms of both prognosis and treatment decision-making. Specifically, it is becoming increasingly important to accurately distinguish biomarkers as ''prognostic'' or ''predictive'', or define them as both. Prognostic biomarkers can be thought of as a measure of the natural history of a disease that is independent of therapeutic intervention (or lack of it) [5]. A population-based register or a placebo/control group from a randomised clinical study is appropriate for evaluating the prognostic value of a biomarker [5]. In contrast, a predictive biomarker differentiates a group with a particular response or lack of response to a therapeutic intervention. In order to establish which patients will benefit most from a treatment, and by how much, the predictive value of a biomarker must be separated from its prognostic value. To do this, experimental and control arms can be stratified by biomarker status and an interaction test performed. It is recommended, in most cases, that biomarkers should be evaluated prospectively rather than retrospectively.

This review considers the current evidence linking biomarker profile to efficacy of epidermal growth factor receptor (EGFR)-targeted therapy in advanced nonsmall cell lung cancer (NSCLC) and the clinical implications of recent findings.

EGFR TYROSINE KINASE INHIBITOR: THE FIRST TARGETED THERAPY IN NSCLC

In NSCLC, activation of the EGFR/HER1 pathway results in a signalling cascade that promotes tumour growth and progression [6]. EGFR is expressed in a large proportion of NSCLC tumours [7], and its associated signalling pathways are frequently dysregulated. These observations provided the rationale for developing small-molecule tyrosine kinase inhibitors (TKIs) targeting EGFR, erlotinib and gefitinib, and EGFR-targeted antibodies, such as cetuximab.

Gefitinib is currently the most widely used EGFR TKI worldwide. It has single-agent activity in patients previously treated with chemotherapy [8, 9], but did not prolong survival compared with placebo in the Iressa Survival Evaluation in Lung Cancer (ISEL) randomised, phase III trial in the second- and third-line setting [10]. When compared with single-agent chemotherapy, it has been shown to be non-inferior to docetaxel, with improved quality of life in a large phase III study of previously treated patients [11]; in a smaller, randomised phase II study of chemotherapy-naïve elderly patients, gefitinib improved quality of life, without progression-free survival (PFS) or OS decrement, compared with vinorelbine [12]. First-line addition of gefitinib to cisplatin and gemcitabine (Iressa NSCLC Trial Assessing Combination Treatment (INTACT)-1) [13] or carboplatin and paclitaxel (INTACT-2) [14] showed no significant difference in response rate (RR) or survival compared with chemotherapy alone.

Erlotinib is the most widely used EGFR TKI in the USA and European Union, and has shown single-agent antitumour activity and symptom improvement in previously treated NSCLC patients [15]. In contrast to gefitinib, second- and third-line erlotinib significantly improved OS compared with placebo in the BR.21 Phase III trial (6.7 *versus* 4.7 months; hazard ratio (HR) 0.70; $p<0.001$) [16]. Like the combination trials with gefitinib, phase III trials combining erlotinib with first-line chemotherapy (Tarceva Lung Cancer Investigation and Tarceva Responses in Conjunction with Taxol and Carboplatin (TRIBUTE)) showed no significant difference in survival between erlotinib and control arms [17, 18]. Finally, the phase III Sequential Tarceva in Unresectable NSCLC (SATURN) trial assessed the efficacy of maintenance erlotinib compared with placebo in patients with advanced NSCLC who did not show disease progression after first-line, platinum-based doublet chemotherapy. This trial demonstrated a significant improvement in PFS for the 437 patients receiving erlotinib, compared with the 447 patients receiving placebo (PFS at 24 weeks 31 *versus* 17%; HR 0.71, 95% CI 0.62–0.82; log-rank $p<0.0001$) [19].

To summarise, both gefitinib and erlotinib are considered to be active single-agent therapies in NSCLC patients treated previously with chemotherapy. Reasons for the discrepancy between the BR.21 and ISEL trial outcomes, when both drugs are chemically and preclinically similar, may be due to dosing: erlotinib is dosed at 150 $mg \cdot day^{-1}$, its maximum tolerated dose (MTD), whereas gefitinib is dosed at 250 $mg \cdot day^{-1}$, about one-third to one-half of its MTD [20]. Other contributing factors could be differences in the populations studied in the two trials, including divergent representation of patients most likely to respond, and differences in the definition of second-line patients, either as those with progressive or those with stable disease after first-line treatment.

EGFR MUTATIONS IN NSCLC: IMPLICATIONS FOR FIRST-LINE TREATMENT WITH EGFR TKIS

A subset of patients responds particularly well to EGFR TKIs. Even in early studies, it was apparent that gefitinib and erlotinib were associated with greater responses in patients with adenocarcinoma, never-smokers, patients from East Asia and females [21]. Somatic activating mutations of the *EGFR* gene have now been identified; these mutations confer an increased susceptibility to EGFR TKI-mediated cell death and probably underlie the increased responses observed in these clinically defined groups [22–24]. Two *EGFR* mutations, the exon 19 deletion and the exon 21 L858R substitution, account for ~90% of all known EGFR kinase domain mutations [25].

