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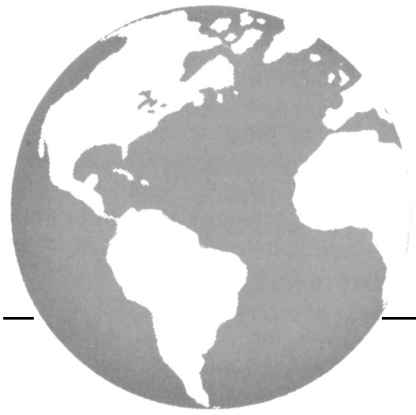

Biomarkers that currently affect clinical practice: *EGFR, ALK, MET, KRAS*

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ABSTRACT

New drugs such as pemetrexed, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, and the Alk inhibitor crizotinib have recently enabled progress in the management of advanced non-small-cell lung cancer (NSCLC). More drugs, especially Met inhibitors, will follow. However, the benefits of these agents are not uniform across the spectrum of NSCLC, and optimizing their utility requires some degree of subgrouping of NSCLC by the presence or absence of certain biomarkers.

The biomarkers of current or imminent value are *EGFR* and *KRAS* mutational status, *ALK* rearrangements, and *MET* immunohistochemistry. As a predictor of benefit for anti-EGFR monoclonal antibodies, *EGFR* immunohistochemistry is also of potential interest.

Some of the foregoing biomarkers (*EGFR, ALK, MET*) are direct drivers of the malignant phenotype. As such, they are, quite rationally, the direct targets of inhibitory drugs. However, *KRAS,* while definitely a driver, has resisted attempts at direct pharmacologic manipulation, and its main value might lie in its role as part of an efficient testing algorithm, because *KRAS* mutations appear to exclude *EGFR* and *ALK* mutations. The indirect value of *KRAS* in determining sensitivity to other targeted agents or to pemetrexed remains controversial. The other biomarkers (*EGFR, ALK, MET*) may also have indirect value as predictors of sensitivity to chemotherapy in general, to pemetrexed specifically, and to radiotherapy and molecularly targeted agents.

These biomarkers have all enabled the co-development of new drugs with companion diagnostics, and they illustrate the paradigm that will govern progress in oncology in the immediate future. However, in NSCLC, the acquisition of sufficient biopsy material remains a stubborn obstacle to the evolution of novel targeted therapies.

KEY WORDS

NSCLC, lung cancer, *EGFR, ALK, KRAS,* prognosis, prediction

1. INTRODUCTION

Metastatic non-small-cell lung cancer (NSCLC) remains, with rare surgical exceptions, incurable. Pending radical new solutions, scientific progress is currently channelled into the conversion of this rapidly lethal disease into a chronic illness. How to make that conversion is conventionally believed to involve "personalized medicine": Tumour biopsies are tested for certain causative and characteristic molecular lesions ("targets"), guiding the selection of customized drugs designed to directly interact with and inhibit those targets.

This paradigm, based on the concept of causality, is deeply seductive, given that it appears to offer the prospect of both efficacy and lack of toxicity. It hews to a fundamentally rational worldview as suggested by its common appellation, "targeted therapy." The molecular lesion is meant to be causally responsible for maintenance of the malignant phenotype and also distinctive, even uniquely characteristic, of the cancer cells. Hence the prospects for both tumour control and selectivity.

The foregoing perspective, while undoubtedly simplistic, nonetheless provides a framework for how four key genes—*EGFR, ALK, MET,* and *KRAS*—will increasingly influence the management of metastatic NSCLC. Those genes, when altered in measurable ways, unquestionably contribute to the pathogenesis of NSCLC, and as such, are widely agreed to be "drivers" [1]. It is rational to seek to inhibit them, even to hope that drugs can be designed that will selectively block the oncogenic varieties while sparing their normal counterparts. But there is no guarantee that oncogenic variants are necessarily druggable, or if they are, that the cancer cell will not eventually find a way around

the inhibition. Furthermore, those genes, although not necessarily mutated *sensu stricto,* may, through altered expression, nonetheless still contribute to the cancer, thus reducing the prospects for selectivity.

Additionally, genetic aberrations such as these may convey useful information beyond the notion of the direct target. Broadly, that information can be classified as prognostic (foreknowledge of probable events in the absence of therapy, which may continue to influence outcomes regardless of therapy) and predictive (indicating the prospects for success of particular therapies), and might be mechanistically related to the aberrant gene, but might also be purely empirical—that is, exhibiting no obvious causal relationship. These genetic alterations, then, are "biomarkers" *sensu lato,* and their utility extends into predicting the future clinical course (even absent therapy) and the selection of drugs (whether those drugs target those particular genes directly or not).

It is better, therefore, to approach *EGFR, ALK, MET,* and *KRAS* and the entire expanding suite of molecular drivers [2] as biomarkers in the broad sense, and not just as direct targets, although the latter status is clearly of major importance.

Although the present review focuses more on the biomarker utility of these genes and less on the technicalities of their measurement, we must emphasize that the acquisition of adequate biopsy material remains problematic in the management of metastatic NSCLC. That problem can partly be addressed by educating respirologists, interventional radiologists, and thoracic surgeons, but sometimes there is no possibility of obtaining other than scant tissue. The reasons include hazard, technical factors, access, patient refusal, and avoidance of delay.

