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The role of molecular pathology in non-small-cell lung carcinoma—now and in the future

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ABSTRACT

In recent years, better understanding of the molecular biology of non-small-cell lung carcinoma (NSCLC) has led to a revolution in the work-up of these neoplasms. As a pathology diagnosis, "NSCLC" without further attempt at subclassification is no longer accepted as a standard of care; separating squamous cell carcinoma from adenocarcinoma and large-cell carcinoma carries implications for prognosis and treatment decisions. Currently, detection of the presence in NSCLC of mutations involving the epidermal growth factor receptor (*EGFR*) gene and fusion of the N-terminal portion of the protein encoded by *EML4* (echinoderm microtubule-associated protein-like 4 gene) with the intracellular signaling portion of the receptor tyrosine kinase encoded by *ALK* (anaplastic lymphoma kinase gene)—that is, *EML4–ALK*—and variants has become routine in many centres because patients having tumours harbouring such alterations might benefit from tyrosine kinase inhibitors as part of their treatment regimen.

The purpose of the present review is to highlight important aspects of the screening for molecular derangements in NSCLC and to briefly discuss the emergence of possible future biomarkers.

KEY WORDS

Non-small-cell lung carcinoma, NSCLC, epidermal growth factor receptor, *EGFR, EML4–ALK,* tyrosine kinase inhibitors, personalized medicine, biomarkers

1. INTRODUCTION

According to the American Cancer Society, approximately 157,300 deaths related to lung cancer (the highest among all cancers) were registered in the United States during 2010. Lung cancer is the leading cancer type for estimated new cancer cases; it also leads in deaths in men and women alike, at 29% and 26% respectively [1]. Non-small-cell lung carcinoma (NSCLC) is the most frequent subtype, representing approximately 85% of all cases, and most patients have locally advanced or distant metastatic disease (stage III/IV) at the time of presentation [2].

Since the early 2000s, greater understanding of the molecular biology of lung cancer, particularly NSCLC, has led to a revolution in the treatment of these neoplasms. Already, searching for specific mutations in individual cases so as to provide the most effective treatment with the least possible occurrence of side effects is a reality. Identification of mutations in the *EGFR* (epidermal growth factor receptor) and *KRAS* genes and, most recently, of rearrangements in the *ALK* gene has been incorporated into routine practice in several centres. These mutations and rearrangements are thought to alter the function or expression of several molecules that can be either located on the cell surface, acting as growth factor receptors, or participating in downstream intracellular pathways. Such derangement of the cellular apparatus ultimately leads to uncontrolled cell growth. The data provided by detection of such mutations can be used to generate prognostic information or to select patients for targeted therapies. In lung oncology, personalized medicine is slowly becoming the norm. Because of the immense amount of data constantly being generated and the rapid flow of information, arriving at a consensus on standardization of molecular testing creates a major challenge for pathologists, clinicians, and molecular biologists.

In this review, we briefly discuss the most common mutations and rearrangements associated with NSCLC, the strategies potentially available to optimize their detection, and future trends in this now ever-changing field.

2. HISTOLOGIC ASPECTS AND COMMON MOLECULAR DERANGEMENTS

2.1 Histologic Aspects

Until recently, nearly all primary epithelial neoplasms of the lung would be classified either as small-cell

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carcinoma or NSCLC, and that classification would suffice for institution by the treating clinician of the accepted standard-of-care treatment. However, three important and previously unrecognized aspects pertaining to treatment and prognosis in NSCLC have been brought to light:

- First, *EGFR* mutations are much more frequently detected in adenocarcinomas (ADCS).
- Second, bevacizumab, a monoclonal antibody that inhibits vascular endothelial growth factor A can induce life-threatening hemorrhage in patients with squamous cell carcinoma of the lung (thereby contraindicating its use in such patients) [3].
- Third, pemetrexed, an inhibitor of purine and pyrimidine synthesis, has demonstrated superior efficacy in patients with ADC or NSCLC "not otherwise specified" than in those with squamous cell morphology [4].

Based on those findings, guidelines for good practice in handling pathology samples from lung neoplasms have begun recommending that small biopsies and cytology specimens be classified whenever possible as squamous cell carcinoma or ADC, and that the use of immunohistochemistry and mucin stains be used in difficult cases, but kept at a minimum to spare tissue for molecular studies [5].