A substantial body of evidence verifies the importance of *EGFR* mutational status in determining which patients are most likely to respond to treatment with erlotinib/gefitinib. Both retrospective studies of second- and third-line EGFR TKIs in unselected populations, as well as prospective studies of first-line EGFR TKI treatment in enriched populations, have been published (table 1). Over a number of studies, the weighted average RR to EGFR TKI treatment in mutation-positive cases was 78%, with most series reporting a RR of more than 60%. In mutation-negative cases, in contrast, the average RR was 10% [38]. This is evidence that EGFR mutations are associated with response to EGFR TKI therapy.

The studies mentioned above include an evaluation of the impact of *EGFR* mutations on survival after gefitinib approval, compared with historical controls (*EGFR* mutants diagnosed and treated before gefitinib approval). A significant association between *EGFR* mutations and prolonged survival was shown with gefitinib [39]. Taken together, these studies suggest that *EGFR* mutational status may be a predictive biomarker. Furthermore, patients with the exon 19 deletion mutation

TABLE 1 Studies evaluating the predictive value of epidermal growth factor receptor (*EGFR*) mutations and responses to erlotinib/gefitinib

First author [ref.]	Patients screened n	*EGFR* mutations n	Patients treated n	Treatment line	Drug	RR % (95% CI) in *EGFR* mutation-positive patients
Asahina [26]	82	20	16	First-line	Gefitinib	75 (48–93)
Yoshida [27]	66	27	21	Mixed	Gefitinib	90.5 (69.6–98.8)
Sunaga [28]	33	21	21	Mixed	Gefitinib	76 (53–92)
Mok [29]	683	261	NR	First-line	Gefitinib	71.2 (NR)
Sequist [30]	98	34	31	First-line	Gefitinib	55 (33–70)
Inoue [31]	99	25	16	First-line	Gefitinib	75 (54–96)
Rosell [32]	2105	350	217	Mixed	Erlotinib	70.6 (NR)
Rosell [33]	NR	123	12	First-line	Erlotinib	90 (NR)
Tamura [34]	118	32	28	Mixed	Gefitinib	75 (58–93)
Sutani [35]	109	38	27	Second-line	Gefitinib	78 (62–94)
Mitsudomi [36]	337	189	175	First-line	Gefitinib	62.1 (NR)
Costa [37] (pooled analysis)	NA	101	101	Mixed	Gefitinib	80.8 (80–99)

RR: response rate; NR: not reported.

have significantly prolonged time to progression and increased survival rate compared with those with the exon 21 L858R point mutation [40, 41]. In addition to evidence that *EGFR* mutational status may have predictive value, retrospective data from randomised, controlled trials, including INTACT and TRIBUTE study results, suggest that *EGFR* mutational status also has prognostic value, with patients harbouring *EGFR* mutations demonstrating prolonged survival compared with those who do not, regardless of treatment group assignment [42, 43].

Prospective studies have assessed the efficacy of first-line EGFR TKIs in patients harbouring *EGFR* mutations. One example is the iTARGET trial, in which patients with advanced NSCLC harbouring *EGFR* mutations (including, but not restricted to, the L858R and exon 19 deletion mutations) received first-line gefitinib [30]. Of 98 patients screened, 34 had *EGFR* mutations and 31 received gefitinib. RR, the primary end-point, was 55%; median PFS was 9.2 months (95% CI 6.2–11.8 months) [30]. This study used clinical characteristics to enrich the patient population for those likely to be *EGFR* mutation-positive, demonstrating that genotype-directed therapy with EGFR TKIs is feasible in a US population, where the overall frequency of *EGFR* mutations is relatively low, compared with Asian populations.

Another prospective study of advanced NSCLC was carried out by the Spanish Lung Cancer Group, in which patients with *EGFR* mutations were selected to receive first-line treatment with erlotinib. Lung tumours from 2,105 patients were screened; *EGFR* mutations were found in 350 (16.6%) of these and 217 received erlotinib, among them, 113 patients received erlotinib as first-line treatment. In these patients, median PFS was 14.0 months (95% CI 11.3–16.7 months) and median OS was 27 months. This study cohort demonstrated that large-scale screening of patients for *EGFR* mutations and customised treatment with EGFR TKIs are feasible [32].

Taken together, the studies described above demonstrate that EGFR TKIs are highly effective in selected patients, with treatment producing improved response rates and PFS compared with chemotherapy. Results from these studies also support the concept that, in a particular patient subgroup, first-line treatment with EGFR TKIs may be the most effective option. A prospective, randomised study to differentiate between the prognostic and predictive value of *EGFR* mutations, and to determine the optimal treatment strategy for different subgroups of NSCLC patients was needed. More recently, the first such study was completed and published.