In the event that the clinician's hand is forced, we therefore provide information correlating the foregoing biomarkers with (usually available) clinical, pathologic, and demographic characteristics. Emphatically, however, it is better to make therapeutic decisions on the basis of a direct test. However, as a definitive solution to this problem, reliable testing based on blood work (that is, analysis of circulating tumour cells or plasma DNA) should soon become available [3,4].

2. *EGFR*

In the early 1960s, Stanley Cohen isolated the mitogen "epidermal growth factor" (EGF) from murine salivary gland [5]. In 1973, the EGF receptor (EGFR) was described [6]; this receptor was later appreciated as the first of a family of 4 human epidermal tyrosine kinase receptors (HER1–4) [7], attended by a broad spectrum of ligands besides EGF, participating in a multifaceted and adaptive signalling network [8] subserving growth and survival. *EGFR,* cloned and isolated in 1984 [9], encodes a 1210-amino-acid transmembrane protein, including an extracellular ligand-binding ectodomain, an anchoring transmembrane domain, and a submembrane tyrosine kinase domain. Ligand activation involves homo-dimerization (or hetero-dimerization with other HER family members), and then activation of the tyrosine kinase domain, resulting in tyrosine autophosphorylation, which enables engagement with 6 or more signalling pathways subserving "cell fate decisions" [8], including the PI3K/Akt and Erk pathways of particular interest in oncology.

Dysregulation of EGFR contributes to a range of cancers and occurs in various ways [10]. In NSCLC, the most important are activating *EGFR* mutations and increased protein expression. Either dysregulation may possibly be associated with increased gene copy number. The uncommon *EGFRvIII* mutation has also been detected in a few squamous cell lung cancers [1]. However it arises, dysregulated *EGFR* activation promotes the malignant phenotype by mediating cell proliferation, raising the apoptotic threshold, increasing cellular motility (and hence metastasis), enhancing neoangiogenesis, and conferring resistance to chemotherapy and radiation.

Although earlier efforts at predicting anti-EGFR therapeutic sensitivity focused on EGFR protein overexpression and *EGFR* gene copy number increment, the most important parameter is whether an activating *EGFR* mutation is present. The mutations are almost exclusively found in lung adenocarcinomas; they are more common in never-smokers or light ex-smokers, women, and patients of East Asian origin. In this demographic, 60%–70% of patients will have a detectable mutation in *EGFR*. Caucasian smokers or ex-smokers with adenocarcinomas have an 8% incidence—enough to mandate testing. All patients with adenocarcinomas should be tested for *EGFR* mutation (Table I) [11–13], although that dictum may need to be softened depending on immunophenotyping. Mutations are associated mainly with papillary and micropapillary adenocarcinomas or non-mucinous bronchioloalveolar adenocarcinomas (rarely with solid adenocarcinomas) and seem mostly to require an immunophenotype positive for thyroid transcription factor 1 (TTF-1).

Nearly all activating *EGFR* mutations occur in exons 18–21. The most important are deletions within exon 19 (more than 20 variants) and point (missense) mutations in exon 21 (usually L858R,

TABLE I Estimated genomic probabilities in adenocarcinoma [11–13]

Variable	*Value by locale*	
	East Asia[a]	*Western world*[b]
Studies (*n*)	6	2
Patients (*n*)	814	116
Never-smokers, *EGFR* M+ (%)	70	37
Ever-smokers, *EGFR* M+ (%)	29	8

[a] Japan, Korea, Taiwan, Hong Kong.
[b] United States, Australia.

occasionally L861Q or L861R). Very occasionally, point mutations involve exon 18 (for example, G719C and others at G719). Generally the tyrosine kinase domain is affected, probably leading to increased ATP binding, with enhanced (and ligand-independent) downstream signalling, especially via the Akt and STAT pathways, affecting cell survival [14]. The resulting condition ("oncogene addiction") is characterized by a dependency of the cancer cell on the mutation. Also implicated is the Erk1/2 pathway, essential to cellular proliferation [15]. The benefits of EGFR blockade may ultimately be mediated by a shift toward apoptosis in the balance of the pro- and anti-apoptotic members of the Bcl-2 family of proteins.

The centrality of EGFR signalling has led to intensive efforts to design therapies aimed at blockade. Two approaches have proved successful: anti-EGFR monoclonal antibodies against the extracellular ligand-binding domain, and small-molecule tyrosine kinase inhibitors (TKIS) to block binding of ATP (upon which signalling depends). The latter have proved much more valuable in NSCLC, although EGFR antibodies have also demonstrated activity.