In terms of specific molecular abnormalities associated with predominant histology patterns, the presence of *EGFR* mutation is associated with ADCS, particularly those containing a non-mucinous bronchioloalveolar ("lepidic") pattern [6] [Figure 1(A)]. As for *EML4–ALK,* ADCS are also the main histologic type, and the most striking correlation is with the presence of a signet-ring component [7] [Figure 1(B)]. Mutations in *KRAS* are also present almost exclusively in ADCS, being strongly associated with the mucinous bronchioloalveolar pattern [8] [Figure 1(C)].

2.2 *EGFR*

The epidermal growth factor receptor (also known as HER1 or ErbB1) is a member of the ErbB receptor tyrosine kinase family, comprising HER1/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. It plays an important role in carcinogenesis and tumour progression through activation mechanisms including overexpression, mutation, and autocrine ligand production. Derangements in the *EGFR* gene are associated with cancer cell proliferation, cell growth, invasion, metastatic spread, apoptosis, and tumour angiogenesis. It accomplishes those actions through activation of the Ras/Raf/Mek/MAPK and PI3K/Akt/MTOR pathways [9].

The two most common *EGFR* mutations are short in-frame deletions of exon 19 and a point mutation (CTG to CGG) in exon 21 at nucleotide 2573 that results in substitution of leucine by arginine at codon 858 (L858R) [10].

Tumours with *EGFR* mutations occur at a higher frequency in East Asians than in non-Asians (30% vs. 8%), in women than in men (59% vs. 26%), in never-smokers than in ever-smokers (66% vs. 22%), and in ADCS than in other NSCLC histologies (49% vs. 2%) [11]. In the United States, activating *EGFR* mutations are estimated to occur in 15% of patients with primary lung ADC [12].

Gefitinib and erlotinib are the first generation of EGFR tyrosine kinase inhibitors (TKIS), which selectively target the intracellular tyrosine kinase domain of EGFR, blocking the downstream signalling of the receptor [2]. Recently, a multicentre phase III trial demonstrated that patients with advanced-stage lung cancer containing *EGFR* mutations and treated with first-line gefitinib (compared with standard chemotherapy) showed improved progression-free survival. That finding led the authors to strongly recommend patient selection based on *EGFR* mutation status [13]. In fact, the American Society of Clinical Oncology recommends *EGFR* mutation testing for patients with advanced NSCLC of the lung who are being considered for first-line therapy with an EGFR TKI [12].

Unfortunately, all responders eventually develop resistance, most commonly because of the emergence of a secondary T790M mutation [14] or amplification of mesenchymal–epithelial transition factor (c-Met) [15].

Tissue samples are usually the specimen of choice. However, the approach of testing small cytology-based specimens, whether enriched for tumour cells by microdissection or not, has been published in various series with varying degrees of success. Tumour concentration and testing technique play an important role in test sensitivity [16–20]. Direct sequencing is the first widely used method for the detection of *EGFR* mutation, but it lacks sensitivity and might miss 25% of positive cases [21]. Several other more sensitive techniques have been applied, including polymerase chain reaction (PCR) single-strand conformation polymorphism [22], TaqMan (Roche Molecular Diagnostics, Pleasanton, CA, U.S.A.) PCR [23], loop-hybrid mobility shift assay [24], Cycleave (Clontech Laboratories, Mountain View, CA, U.S.A.) PCR [25], PCR restriction fragment length polymorphism and length analysis [26], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry genotyping [27], peptide nucleic acid–locked nucleic acid PCR clamp [28], Scorpions (DxS Limited, Manchester, U.K.) amplified refractory mutation system [29], denaturing high-performance liquid chromatography [30], single-molecule sequencing [31], mutant-enriched PCR [32], and Smart Amplification Process (DNAFORM, Yokohama City, Japan) [33]. These techniques not only vary in sensitivity, but also differ in terms of the detection of new and already-known mutations and the comprehensive detection of deletions and insertions [21].