The results from the Iressa Pan-Asia Study (IPASS) of first-line gefitinib *versus* carboplatin/paclitaxel in 1,217 clinically selected patients with advanced NSCLC [29] have considerable implications for clinical practice. Eligible patients were never- or light ex-smokers with adenocarcinoma histology; the overall rate of *EGFR* mutations in the 437 evaluable patients with available tissue was 59.7%. Gefitinib had a superior PFS compared to chemotherapy, exceeding the primary end-point of the trial, which was to show noninferiority. The molecular subgroup analysis demonstrated that patients with *EGFR* mutations had superior PFS in the gefitinib arm compared with those in the chemotherapy arm (HR 0.48, 95% CI 0.36–0.64; $p<0.001$; treatment by *EGFR* mutations status interaction test, $p<0.0001$) [29]. A crucial observation from this study is taken from the patients whose tumours had wild-type *EGFR*. In those patients, all of whom had clinical characteristics typical of gefitinib responders, those receiving gefitinib had a marked decline in PFS compared with those who received chemotherapy (HR 2.85, 95% CI 2.05–3.98; $p<0.001$) [29]. This argues strongly that mutational testing should become standard practice at diagnosis, at least for adenocarcinoma patients with a never- or low smoking history, as clinical predictors are insufficient to optimise treatment. Such patients should be treated with first-line EGFR TKI therapy if their tumours harbour activating *EGFR* mutations, given the

demonstrated PFS benefit, and chemotherapy should be the preferred therapy for those patients with wild-type *EGFR*. OS analysis on the IPASS trial is not yet completed. However, other studies support its conclusions; for example, in a smaller phase III study comparing first-line gefitinib with carboplatin/paclitaxel in patients known to have *EGFR* mutation-positive advanced NSCLC, PFS was significantly prolonged in the gefitinib group in an interim analysis (10.4 *versus* 5.5 months; HR 0.4; log rank p<0.001) [44]. This was also confirmed by another, more recently published phase III trial comparing first-line gefitinib with cisplatin plus docetaxel in NSCLC patients harbouring *EGFR* mutations. The gefitinib group had significantly prolonged median PFS compared with the patients receiving cisplatin plus docetaxel (9.2 *versus* 6.3 months; HR 0.489; log rank p<0.0001) [36].

It is important to note that IPASS was an Asian study, and that activating *EGFR* mutations occur at a lower frequency in Caucasian populations (~40 and ~10%, respectively). Some feel that this may play a role in determining the uptake of mutational testing before first-line therapy, although it could be argued that it is more important to perform the definitive test in a population with a lesser chance of mutation. In addition to its implications for therapy choice, IPASS also set a new standard for the collection and analysis of biomarker data within large-scale clinical studies; this has an important bearing on tissue collection and analysis in future studies. Further prospective clinical trials are needed to confirm these findings in a study population that is not entirely Asian, validate that the same trend is seen with other chemotherapeutics (*e.g.* pemetrexed), and to examine whether the sequence of chemotherapy and EGFR TKI therapy in patients with mutations influences survival and other outcomes. For example, in a recently published retrospective study including 152 NSCLC patients with exon 19 deletions or L858R, those receiving first-line gefitinib had a significantly higher RR than chemotherapy-treated patients (76 *versus* 54%; p=0.005). However, OS and PFS did not differ significantly between chemotherapy-naïve and -pretreated groups (p=0.207 and p=0.804, respectively) [45]. It is also important to note that patients with *EGFR* mutations also have a higher RR to chemotherapy compared to patients with wild-type *EGFR*. This was demonstrated by a phase III, open-label study investigating the efficacy of gefitinib compared with carboplatin plus paclitaxel in patients with NSCLC. During that study, *EGFR* mutation-positive patients were shown to have a higher objective RR to carboplatin/paclitaxel chemotherapy than patients with wild-type *EGFR* (47.3 *versus* 23.5%) [29]. Physicians need to consider this information alongside data from mutational testing and the overall state of health of the patient when deciding on first- and second-line therapy until more conclusive evidence is available; in the long term, data on patient selection may also have an impact on social security reimbursement in European countries. In addition to these considerations, the time to initiation of therapy with EGFR TKIs requires clarification in cases of aggressive disease in which it may not be appropriate to wait for the results of *EGFR* mutation testing. Furthermore, as it is likely that most patients will, at some point, receive treatment with an EGFR TKI, the risk of patients with *EGFR* mutations experiencing side-effects from first-line chemotherapy that preclude further treatment, or of new metastases occurring at progression, should always be considered when selecting a first-line treatment.