Curiously, small-molecule TKIS (gefitinib and erlotinib) were designed before the elucidation, in 2004 by three American groups, of the *EGFR* mutation [2,16,17]. The small subset of metastatic NSCLC patients who had responded dramatically to single-agent TKI therapy prompted the search for an explanation, culminating in discovery of the mutations. These mutations not only confer oncogene addiction, but also fortuitously show markedly increased affinity for gefitinib or erlotinib because of residue repositioning around the binding cleft [18]. It soon became apparent that almost all the dramatic responses had occurred in patients whose cancers harboured one of these activating (and sensitizing) *EGFR* mutations; however, EGFR-TKI can also, to a lesser extent, benefit patients without those mutations: that is, the *EGFR* "wild-type" (*EGFR* WT) patients, whose cancers are presumably driven by upregulated signalling (from overexpression of the normal protein, for instance).

Small (mainly East Asian) studies of EGFR-TKI monotherapy with gefitinib rapidly confirmed high objective response rates (55%–91%) in patients with cancers harbouring a mutation [19–26]. A large non-randomized 217-patient Spanish-led experience [27] with erlotinib was published in 2009. The objective response rate (ORR) of 70.6%, the progressive disease rate of just 10.2%, the prolonged progression-free survival (PFS) of 14 months, and the overall survival (OS) of 27 months suggested that responsiveness in mutation-positive patients was not a function of ethnicity and that erlotinib might be superior to gefitinib. Furthermore, Caucasian patients demonstrated a spectrum of *EGFR* mutational subtypes similar to those seen in East Asian patients. Those phase II trials led to six large randomized trials comparing first-line EGFR-TKI with then-standard platinum-doublet third-generation chemotherapy in proven *EGFR* mutation–positive patients (*EGFR* M+) or in populations enriched for mutation positivity (Table II).

The randomized studies (IPASS, WJTOG 3405, NEJ002, First-SIGNAL, OPTIMAL, and EURTAC) uniformly revealed that, compared with chemotherapy, first-line TKI consistently resulted in a higher ORR and longer PFS; however, OS was not prolonged because of extensive crossover from chemotherapy to TKI upon progression. Because TKI and chemotherapy appear non-cross-resistant, those who receive a second-line TKI benefit as much as those who receive it in the first line [27,35]. However, because of unavoidable attrition (35% in IPASS), it is desirable to treat with a TKI up front if possible in *EGFR* M+ patients, notwithstanding a modest delay to secure a test result. However, as revealed by IPASS, the one trial to accrue and analyze both *EGFR* M+ and WT patients, the opposite is even more true. Clearly, in *EGFR* WT disease (approximately 40% of the East Asian IPASS population of never-smokers or light ex-smokers), gefitinib appears virtually devoid of useful activity (ORR: 1.1%) and may be associated with passive harm because of the opportunity cost of delaying active chemotherapy.

In the trials, patients with exon 19 deletions and exon 21 point mutations did not have markedly different outcomes on TKI (the former perhaps conferring a modestly better outcome). Also, erlotinib is probably not markedly different from gefitinib in outcome, and (from IPASS) *EGFR* mutation positivity is prognostic for inherently longer survival. There is a suggestion (again from IPASS) that, compared with *EGFR* WT patients, those who are *EGFR* M+ respond better to chemotherapy, although the ORR in IPASS for M+ patients (47%) was outside the range for chemotherapy in the other five randomized trials (15%–37%). However, IPASS did establish that first-line chemotherapy in *EGFR* M+ patients was much more active than TKI in *EGFR* WT patients, implying that *EGFR*-unknown patients should receive first-line chemotherapy rather than "empirical TKI."

A subsequent randomized trial of post-chemotherapy maintenance erlotinib compared with placebo (SATURN) exhibited a dramatic benefit for the *EGFR* M+ subset [hazard ratio (HR): 0.10] in PFS, but not in OS, again because of crossover. Interestingly, the *EGFR* WT patients did experience an OS advantage—but only if the best response on prior first-line chemotherapy was stable disease, not complete or partial response [36].

Mutations in *EGFR* also occur in exon 20, especially T790M, which inserts a bulky methionine over the ATP binding cleft, blocking access to first-generation EGFR-TKI (but not to ATP) [37]. This "gatekeeper" T790M mutation occurs only within a pre-existing sensitizing mutation, either del 19 or exon 21, and seemingly causes up to 50% of the resistance inevitably occurring in all *EGFR* M+ patients on first-generation TKI (gefitinib or erlotinib). Novel EGFR-TKI (for example, afatinib, dacomitinib)

TABLE II Randomized trials of chemotherapy compared with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) in mutation-positive patients

Reference (study name)	*Regimen*	*ORR (%)*	*Statistic*	p *Value*	*PFS (months)*	*HR*	p *Value*	*OS (months)*	*HR*	p *Value*
Lee *et al.,* 2009 [28]; Ku *et al.,* 2011 [29] (First-SIGNAL)	Cisplatin–gemcitabine vs. gefitinib	38 85		0.002	6.7 8.4		0.0084	26.5 30.6	HR?	0.648
Maemondo *et al.,* 2010 [30] (NEJ 002)	Carboplatin–paclitaxel vs. gefitinib	31 74		<0.001	5.4 10.8	0.30	<0.001	23.6 30.5	Not available	0.31
Mitsudomi *et al.,* 2010 [31] (WJTOG 3405)	Cisplatin–docetaxel vs. gefitinib	32 62		<0.001	6.3 9.2	0.49	<0.0001	Not reached 30.9	1.64	0.211
Fukuoka *et al.,* 2011 [32]; Mok, 2011[a] (IPASS)	Carboplatin–paclitaxel vs. gefitinib	47 71		<0.001	6.3 9.5	0.48	<0.001	21.9 21.6	1.0	0.99
Zhou *et al.,* 2011 [33] (OPTIMAL)	Carboplatin–gemcitabine vs. erlotinib	36 83		<0.0001	4.6 13.1	0.16	<0.0001	Not available	Not available	
Rossell *et al.,* 2012 [34] (EURTAC)	Platinum–gemcitabine or platinum–docetaxel vs. erlotinib	15 58	OR: 7.5	<0.0001	5.2 9.7	0.37	<0.0001	19.5 19.3	1.047	0.87