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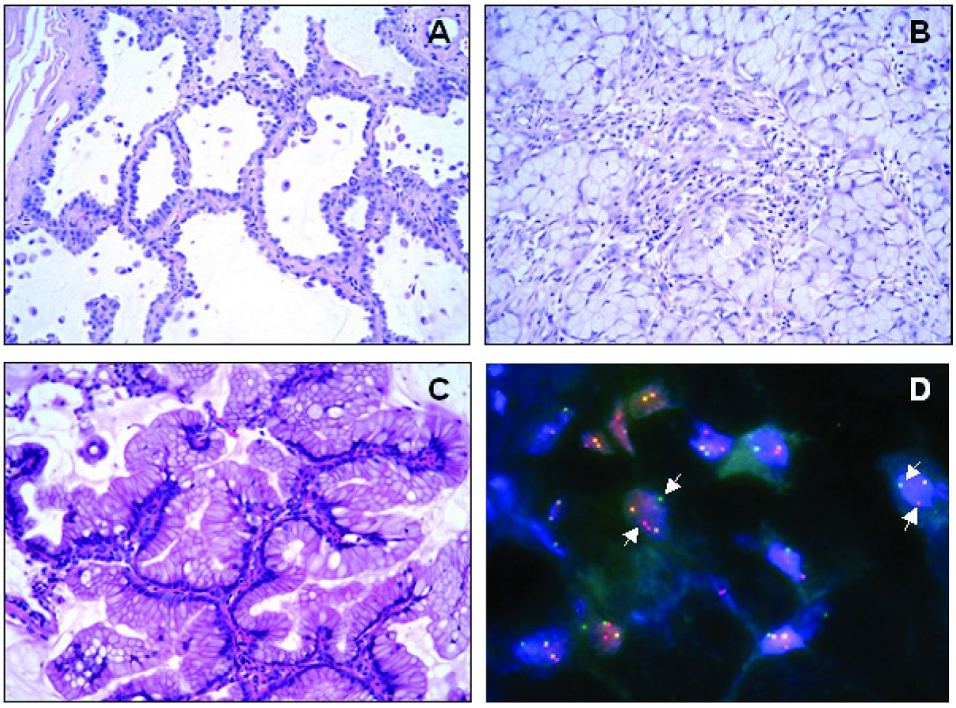

FIGURE 1 *Certain adenocarcinoma patterns are more frequently associated with specific mutations. Here, three patterns are shown: (A) lepidic (non-mucinous bronchioloalveolar), (B) signet-ring, and (C) mucinous bronchioloalveolar. These patterns correlate with abnormalities in the EGFR, ALK, and KRAS genes respectively. (D) A break-apart EML4–ALK fluorescence in-situ hybridization assay reveals split signal (arrows).*

2.3 *ALK* Gene Rearrangements

In generating a retroviral complementary DNA (cDNA) expression library from a lung ADC sample, Soda *et al.* noted that one of the amplified cDNAs had 3926 base pairs and contained an open reading frame for a protein of 1059 amino acids, with the amino-terminal portion of the protein being identical to that of human *EML4,* and the carboxy-terminal portion being identical to the intracellular domain of human *ALK.* That finding led to the conclusion that the cDNA was derived from a fusion product of the genes *EML4* and *ALK.* The *EML4–ALK* fusion was shown to result from a small inversion within chromosome 2p. The transforming potential of *EML4–ALK* was demonstrated using expression plasmids for *EML4–ALK,* which were introduced into mouse 3T3 fibroblasts, where transformed foci were readily identified. Additionally, transfected 3T3 cells in nude mice expressing *EML4–ALK* were able to form subcutaneous tumours. In this foundational work, *EML4–ALK* fusion transcript was detected in 5 of 75 NSCLC patients (6.7%) [34].

Translocations involving the *ALK* gene have already been identified in a subset of T-cell lymphomas named anaplastic large-cell lymphomas and in an unusual mesenchymal neoplasm known as inflammatory myofibroblastic tumour or inflammatory pseudotumour [35–37]. In these situations, *ALK* is associated with several other partner fusion genes, including, in anaplastic large-cell lymphoma, *NPM, TPM3, CLTC, ATIC,* and *TFG* [35,38–41], and in inflammatory myofibroblastic tumours, *TPM3* and *TPM4* [42]. Several *EML4–ALK* fusion variants—and also other rare fusion partners such as *KIF5B*—have been described in NSCLC, all harbouring transforming activity [34,43,44].

Initially, it was thought that *EML4–ALK* fusions were restricted to NSCLC. Reverse-transcriptase PCR (RT-PCR) was attempted, without success, to detect the fusion messenger RNA in a variety of neoplasms, including acute myeloid leukemia, non-Hodgkin lymphoma, and gastric and colorectal carcinomas [34]. However, in one study using RT-PCR screening of patient samples, *EML4–ALK* fusion was detected in 2.4% of breast and 2.4% colorectal carcinomas. In the same study, 11.3% of NSCLCs tested showed *EML4–ALK* fusion [45].