RESISTANCE TO EGFR TKIS: NEED FOR A NEW GENERATION OF TARGETED THERAPY

Although patients with *EGFR* mutations initially tend to have a good therapeutic response to erlotinib or gefitinib, prolonged administration of either drug invariably leads to secondary resistance, with patients experiencing relapse or tumour progression [25].

To date, two principal mechanisms have been identified that underlie secondary resistance (fig. 1). One is a resistance mutation in the *EGFR* gene, T790M [47, 48], which impairs the binding of the reversible TKIs erlotinib or gefitinib to the adenosine triphosphate binding pocket of the EGFR tyrosine kinase, rendering them ineffective [49]. T790M occurs in ~50% of patients with acquired resistance to gefitinib/erlotinib [47, 50]. Some studies have suggested that, rather than causing

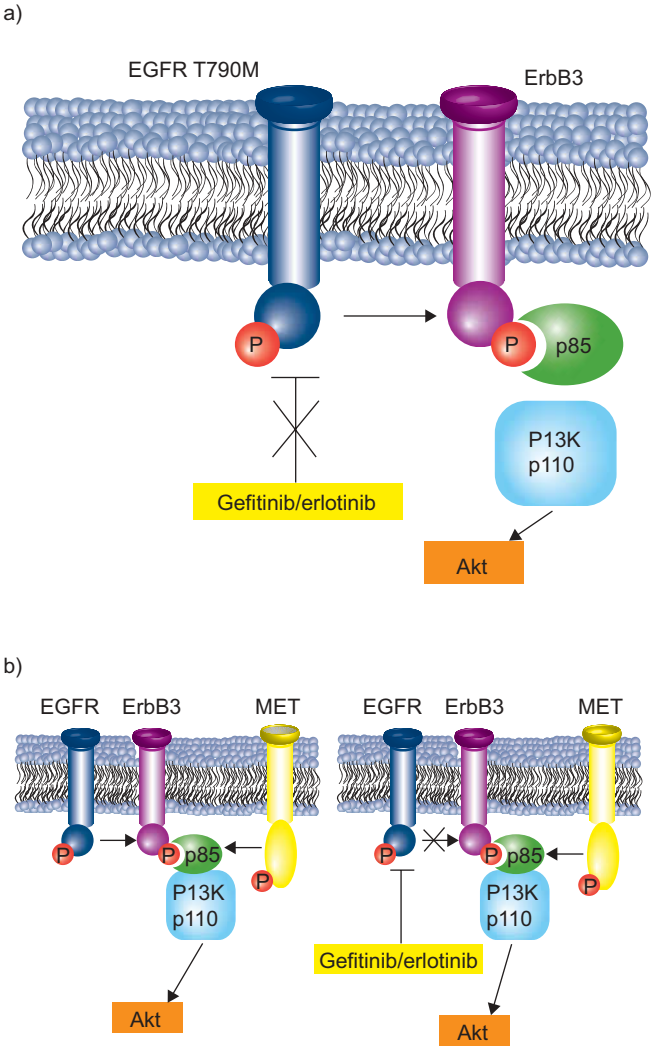

FIGURE 1. a) The T790M mutation prevents erlotinib/gefitinib from effectively inhibiting phosphorylation of epidermal growth factor receptor (EGFR). b) MET amplification activates phosphoinositide 3-kinase (PI3K) signalling *via* ErbB3, independently of EGFR. Reproduced from [46] with permission from the publisher.

the mutation to arise, treatment with TKIs simply selects for the resistant clones. Molecular characterisation of tumour tissue from 27 patients with metastatic NSCLC using an ultrasensitive, allele-specific assay revealed that low levels of T790M *EGFR* were present in 38% of patients. The presence of the T790M mutation was associated with a significantly shorter PFS with EGFR TKI therapy compared with patients who did not have detectable levels of T790M at baseline, although it did not preclude response [51]. Although other mutations in exons 19–21 have been identified that also confer resistance to EGFR TKIs [52], T790M is the most common.

Irreversible TKIs that bind covalently with the catalytic pocket of EGFR are believed to provide a sustained blockade of EGFR signalling and may also retain activity against tumours that harbour resistance mutations, such as EGFR T790M. Several such agents are under clinical development for the treatment of various tumour types, including EKB-569 [53], CI-1033 [54], PF-00299804 [55] and BIBW 2992 [56] (table 2). In NSCLC, it is crucial to perform studies of these drugs in patients with *EGFR* mutations, both in those naïve to therapy with first-generation TKIs, such as gefitinib and erlotinib, and in those who have progressed through prior TKI therapy. Preliminary phase II

TABLE 2 Irreversible epidermal growth factor receptors (EGFR) inhibitors and MET inhibitors in clinical development in nonsmall cell lung cancer (NSCLC)