[a] Mok T. Novel therapies [part of mini-symposium M12]. Presented at the 14th World Conference on Lung Cancer; Amsterdam, Netherlands; July 3–7, 2011.

ORR = objective response rate; PFS = progression-free survival; HR = hazard ratio; OS = overall survival; OR = odds ratio.

bind despite the T790M mutation, but can also bind to other HER receptors. These drugs can undoubtedly benefit patients failed by first-generation TKI, but whether T790M binding is responsible remains uncertain. The LUX-Lung series of trials with afatinib are illustrative; LUX-Lung 1 randomized patients who had received prior platinum chemotherapy and who had progressed after 12 or more weeks on erlotinib or gefitinib to either afatinib or placebo. The PFS, ORR, and symptom control outcomes strongly favoured afatinib, but OS was not significantly different (79% of patients on the placebo arm received further lines of treatment). The large single-arm phase II LUX-Lung 2 trial included *EGFR* M+ patients at either first or second line. The ORR was 60%, but the PFS was an impressive 14 months. Results of LUX-Lung 3, which is now accrued and which randomized *EGFR* M+ patients in the first line to afatinib or cisplatin–pemetrexed, are imminent and could lead to regulatory application.

Other resistance mechanisms to TKI in M+ patients include *MET* amplification (5%–20%) and, occasionally, epithelial–mesenchymal transition and even transformation to a small-cell phenotype [38]. However, progressive disease on a first-generation TKI according to the formal Response Evaluation Criteria in Solid Tumors does not necessarily mean exhausted utility, because abrupt TKI cessation can, in about 20% of patients, induce a significant "flare" phenomenon that responds to immediate re-introduction of the same TKI [39]. Also, rechallenge with the same TKI after a "holiday" (during which chemotherapy may be given) is increasingly recognized as valuable [40,41]. There is an unmet need for biomarkers to guide the management of patients who experience technical progressive disease in front-line TKI, and there is evidence that resistance may differentially affect some metastases and not others—that is, clonal metastasis [42,a].

Updated IPASS biomarker analysis [32] clearly showed that measurement of *EGFR* gene copy number

[a] Zhong WZ. Genomic heterogeneity between primary tumor and its metastases. Presented at the 3rd International Thoracic Oncology Congress Dresden; Dresden, Germany; September 13–15, 2012.

by fluorescence *in-situ* hybridization (FISH) or of EGFR expression by immunohistochemistry (IHC) does not substitute for a mutation test. However, high copy number or IHC expression seems to be a weak surrogate for *EGFR* mutation positivity.

The NCIC BR.21 trial enrolled second- or third-line metastatic NSCLC patients who had exhausted their chemotherapy options. It showed an OS benefit for erlotinib compared with placebo. A limited biomarker analysis suggested that high *EGFR* copy number by FISH (because of either gene amplification or high polysomy) [43] predicted a higher ORR (21% vs. 5%) and an improved OS benefit from erlotinib (HR: 0.43 vs. 0.80 in FISH-negative patients). The FISH-positive control subjects had the worst OS, but the most benefit from erlotinib, and compared with mutational status, FISH seemed to influence OS more [44].

Erlotinib was administered to more than 7000 patients in the large, open-label TRUST study (0–2 prior chemotherapies), with the German centres reporting their biomarker data. *EGFR* mutations and FISH positivity predicted response. Positivity by FISH also predicted PFS and OS. The EGFR IHC positivity weakly correlated with PFS and OS. Interestingly, 22% of patients were both IHC-positive and FISH-positive; about half to two thirds were IHC-positive, but FISH-negative; and 11%–21% were IHC-negative and FISH-negative, independent of histology [45].

In nonrandomized studies such as TRUST, and even in randomized studies not using a placebo control, it is impossible to disentangle prognostic and predictive factors for PFS and OS; in this respect, BR.21 is highly valuable—as is ISEL[46,47], a similar study that compared gefitinib with placebo, but in a more refractory population. In ISEL, which showed a nonsignificant benefit for gefitinib compared with placebo, high *EGFR* copy number predicted an OS treatment effect (HR: 0.61 compared with placebo). An interaction test was significant ($p = 0.045$), indicating a genuinely different effect by copy number. The same applied to IHC status. *EGFR* mutations substantially predicted response (37.5% vs. 2.6%), but the data were too few to adjudicate survival effects. Results in the ISEL placebo group also suggested that FISH positivity was an adverse prognostic indicator (median survival time: 4.5 months vs. 6.4 months; HR: 1.41).