The prevalence of *EML4–ALK* varies in different studies, with 4% being probably the most accurate figure. The presence of *EML4–ALK* appears to be mutually exclusive for *EGFR* and *KRAS* mutations [46,47]. Patients are usually nonsmokers or light smokers and tend to be younger than the average patient with NSCLC. They frequently present at an advanced clinical stage, and the tumours demonstrate ADC with a solid pattern and signet-ring cells [48,49]. In other studies looking at clinicopathologic correlations, the younger age of patients with lung ADCs positive for *EML4–ALK* was emphasized. However, in a

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departure from previous reports, these patients were characterized by less differentiation and an acinar-predominant pattern [47].

Fluorescence *in-situ* hybridization (FISH) is regarded as the preferred standard method for detecting *ALK* rearrangement [Figure 1(D)]. Recently, the U.S. Food and Drug Administration approved crizotinib (Xalkori: Pfizer, Mission, KS, U.S.A.) to treat certain patients with locally advanced or metastatic NSCLC expressing the abnormal *ALK* gene. Crizotinib, an orally available small-molecule inhibitor of the Alk tyrosine kinase, is being approved with a companion diagnostic kit from Abbott Molecular (Abbott Park, IL, U.S.A.) [50,a].

The use of RT-PCR, immunohistochemistry, and chromogenic *in-situ* hybridization has also been described in clinical samples with variable sensitivity [51,52]. Several studies have addressed the role of immunohistochemistry in the detection of *ALK*-positive cases. The sensitivity of the immunohistochemistry assay depends on several factors, including the antibody clone and the detection method used [47,48,52–54].

In 2010, only 3 years after the first description of *EML4–ALK* fusion in NSCLC, the results of a clinical trial that enrolled 82 *ALK*-positive patients were made public. In that study, patients found to be *ALK*-positive by FISH were evaluated for the therapeutic efficacy of crizotinib. The results were impressive, including an overall response rate of 57% (46 partial responses and 1 complete response) and 33% stable disease (27 patients). Of the 82 patients, 63 (77%) were continuing to receive crizotinib at the time of data cutoff, and the estimated probability of 6-month progression-free survival was 72%. Side effects were considered minor [55]. The results of this trial have underscored the importance of establishing strategies for identifying *ALK*-positive patients.

2.4 *KRAS*

Kirsten rat sarcoma viral oncogene homolog (*KRAS*), a member of the *ras* gene family, encodes a small guanosine triphosphate GTPase that cycles between inactive GDP-bound and active GTP-bound conformations [56,57]. An important downstream signalling target of EGFR, KRAS has also been implicated in the development and prognosis of several cancers such ADCs of colon, lung, and pancreas [58–60]. Mutations that cause the loss of KRAS GTPase activity render the protein constitutively GTP-bound, resulting in sustained activation of downstream components and persistent proliferation signal [59,61].

In lung cancers, *KRAS* mutations occur primarily at codons 12 and 13; mutations at codons 10 and 61 are less frequently seen [56,62]. The most common *KRAS* mutation in smoking patients with NSCLC is a G-to-T transition (84%) resulting in substitution of cysteine (47%), valine (24%), aspartate (15%), or alanine (7%) for wild-type glycine [62,63].

Mutations in *KRAS* have been found in 15%–30% of patients with NSCLC and are considered to be one of the more frequent mutations in these tumours [64–66]. As with *EGFR* mutations, *KRAS* mutations are detected mainly in lung ADCs and are less frequently observed in squamous cell carcinomas of the lung [67,68]. By contrast with lung ADCs harbouring *EGFR* mutations, tumours having *KRAS* mutations are seen at a higher frequency (20%–30%) in Caucasian patients than in East Asian patients (5%) [67,69]. Also, compared with *EGFR* mutations, *KRAS* mutations are more common in current or former smokers than in never-smokers, although the absence of a history of tobacco use does not eliminate the possibility of such abnormalities [70–72].