Agent	Target	Development phase	Ongoing phase II/III studies
Irreversible EGFR inhibitors			
EKB-569	EGFR	II	Second-/subsequent-line EKB-569 in platinum- and docetaxel-refractory patients with advanced NSCLC (study completed)
CI-1033	EGFR, HER2 and HER4	II	Second-/subsequent-line CI-1033 in patients with advanced/metastatic NSCLC who have failed prior platinum-based combination chemotherapy (study completed)
BIBW 2992	EGFR and HER2	II/III	Phase II: single-arm study of BIBW 2992 monotherapy in *EGFR* FISH-positive patients Phase II: single-arm study of BIBW 2992 monotherapy in *EGFR* mutation-positive patients Phase II single-arm study of BIBW 2992 monotherapy in patients with *EGFR* mutations, HER2/neu mutations or *EGFR* FISH-positive tumours with no *EGFR* mutations Phase IIB/III: BIBW 2992 in patients with NSCLC who have received 1–2 chemotherapy regimens (including one platinum-containing regimen) and either gefitinib or erlotinib for a period of $\geqslant$12 weeks Phase III: First-line BIBW 2992 *versus* pemetrexed/cisplatin in patients with lung adenocarcinoma bearing activating *EGFR* mutations
XL647	EGFR, HER2 and VEGFR2	II	Open-label study of XL647 monotherapy in previously untreated NSCLC patients Open-label study of XL647 monotherapy in NSCLC patients who have progressed after previously responding to gefitinib/erlotinib
PF-00299804	Pan-HER	II/III	Open-label study of PF-00299804 monotherapy in NSCLC patients who have progressed after chemotherapy and erlotinib Open-label study of PF-00299804 monotherapy in patients with adenocarcinoma who are either non-smokers or former light smokers PF-00299804 *versus* erlotinib in patients with advanced NSCLC who have progressed after 1 or 2 prior chemotherapy regimens PF-00299804 in patients with advanced NSCLC that has not responded to standard therapy
MET inhibitors			
MET-Mab (antibody)	MET	II	MET-Mab plus erlotinib *versus* erlotinib plus placebo in second-/third-line NSCLC
ARQ197 (small molecule; only non-ATP inhibitor)	MET	I/II	Randomised study of ARQ197 plus erlotinib *versus* erlotinib plus placebo in patients with advanced/metastatic NSCLC who have progressed after one chemotherapy regimen
XL184 (small molecule)	MET, VEGFR2, RET	I/II	XL184 with or without erlotinib in patients with NSCLC who have progressed after previously responding to erlotinib

Information on ongoing studies is current according to http://clinicaltrials.gov/ as of February 4, 2010. ATP: adenosine triphosphate; HER: human EGFR; VEGFR: vascular endothelial growth factor receptor; FISH: fluorescence *in situ* hybridisation.

results from 67 patients with *EGFR* mutations receiving BIBW 2992 as a second-line treatment show that 66% achieved a partial response, with 51% of patients remaining progression-free at 12 months [57]. If irreversible EGFR TKIs prove to be as effective as or superior to gefitinib and erlotinib, then defining their role in treating or preventing acquired resistance is of great interest.

The second major mechanism of acquired resistance is MET amplification, observed in ~20% of patients with NSCLC who develop resistance to EGFR TKIs [58]. MET amplification activates phosphoinosite 3-kinase signalling *via* ErbB3, independently of EGFR. This allows signalling downstream of EGFR to continue, despite the presence of EGFR inhibitors [59]. MET amplification occurs independently of the T790M mutation, although both can occur simultaneously in the same patient [58, 60]. A number of therapeutic strategies for the inhibition of MET or its ligand, hepatocyte growth factor, are currently under investigation in early-phase clinical trials (table 2) [61].

In general, combination treatment with EGFR TKIs and other agents targeting downstream or redundant pathways may have considerable clinical potential; combination treatment with the mTOR (mammalian target of rapamycin) inhibitor rapamycin and irreversible EGFR TKIs has shown activity in preclinical *in vivo* experiments in EGFR L858R/T790M mouse models [62].

With increasing knowledge about the molecular mechanisms of acquired resistance to EGFR TKIs, the clinical implications should be considered: will repeat mutational testing be required during the course of a patient's treatment? If so, are repeat biopsies needed or can sensitive methods be devised that allow mutations to be tested for in blood samples? Which samples are most informative, those from the primary tumour or those from metastases? In which order should treatments be administered to optimise response? Which agents are effective once the first-generation EGFR TKIs erlotinib and gefitinib are no longer effective?

EGFR COPY NUMBER IN NSCLC: A MORE OPEN QUESTION THAN *EGFR* MUTATION

In addition to *EGFR* mutations, other techniques for identifying patients who may benefit from treatment with EGFR TKIs have been studied. The most notable of these is *EGFR* fluorescence *in situ* hybridisation (FISH), which indicates whether there is an overall increase in *EGFR* gene copy number [63]. FISH results have been shown to correlate with increased sensitivity to gefitinib or erlotinib and increased survival [63–65].