The BR.21, TRUST, and ISEL trials seem to imply utility for FISH and IHC as well as for *EGFR* mutational status, especially in Caucasian patients, in whom FISH positivity is more common than is mutation in unselected patients. In ISEL, 30.8% were FISH-positive and 12.1% were M+. Of the entire population, 20.2% were East Asian. In BR.21 (only 12% East Asian), 38% were FISH-positive and 18% were *EGFR* M+.

Ellis *et al.* performed a meta-analysis on the BR.21 and SATURN trials, two post-first-line trials, each with a placebo arm. Those authors concluded that EGFR IHC positivity is prognostic (weakly) for longer PFS and OS, that *EGFR* FISH status was not prognostic, and that *EGFR* mutations may be prognostic for OS (perhaps confounded by crossover). Neither IHC nor FISH were recommended for "routine" prediction of erlotinib sensitivity; mutation positivity implied a better PFS on erlotinib, but mutation negativity did not preclude a benefit, and therefore *EGFR* mutation testing was not valuable after the first line [48]. In that analysis, some results for IHC, FISH (especially), and mutation status appeared to be discrepant between BR.21 and SATURN. In particular, FISH positivity was both predictive and negatively prognostic in BR.21, but not in SATURN. Notably, the BR.21 and SATURN patient populations were dissimilar.

The utility of FISH in the context of EGFR-TKI, especially in *EGFR* WT patients of any histology, should not be discounted for both prognosis and prediction.

Anti-EGFR monoclonal antibodies, especially cetuximab, added to chemotherapy in metastatic NSCLC generally produce modestly positive results. The FLEX study considered the addition of cetuximab to cisplatin–vinorelbine in EGFR IHC-positive metastatic NSCLC. Median OS was increased by 1.2 months (HR: 0.871; $p = 0.044$) [49]. However, application of a scoring system ("H-score," continuous scale 0–300) revealed that 31% scored high (>200) and that the high-scoring patients (either histology) monopolized the OS benefit (9.6 months vs. 12.0 months; HR: 0.73; $p = 0.01$). The low-score HR was 0.99. The interaction test was significant ($p = 0.044$) [50]. The Southwest Oncology Group 0819 study is attempting to prospectively confirm that result with cetuximab and carboplatin–paclitaxel–bevacizumab.

Technical aspects of *EGFR* testing are beyond our scope [51]; however, microdissection and sequencing (Figure 1) may represent the current clinical standard. Allele-specific amplification—for example, Scorpions ARMS (DxS Limited, Manchester, U.K.)—is an alternative. Experimental mutation-specific antibodies are highly specific (97%–100%) and moderately sensitive (74.2%–100%) [52–55]. Detection of mutations in circulating tumour cells [56,57] or even circulating DNA [58,59] is rapidly being perfected.

In Canada (Table III), *EGFR* testing has been centralized in 5 laboratories, which might use different methodologies (for example, restriction fragment length polymorphism analysis, sequencing; Figure 1) and for which a minimum of five 5-μm sections are required, each with more than 100 tumour cells per section, and with the tumour cells representing more than 25% of the nucleated cells. Specimens are preferably microdissected and are better derived from core biopsies, although cell blocks and generous fine-needle aspirates may be adequate. Neither IHC nor FISH are routinely obtained.

3. *ALK*

As a driver oncogene, *ALK* (the anaplastic lymphoma kinase gene) was initially discovered in a

TABLE III Testing for *EGFR* mutation and selected uses of epidermal growth factor receptor inhibitors by province in Canada

Province	EGFR *testing*	*First-line gefitinib* (EGFR *M+)*	*Maintenance erlotinib*[a]
BC	√	√	√
AB	√	√	X
SK	AZ[b]	Case by case	X
MB	AZ	Case by case	X
ON	AZ	√	X
QC	√	√	X
NS	AZ	X	X
NL	AZ	X	X
PE	AZ	X	X

[a] Not restricted by *EGFR* status.
[b] Paid for by pharmaceutical company.
AZ = AstraZeneca Canada.

chromosomal rearrangement in anaplastic large-cell lymphoma [60]. In 2007, Soda *et al.* described *ALK* activation in a subset of NSCLC that exhibited a "small inversion" in chromosome 2, fusing the normally separated *EML4* (echinoderm microtubule-associated protein-like 4 gene) with *ALK* [61]. This *EML4–ALK* fusion transcript was detected in 5 of 75 Japanese NSCLC patients and in none of 261 patients with "other" cancers. Interestingly, although some *EGFR* (and *KRAS*) mutations were also found in the NSCLC cohort, none overlapped with the patients positive for *EML4–ALK*. The oncogenicity of the transcript was confirmed by transfection of expression plasmids into 3T3 cells, transforming them and subsequently showing tumorigenicity in nude mice. Although variants of the fusion transcript have been identified, in each case oncogenicity requires intact kinase function of *ALK*.