Mutant *KRAS* plays an important role both in tumour development and in resistance to therapy. In colorectal cancer, it is well known that the presence of *KRAS* gene mutations is independently associated with poorer prognosis and is predictive of resistance to anti-EGFR therapies [73–76]. However, in NSCLC, the value of *KRAS* status as a predictive biomarker for anti-EGFR therapy is less clear. Although several studies have attempted to investigate the prognostic impact of *KRAS* mutations on survival and recurrence rates in NSCLC, the results are difficult to interpret because of differences in histologic inclusion criteria (all NSCLC vs. ADC only), small sample size, and tumour stage [65,66]. One group demonstrated that NSCLC harbouring *KRAS* codon 12 mutation carries a strong and unfavourable prognostic factor and is associated with mortality and inferior disease-free survival [77]. In addition, a meta-analysis of more than 53 studies showed that *KRAS* mutations are a factor correlated with poor survival in patients with NSCLC [2,78]. Interestingly, Ihle *et al.* recently found that NSCLC patients whose tumours harbour either mutant *KRAS*-Gly12Cys or mutant *KRAS*-Gly12Val had worse progression-free survival outcomes than did those with all other mutant *KRAS* proteins or with wild-type *KRAS* [62].

The predictive value of *KRAS* status for response to conventional chemotherapy, adjuvant therapy, or targeted therapy is far from being established. When *KRAS* mutations were tested prospectively in clinical studies such as TRIBUTE, JBR.10, and IFCT-0002, they were shown not to be significantly associated with chemoresistance, nor to be predictive of a differential benefit from adjuvant chemotherapy [57,79,80]. A recent prospective biomarker-driven study conducted in 889 patients included in a phase III trial comparing placebo with sequential erlotinib maintenance in unresectable NSCLC (SATURN, BO18192) showed that the presence of *KRAS* mutations was not predictive

[a] Vysis ALK Break Apart FISH Probe Kit, with the Vysis Paraffin Pretreatment IV and Post Hybridization Wash Buffer Kit, ProbeChek ALK Negative Control Slides, and ProbeChek ALK Positive Control Slides.

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for erlotinib efficacy and was significantly associated with reduced progression-free survival [81].

2.5 *BRAF*

BRAF encodes a nonreceptor serine/threonine kinase that is a member of the Ras/MAPK signaling pathway downstream of Ras protein. Upon activation, *BRAF* directly phosphorylates *MEK,* which in turns phosphorylates *ERK,* thereby regulating cellular responses to growth signals [82,83].

Davies *et al.* were the first to identify somatic activating mutations in the *BRAF* gene by screening 923 cancer samples. Missense mutations were found in approximately 60% of melanomas and 15% of colorectal cancers. Furthermore, mutations of *BRAF* were identified in other human cancers at smaller percentages, including in NSCLCs at 3%. The same study also showed that most *BRAF* mutations (89%) substitute a Glu for Val at residue 600 (V600E) in the activation segment of the kinase domain. Interestingly, all *BRAF* mutations found in NSCLCs during the study were non-V600E; by contrast, in melanoma, 91% of mutations involved V600E [83]. Accordingly, Brose *et al.* identified *BRAF* mutations in 5 of 179 NSCLC cases (3%) and found that all mutated samples but 1 had non-V600E mutations [64].

Recently, Caucasian patients with NSCLC were investigated to determine the prevalence, distribution, and prognostic role of *BRAF* mutations. In this large cohort, mutations of *BRAF* were found predominantly in lung ADCs (97.3%), with approximately 57% being V600E and 43% being non-V600E. The V600E mutations were demonstrated to be significantly more prevalent in women than in men, and in never-smokers than in smokers or former smokers, although all non-V600E mutations were found in tobacco users. Furthermore, V600E-mutated NSCLC showed a more aggressive tumour histotype characterized by micropapillary features and associated with poor prognosis [84]. Similarly, Paik *et al.* found that the frequency of non-V600E mutations was higher in NSCLC than in melanoma, which could reflect a carcinogenic effect caused by tobacco [85].

BRAF mutations were shown to be mutually exclusive with *EGFR* mutations within exons 18–21, *KRAS* codon 12 mutations, *ERBB2* codon 20 mutations, and translocations in *ALK* [85,86].

3. FUTURE OF NSCLC

In analyzing the future of molecular testing and adoption of personalized treatments in NSCLC, four aspects need consideration:

- Implementing strategies for routine testing of known biomarkers associated with specific treatments (currently *EGFR* and *ALK*)
- Identifying new biomarkers and their potential inhibitors
- Understanding the development of resistance by tumours
- Funding research in lung oncology

Identification of defects in lung cancer tyrosine kinase signalling has been accomplished through the phosphoproteomic approach, revealing (in addition to the already known oncogenic kinases EGFR and c-Met, and Alk and Ros fusion proteins) other activated tyrosine kinases, including PdgfrA and Ddr1, not previously implicated in the genesis of NSCLC [87].