Results from both the BR.21 and ISEL trials suggested that patients with increased gene copy number according to FISH had improved survival with EGFR TKI therapy, compared with placebo (BR.21 HR 0.43, 95% CI 0.23–0.78 ($p=0.004$); ISEL HR 0.61, 95% CI 0.36–1.04 ($p=0.067$)) [66, 67]. However, biomarker analyses of the SATURN study indicate that increased *EGFR* copy number according to FISH does not have adequate predictive power to enable selection of patients for early second-line treatment with erlotinib over placebo [68]. Furthermore, in randomised trials comparing an EGFR TKI to chemotherapy, *EGFR* gene copy number by FISH has not always been associated with improved results on the TKI arm (table 3). Finally, in the Iressa NSCLC Trial Evaluating Response and Survival Versus Taxotere (INTEREST) study, no significant difference in OS between treatment arms was detected for any of the biomarkers assessed, including *EGFR* FISH, and *EGFR* mutation was more powerful than *EGFR* FISH analysis in predicting objective response and PFS in patients receiving gefitinib [70].

To date, one prospective clinical trial has selected patients for gefitinib therapy based on *EGFR* copy number according to FISH. Results from the phase II ONCOBELL study show that of 37 patients with sufficient tumour tissue for analysis, 25 (69.4%) were *EGFR* FISH-positive. Patients who had *EGFR* FISH-positive status had a significantly higher RR than *EGFR* FISH-negative patients (68.0 *versus* 9.1%; $p<0.001$). *EGFR* FISH-positive patients also had a significantly longer time to progression than *EGFR* FISH-negative patients (7.6 *versus* 2.7 months, respectively; $p=0.02$). These data suggest that *EGFR* FISH analysis may, indeed, predict response to gefitinib [71].

In conclusion, *EGFR* gene amplification together with *EGFR* mutation is a common finding and usually affects the mutant allele [72]. It is probable that the predictive value of *EGFR* FISH for EGFR TKI effectiveness is more likely a result of its association with *EGFR* mutations. In some cases, EGFR protein overexpression may result from *EGFR* amplification alone, but its impact on response to EGFR TKIs remains debatable.

KRAS MUTATIONS IN NSCLC: DO THEY HAVE PREDICTIVE OR PROGNOSTIC VALUE?

Somatic mutations in the oncogene *KRAS* have been associated with lack of primary response to EGFR TKIs in several studies. It is thought that mutations in codons 2, 12, 13 and 61 lead to constitutive activation of the RAS protein, which may allow tumour cells to grow independently of EGFR signalling and, thus, render them resistant to EGFR TKIs [73]. Mutations in *KRAS* occur in ~25% of European patients with adenocarcinoma, although they are less common in Asian patients [74]. Increased frequency of *KRAS* mutations have been shown not to be significantly associated with age, sex or smoking history [73]. Therefore, seeing clinical characteristics only to identify those patients who have a very limited chance of responding to treatment with EGFR TKIs is not the best option, and molecular testing will be required.

Analysis of 206 tumours from the BR.21 study showed that 15% had mutations in codons 12 or 13 of *KRAS*. These patients did not appear to derive any benefit from erlotinib therapy, whereas patients with wild-type *KRAS* did appear to gain a survival benefit (HR 0.69; $p=0.03$) [67]. In the TRIBUTE study, 55 out of 264 (21%) patients had *KRAS* mutations, and those with *KRAS* mutations in the erlotinib arm exhibited significantly shorter OS than those in the chemotherapy-only arm (HR 2.1, 95% CI 1.1–3.8; $p=0.019$) [43]. Preliminary results from 246 patients with sequenced tumour specimens receiving erlotinib in the prospective Evaluation of the EGFR Mutation Status for the Administration of EGFR-TKIs in Non Small Cell Lung Carcinoma (ERMETIC) cohort show that *KRAS* mutations have no significant impact on PFS but negatively affect survival, whereas *EGFR* mutations strongly predict prolonged

TABLE 3 Impact of epidermal growth factor receptor (*EGFR*) gene copy number and *EGFR* mutations in nonsmall cell lung cancer (NSCLC) treated by EGFR tyrosine kinase inhibitors