It was soon revealed that *EML4–ALK* lung carcinogenesis extended beyond Asia, characteristically occurring in middle-aged patients, usually never-smokers of either sex, and presenting as adenocarcinoma, especially the acinar histology in East Asia or the signet-ring or cribriform morphology in the West. This variant is always positive for TTF-1 [62,63]. Furthermore, mutual exclusivity between *EML4–ALK* and *EGFR* and *KRAS* mutations has been confirmed [64].

The interest in *EML4–ALK* that has elevated its importance above its 2.5% incidence in NSCLC is its relatively specific and well-tolerated inhibitor, crizotinib. Crizotinib, originally in development as a Met inhibitor [65], is also a potent Alk inhibitor. Entering human studies in 2006, the maximum tolerated dose was established as 250 mg twice daily. While that trial was open, the Morris *et al.* study was published, and the first *EML4–ALK* patient enrolled (receiving 300 mg orally, twice daily) enjoyed a rapid and dramatic response. Subsequently, intensive efforts were made to

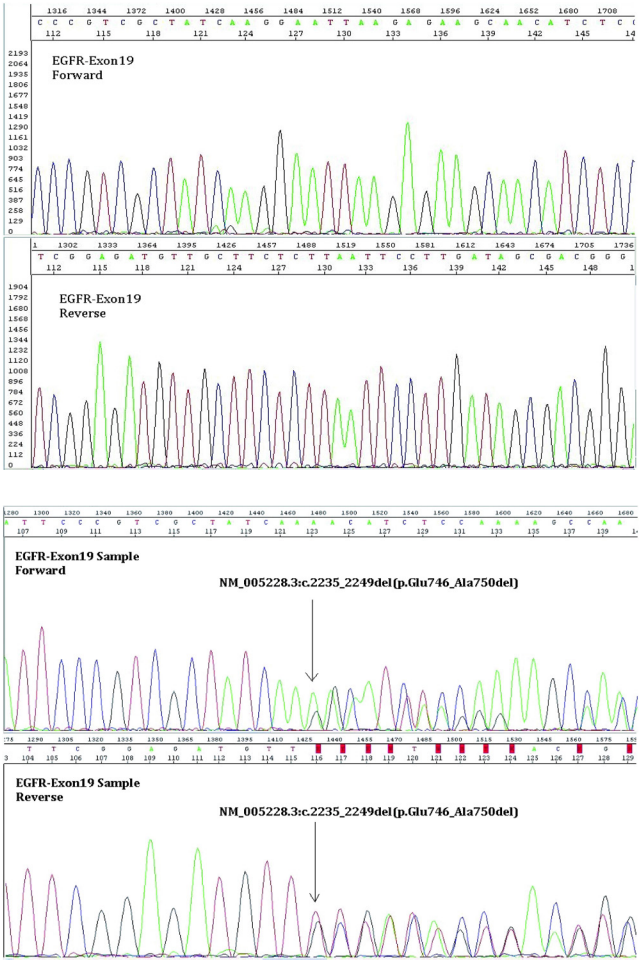

FIGURE 1 The DNA extracted from the macrodissected tissue specimen was amplified using primers specific for exon 19 of the EGFR *gene. The polymerase chain reaction product was then purified and sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.). The sequences obtained were then compared with the U.S. National Center for Biotechnology Information reference sequence (EGFR: NM_005228.3). In this case, a deletion is observed to span nucleotides 2235–2249, which results in the deletion of amino acids at positions 746–750 (inclusive) in exon 19 of the* EGFR *gene.*

recruit NSCLC patients based on *ALK* rearrangements, and a high ORR was confirmed (10 responders in the first 19 patients), as reported in 2009 [66]. A further trial in 82 *ALK*-rearranged NSCLC patients appeared in 2010, showing a 57% ORR and 33% of patients with stable disease [67]. Further trials reported in 2011 that involved 119 (A8081001) and 136 (PROFILE 1005) patients led to conditional approval of crizotinib (Xalkori: Pfizer, Mission, KS, U.S.A.) by the U.S. Food and Drug Administration (FDA) [67,68] and by Health Canada more recently.

In those 255 patients (median age: 51 years; 48% men; 63% Caucasian, 30% Asian; 70% never-smokers, 28% former smokers; 96.5% with adenocarcinoma),

the ORRs were 61% (A8081001) and 50% (PROFILE 1005[b]). The PFS is expected to be ± 10 months (A8081001), with the OS still uncertain. In patients progressing on the chemotherapy arm of the randomized second-line trial of pemetrexed or docetaxel compared with crizotinib (PROFILE 1007), PROFILE 1005 confirmed a very high ORR[b]. An ongoing phase III trial, PROFILE 1014, is investigating first-line crizotinib compared with platin–pemetrexed.

The best detection method for *ALK* rearrangements in NSCLC is debatable. The current clinical standard—the Break Apart FISH Probe kit (Abbott Molecular, Abbott Park, IL, U.S.A.), Figure 2—uses fluorescent green (5′) and red (3′) signals on loci in chromosome 2, normally so close together that they may fuse visually. Positivity consists of separation of these two markers by more than 2 signal diameters, or a red signal alone, in more than 15% tumour cells, counting more than 50 tumour cells. This is the companion assay approved by the FDA with crizotinib. Suitable for formalin-fixed, paraffin-embedded specimens, it is technically demanding and expensive, encouraging development of alternative methodologies, for example, reverse-transcriptase polymerase chain reaction (requiring knowledge of known fusion variants), DNA sequencing, or IHC. Immunohistochemistry, potentially with augmentation, may become a standard-of-care, high concordance with FISH having been established for IHC 3+ or IHC 0 [69]. Intermediate IHC scores may, however, still require FISH. Several different antibodies are in development [64].