Several other molecules, such as the receptor tyrosine kinase c-Met and its ligands [15,87], hepatocyte growth factor, vascular endothelial growth factor and its receptor, HER2, PIK3CA, BRAF, and insulin-like growth factor 1 receptor (IGF-1R) that are involved in cell signalling, are currently being investigated.

Abnormalities in the receptor tyrosine kinase c-Met and PIK3CA have been shown to play a role in predicting resistance to TKI therapy [15,88].

Alterations in HER2, with different prevalences depending on the technical assay used, have been demonstrated in NSCLC [89]. Those observations led the authors to the conclusion that inhibitory monoclonal antibodies might play a role in the treatment of a subgroup of NSCLC patients.

Evidence has also implicated IGF-1R, its ligands IGF-1 and -2, and their related downstream signalling in the development of cancer and also in a mechanism of resistance development in EGFR-targeted therapy [90,91]. The development of a potential therapeutic strategy targeting IGF-1R was initiated by the development of a human antibody that binds the receptor with high affinity, consequently inhibiting ligand attachment, and preventing downstream signalling of the MAPK/PI3K/Akt pathways. However, the association of a monoclonal antibody to IGF-1R and erlotinib in unselected NSCLC patients did not appear beneficial, likely because of high drug toxicity, which prevented optimal dosage [92–94].

Several others potential biomarkers of interest are thought to have a predominant role as indicators of chemotherapy sensitivity. That group includes Ki-67, p27, p16, the cyclin-dependent kinases, ERCC1, BRCA, tubulin III, Rrm1, and Tp53. Recently, a consensus meeting of Canadian lung cancer oncologists and pathologists found that the evidence is currently insufficient to support routine testing for those markers [95].

Other approaches are also been studied, and among those, MAGE-A3 (melanoma antigen-A3) deserves to be mentioned. The phase III study MAGRIT (MAGE-A3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy) is investigating the efficacy of MAGE-A3 antigen-specific cancer immunotherapy in preventing cancer relapse, when treatment is given after tumour resection in patients with MAGE-A3–positive stages IB, II, and IIIA NSCLC. Approximately

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30% of NSCLC tumours are MAGE-A3–positive. A tumour-specific antigen, MAGE-A3 is expressed by a variety of cancer cells, but not by normal ones. The strict tumour-specific expression of MAGE-A3 has prompted immunotherapeutic trials [96]. Non-small-cell lung carcinoma is among the tumours that express MAGE-A3.

Today, the general consensus among oncologists appears to be that, whenever possible, tissue rather than cytology specimens should be obtained in NSCLC. However, it is possible to evaluate *EGFR* mutation status by drawing blood from patients with NSCLC, particularly those at advanced stages. The test is accomplished with the use of a microfluidic device containing microposts coated with antibodies against epithelial cells. The *EGFR* mutational analysis is performed on DNA recovered from circulating tumour cells [97].

Research funding is crucial for the development of any science. Unfortunately, the reality of funding in lung research is far from optimal. The excerpt that follows is reproduced from a recent article published by *The New York Times* that emphasizes the perennial underfunding of cancer research [98]:

> The big loser in the cancer funding race is lung cancer. It is the biggest cancer killer in the country, yet on a per-death basis receives the least [U.S. National Cancer Institute] funding among major cancers. In 2006, the NCI spent $1,518 for each new case of lung cancer and $1,630 for each lung cancer death, according to data from the institute and the American Cancer Society.
>
> Among the big cancers, breast cancer receives the most funding per new case, $2,596 — and by far the most money relative to each death, $13,452. Notably, prostate cancer, the most common cancer, receives the least funding per new case at just $1,318. But on a per-death basis it ranks second, with $11,298 in NCI funds.

The situation described likely derives from a lack of public mobilization; lung cancer nongovernmental organizations are scarce. Survivors represent a small fraction of the patients affected by the disease, and they are frequently dogged by the "guilt effect" for having smoked and therefore having voluntarily exposed themselves to the potential risks. However, there is hope that the development of new personalized strategies in NSCLC, based on targeting specific molecular alterations, will eventually lead to prolonged survival. Those survival improvements in turn will influence public awareness. Consequently, those two factors could synergistically lead to heightened interest in pulmonary research, bringing about changes that will perhaps forever alter the history of human lung cancers.

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5. CONFLICT OF INTEREST DISCLOSURES

GDAB has consulted for Pfizer and Boehringer Ingelheim. AS has consulted for AstraZeneca and Amgen. EFB has no relevant financial conflicts of interest to declare.

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