Study	Drug (dose)	Samples or gene copy evaluations/gene mutation evaluations n	End-points analysed by biomarker	HR for FISH-positive patients (95% CI)	HR for mutation-positive patients (95% CI)
IDEAL/INTACT [42]	Gefitinib (250 and 500 mg·day^{-1}) *versus* gefitinib 250 and 500 mg·day^{-1} plus gemcitabine/cisplatin or plus carboplatin/paclitaxel	821	INTACT: OS Others: NR	INTACT: 2.03 (0.67–6.13) Others: NR	INTACT: 1.77 (0.50–6.23) Others: NR
TRIBUTE [69]	Erlotinib (150 mg) plus carboplatin/paclitaxel *versus* carboplatin/paclitaxel plus placebo	245	OS	1.52 (0.94–2.46)	NR
BR.21 [65]	Erlotinib (150 mg) *versus* placebo	125/110	OS	0.44 (0.23–0.82)	0.77 (0.40–1.50)
ISEL [66]	Gefitinib (250 mg) *versus* placebo	370/215	OS	0.61 (0.36–1.04)	Evaluation limited owing to low number of deaths in *EGFR*-mutation positive patients
IPASS [29]	Gefitinib (250 mg) *versus* carboplatin/paclitaxel	NR	PFS	NR	0.78 (0.50–1.20)
INTEREST [70]	Gefitinib (250 mg) *versus* docetaxel	374/297	OS, PFS, RR	OS: 1.00 (0.80–1.25)	OS: 0.97 (0.76–1.25)
INVITE [12]	Gefitinib (250 mg) *versus* vinorelbine	158/65	PFS, OS	OS: 2.88 (1.21–6.83)	OS: NR owing to low patient numbers
SATURN [68]	Erlotinib (150 mg) *versus* placebo (maintenance therapy after first-line chemotherapy)	488/437	PFS	0.68 (0.51–0.90)	0.10 (0.04–0.25)
ONCOBELL [71]	Gefitinib 250 mg·day^{-1}	37	TTP	NR	NR

HR: hazard ratio; FISH: fluorescence *in situ* hybridisation; IDEAL: Incremental Decrease in Clinical Endpoints Through Aggressive Lipid Lowering; INTACT: Iressa NSCLC Trial Assessing Combination Treatment; TRIBUTE: Tarceva Responses in Conjunction with Taxol and Carboplatin; ISEL: Iressa Survival Evaluation in Lung Cancer; IPASS: Iressa Pan-Asia Study; INTEREST: Iressa NSCLC Trial Evaluating Response and Survival Versus Taxotere; INVITE: Iressa in NSCLC *versus* Vinorelbine Investigation in the Elderly; SATURN: Sequential Tarceva in Unresectable NSCLC; NR: not reported; OS: overall survival; PFS: progression-free survival; RR: response rate; TTP: time to progression.

PFS compared with wild-type *EGFR*, including all clinical and molecular markers [75].

As *EGFR* and *KRAS* mutations appear to be mutually exclusive [76–78], the possibility of defining these two biomarkers as predictors of response and resistance to EGFR TKIs, respectively, is generally accepted by many physicians, although simultaneously occurring mutations in *EGFR* and *KRAS* have been observed very rarely in tumours [43].

ANTIBODIES TO EGFR IN NSCLC: WAITING FOR A PREDICTIVE BIOMARKER?

Cetuximab, a humanised monoclonal antibody that prevents ligand binding to the extracellular domain of EGFR, has shown encouraging results in NSCLC in combination with standard chemotherapy, in both the first- and second-line settings [79–83]. In the First-Line ErbituX in Lung Cancer (FLEX) study, a randomised, phase III study of cetuximab combined with cisplatin/vinorelbine (CV) *versus* CV alone in the first-line treatment of patients with EGFR immunohistochemistry (IHC)-positive advanced NSCLC, patients receiving cetuximab had statistically longer OS (primary end-point) than those receiving CV alone (11.3 *versus* 10.1 months; HR 0.871; p=0.044). There was no significant difference in PFS between treatment groups [82]. The role of *EGFR* copy number, *KRAS* mutation status and *EGFR* IHC values in the FLEX study have recently been reported [84]. A benefit from cetuximab treatment was seen regardless of either *EGFR* copy number, as assessed by FISH, or *KRAS* mutation status [84]. Currently, only a clinical characteristic is associated with increased PFS with cetuximab in FLEX: the early occurrence of skin rash. However, it is not thought that *EGFR* mutations play a crucial role in cetuximab activity as they do in EGFR TKI treatment, and cross-resistance with EGFR TKIs is unlikely to occur.

MUTATION TESTING: THE NEED FOR STANDARDISATION

Standardisation of sampling and test methodologies is essential to remove bias, allow comparison across trials and further our understanding of which patients may benefit from specific treatments. However, such efforts are hampered by a lack of

consensus between centres on optimal methods and practical limitations, including tissue availability. Going forward, it is crucial to identify, standardise and validate methods of sampling and testing that are practicable across a wide number of hospital laboratories, and create evidence-based practice guidelines to facilitate comparison of test results between studies. The mutation status of *EGFR* is generally determined from samples taken by surgical resection, biopsy or fine-needle aspiration before treatment begins [27, 85–88]. Although minimally invasive fine-needle aspiration procedures have safety advantages for the patient, larger tissue samples, such as those provided by core biopsies, may allow more informative and reliable mutation testing. A further consideration is tumour heterogeneity: it remains unclear whether isolated biopsy samples are truly representative of the overall tumour and whether samples taken from a primary tumour may have a different profile than metastatic sites.