Already, a crizotinib resistance mechanism has been identified, a "gatekeeper" mutation L1196M [70–72]. The gene *ROS* is also a target of crizotinib. Activation of *ROS* can be found in about 1.7% of NSCLC and can be assayed for; crizotinib appears to have marked activity in these cases [73].

Pemetrexed may have exceptional activity in *ALK*-rearranged NSCLC [74], with a response (in monotherapy or in combination with a platin) of 42% and a PFS of 9 months. Other publications have appeared in support [75,76], but more recently, those findings have been questioned. The ongoing PROFILE studies should be informative.

4. *KRAS*

The *ras* oncogenes were identified as cellular homologues of the Harvey and Kirsten strains of a mouse sarcoma virus [77]. Normally, Ras functions in signal transduction downstream of transmembrane receptor tyrosine kinases, especially EGFR, to which it is recruited by adaptor molecules, after binding of growth factors such as EGF and transforming growth factor α to the receptor tyrosine kinase. Activation of Ras occurs through GTP binding, and as an intrinsic GTPase, it catalyzes GTP breakdown, enabling (then switching off) downstream signalling predominantly via the Raf/Mek/Erk downstream signal transduction pathway (the classical MAPK pathway). Erk activates transcription of genes mediating mitosis (and cell survival). At least 9 other pathways may be stimulated by Ras, including PI3K/Akt, a survival pathway [78,79].

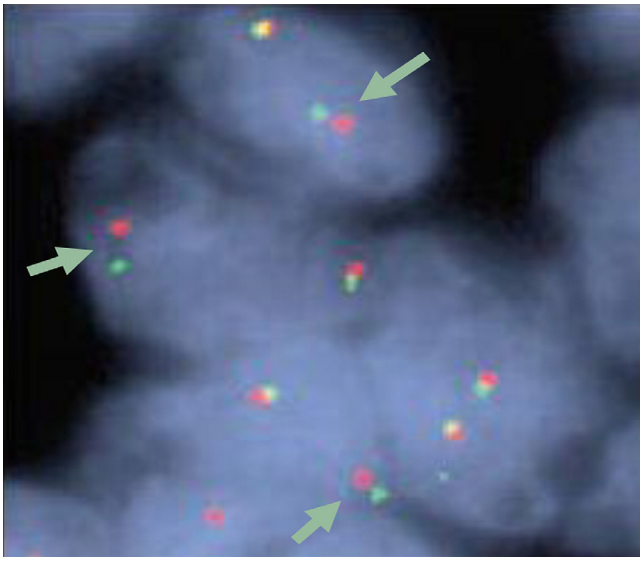

FIGURE 2 An example of a positive test with the Break Apart FISH Probe kit (Abbott Molecular, Abbott Park, IL, U.S.A.). A red and a green probe are hybridized to regions flanking the ALK *translocation breakpoint; these probes will be separated by an intervening fusion of a translocated fragment (for example,* EML4*). That intervening fusion can be clearly seen here in several cells (arrows). In the other (normal) allele, the red and green probes are not separated, even appearing yellow (an artefact of visual overlap). The assay is designated positive if 15% or more of 50 or more cell nuclei demonstrate the split signal or an isolated red signal.*

Of the *KRAS* mutations in NSCLC, 97% occur in exon 2, codon 12 or 13 [80]. These missense mutations impair the functionality of *ras* GTPase, locking the Ras signalling in active mode. Paradoxically, although the mutations inactivate Ras, the result is persistent signal activation. That persistence is one reason that Ras has been difficult to "drug"; it requires reactivation, not inactivation, to switch the signalling off.

Mutations of *ras* in NSCLC occur predominantly in "smoking adenocarcinoma" patients (30%–40%, Table I). In those patients, the mutations are G-to-T or G-to-C transversions (that is, pyrimidine swapped for purine); recently however, in never-smokers with adenocarcinoma, "transition" mutations [G to A (purine for purine)] have occasionally been found (approximately 15%), also probably oncogenic [81]. The mutation subtype may

[b] Riely GJ, Kim DW, Crino L, *et al.* Phase 2 data for crizotinib (PF-02341066) in *ALK*-positive advanced non-small cell lung cancer (NSCLC): PROFILE 1005 [abstract 1618]. Presented at the 14th World Conference on Lung Cancer; Amsterdam, Netherlands; July 3–7, 2011.

alter downstream signal activation, with potential implications [82] for prognosis. This heterogeneity may explain some of the conflicting data that characterize *KRAS* clinical research.

Currently, *KRAS* itself remains undruggable despite decades of effort. Attention has recently focused on inhibition of the Ras-contingent downstream signalling (especially Raf and Erk) or exploitation of synthetic lethality [83].

Whether *KRAS* mutations influence EGFR-TKI responsiveness is contentious, and current Canadian recommendations discourage *ras* testing [48]. Studies indicating no benefit [44,84] have to be balanced by studies indicating that *KRAS* mutations are compatible with some benefit in, for example, maintenance—as with SATURN [85]. However, *KRAS* mutations may indicate a short PFS in the control arm and may therefore be adversely prognostic regardless of treatment.

The negative predictive effect of *KRAS* for treatment with anti-EGFR antibodies in colorectal cancer does not carry over to NSCLC treated with cetuximab; consider FLEX, for example, in which *KRAS* mutations were neither predictive nor prognostic [86]. However, *KRAS* mutations may sensitize tumours to antifolates such as pemetrexed [74,87], possibly by upregulation of miR-181c, a micro RNA that can downregulate *KRAS*. Those observations require confirmation, given the high frequency of *KRAS* mutations in adenocarcinoma associated with smoking.

Currently, the chief value of *KRAS* lies in providing information about the other biomarkers that are directly druggable—that is, *EGFR* and *ALK*. The presence of mutated *KRAS* rules out *ALK* and *EGFR,* and *KRAS* may therefore form part of an efficient pathway in a testing algorithm.

5. *MET*

Met is a receptor tyrosine kinase often expressed in epithelium. Its paracrine ligand, hepatocyte growth factor ("scatter factor"), is produced by stromal cells. Met signals via Ras, PI3K/Akt, and STAT, affecting mitosis, survival, angiogenesis, migration, invasion, and as implied, mesenchymal–epithelial transversion. Upregulation in cancer cells results in "invasive growth" [88]. Amplification of *MET* is documented in 4.1% of North American lung adenocarcinomas, but *MET* overexpression maybe more common[c]. Mutations in *MET* occur rarely.

Upregulation of *MET* may depend on prior exposure to therapy and may mediate resistance to it. Several studies indicate that *MET* amplification is responsible for ±20% of resistance to EGFR-TKI [89–92], prompting the development of Met-inhibitory strategies. Tivantinib (ARQ 197) is currently in phase III trial (MARQUEE) based on a successful randomized phase II study (erlotinib ± tivantinib). Non-squamous and *KRAS* M+ patients benefited most. MetMAb (Hoffmann–La Roche, Mississauga, ON), an anti-Met monoclonal antibody, achieved significant PFS and OS benefit in a randomized phase II trial (OAM 4558g) with a similar "erlotinib ± experimental drug" design, but only in high expressors of *MET* (Met IHC 2+ or 3+). Detection by IHC (that is, expression) may be more reliable than detection by FISH (that is, amplification) in predicting MetMAb benefit. The effect in low expressors of Met appeared actually harmful, highlighting the importance of a companion diagnostic as MetMAb proceeds into phase III.

Crizotinib, although approved for *ALK*-rearranged metastatic NSCLC, is also a good Met inhibitor. An anecdotal report [93] of a rapid, durable response to crizotinib in a *MET*-amplified NSCLC patient with normal *ALK,* suggests that crizotinib may be suitable for that situation as well as for *ALK* rearrangements, as already shown for other types of cancer with *MET* amplification [94,95].

MET will likely be the next major biomarker in metastatic NSCLC, given the speed with which the foregoing drugs (and others) [88] are approaching the clinic. How best to integrate them into the increasingly complex metastatic NSCLC algorithm will require substantial investment, but will likely pay major dividends.

6. SUMMARY

EGFR and *ALK* are biomarkers of current relevance in the management of non-squamous metastatic NSCLC and definitely predict a higher likelihood of benefit from EGFR-TKI and crizotinib respectively. Across Canada, efforts to promote access to testing require intensification. *KRAS* testing remains controversial—but interesting in the research setting and in testing algorithms as an efficiency tactic, because *KRAS* mutations are common and almost entirely rule out *EGFR* mutations and *ALK* rearrangements. *MET* amplification—or more likely, Met IHC—is required to optimize the development and clinical deployment of Met-directed therapies. Subject to confirmation, EGFR IHC ("H-score") might allow for the selection of patients benefiting from anti-EGFR monoclonal antibodies such as cetuximab.

The problem of inconsistent access to adequate tissue remains an important obstacle to the evolution of personalized medicine in metastatic NSCLC. The solution lies partly in the ongoing development of serum-based molecular assays, but for now, it lies in the education of interventional radiologists, thoracic surgeons, and respirologists, because optimal treatment of metastatic NSCLC is highly contingent on an adequate biopsy.

[c] Varella–Garcia M, Iafrate J, Pao W, *et al. ALK* fusion and *MET* amplification as molecular biomarkers and therapeutic targets in advanced lung adenocarcinomas in the Lung Cancer Mutation Consortium [abstract 1348]. Presented at the 14th World Conference on Lung Cancer; Amsterdam, Netherlands; July 3–7, 2011.

7. CONFLICT OF INTEREST DISCLOSURES

The authors declare consultancy work for Lilly and Roche; work on advisory boards for Pfizer, AstraZeneca, and Roche; membership in a speakers' bureau for Lilly.

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