If a high fraction of neoplastic cells are present in a biopsy sample, direct sequencing to determine *EGFR* mutation status has been regarded as the gold standard [89]. Limitations in the feasibility of genomic DNA sequencing arise when tumour material available for PCR or RT-PCR is limited. In addition, direct sequencing techniques are relatively costly and time-consuming.

The fixative used in pathologic preservation and the age of the samples can also affect the quality of sequencing test results. Formalin fixation can cause nucleic acid degradation, decreased amplicon length and PCR artefacts [90]. For example, in the molecular analysis of samples from the BR.21 trial of second- and third-line erlotinib, a large proportion of *EGFR* mutations were misidentified as uncommon novel transitions, an error caused by *post mortem* deamination of cytosine or adenine. These small aberrations can be artefactually amplified from low concentrations of tumour DNA and interpreted as significant when a small or old sample is analysed, whereas such deaminated sites are diluted and not detected when larger amounts of tumoural DNA are used [89, 91].

Biopsy samples with a large proportion of non-neoplastic cells are more suited to allele-specific assays, although these can only be used to assess the presence of a small number of predefined mutations. PCR-based assays are often the preferred choice here, due to their sensitivity, specificity, robustness and relative cost-effectiveness compared with direct sequencing. Because PCR-based assays look for predefined variants, they avoid the time-consuming steps of tissue microdissection and multiple rounds of DNA extraction, enabling their routine use in the clinical setting at acceptable cost. However, allele-specific PCR-based tests can only amplify known mutations in the selected *EGFR* regions. There are a plethora of different methods that have been published to identify *EGFR* mutations [35, 51, 92, 93].

Novel techniques are being developed to improve the feasibility of *EGFR* mutation testing from nontissue-based samples. Noninvasive testing of *EGFR* mutation status using serum samples and captured circulating tumour cells are under investigation [51, 94]. For example, the SMart Amplification Process (SMAP) is a single nucleotide polymorphism-based diagnostic assay that can be used to detect *EGFR* alterations from blood samples. HOSHI *et al.* [95] adapted the SMAP technology to target three known hotspots for activating *EGFR* mutations, identifying the mutations with a high sensitivity within 30 min directly from blood samples. In addition, mutation-specific antibodies, which detect deletions in exon 19 and the L858R mutation in exon 21, have been developed and have shown high sensitivity and specificity when tested in paraffin-embedded tumour samples from NSCLC patients [96]. To simplify *EGFR* mutation testing and ease patient selection, one option is inclusion of a standardised, registered companion diagnostic test.

It remains a challenge to ensure that testing methods are used consistently and to encourage the realisation of biomarker-directed treatment in NSCLC. Efforts are ongoing: for example, the French National Cancer Institute has implemented a 2-yr, multicentre, prospective study (ERMETIC). The primary objective of this study is to evaluate the ability of each of the participating 16 centres to perform biomarker assays, including *EGFR* exons 18–21 and *KRAS* exon 2 sequencing in paraffin-embedded tissues, as determined by the concordance of results between centres with those of an external molecular reference laboratory. After a pilot phase, during which all centres became familiar with the sequencing techniques involved, a prospective analysis has been undertaken of tumour samples from 522 EGFR TKI-naïve patients with stage IV NSCLC who received erlotinib at these centres. The objective of this part of the study was to assess the effectiveness of *EGFR* sequencing in identifying patients who are likely to benefit from treatment with erlotinib [75].

CONCLUSIONS

Having established the current state of evidence regarding genetic profiling and targeted therapy in NSCLC, what clinical implications can we draw? For now, EGFR TKIs should not be given as first-line treatment in the absence of an *EGFR* mutation test. However, we can now realistically envisage *EGFR* mutational testing becoming standard practice in NSCLC diagnostics, especially in patients with appropriate clinical predictors, such as never- but also former smoking patients. As this practice becomes increasingly common, important considerations include the timing of testing and standardisation of the methodology used; future efforts should be directed at developing a more practical test for *EGFR* mutations. For patients with *EGFR* mutations, the issue of secondary resistance must be addressed, and the sequence of chemotherapy in treatment paradigms that include EGFR TKIs must be more clearly defined. For patients with *KRAS* mutations, alternative targeted therapies may be more appropriate than EGFR TKIs and should be investigated further. For patients with neither *EGFR* nor *KRAS* mutations, representing the largest proportion of NSCLC patients, further studies to establish the best treatment options are still needed. However, it is likely that, because of what we have learned about *EGFR* mutations and EGFR TKIs over the past decade, development of future targeted therapies will include earlier investigation into the genotype of good responders and efforts will be focused on defining particular populations that benefit the most from treatment.

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STATEMENT OF INTEREST

Statements of interest for all authors can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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