



The role of the molecular footprint of EGFR in tailoring treatment decisions in NSCLC

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ABSTRACT

The majority of patients with non-small-cell lung cancer (NSCLC) present with advanced disease, with targeted therapies providing some improvement in clinical outcomes. The epidermal growth factor receptor (EGFR) tyrosine kinase (TK) plays an important role in the pathogenesis of NSCLC. Tyrosine kinase inhibitors (TKIs), which target the EGFR TK domain, have proven to be an effective treatment strategy; however, patient responses to treatment vary considerably. Therefore, the identification of patients most likely to respond to treatment is essential to optimise the benefit of TKIs. Tumour-associated activating mutations in *EGFR* can identify patients with NSCLC who are likely to have a good response to TKIs. Nonetheless, the majority of patients relapse within a year of starting treatment. Studies of tumours at relapse have demonstrated expression of a T790M mutation in exon 20 of the *EGFR* TK domain in approximately 50% of cases. Although conferring resistance to reversible TKIs, these patients may remain sensitive to new-generation irreversible/pan-erb inhibitors. A number of techniques have been employed for genotypic assessment of tumour-associated DNA to identify *EGFR* mutations, each of which has advantages and disadvantages. This review presents an overview of the current methodologies used to identify such molecular markers. Recent developments in technology may make the monitoring of changes in patients' tumour genotypes easier in clinical practice, which may enable patients' treatment regimens to be tailored during the course of their disease, potentially leading to improved patient outcomes.

BACKGROUND

Epidemiology of non-small-cell lung cancer

Lung cancer is the most prevalent life-threatening cancer worldwide¹ with more than 80% being non-small-cell lung cancer (NSCLC). Approximately 70–80% of patients with NSCLC present with locally advanced or metastatic disease and, if untreated, have a 1-year survival of ~15%. Palliative chemotherapy improves cancer-related symptoms and increases the 1-year survival rate by approximately 10%.² The pathogenesis of lung cancer involves the accumulation of several molecular abnormalities over time. Alterations in gene sequence or expression can occur in the cell-signalling and regulatory pathways involved in cell-cycle control, apoptosis, proteasome regulation and angiogenesis. Genetic changes include mutations, gene silencing through epigenetics, gene amplification or deletion and whole chromosome gains or losses. Alterations in receptor tyrosine kinases

(TKs), such as the epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor 1, include overexpression, amplification or mutations.³ The development of molecular targeted therapies aimed at these molecular alterations has generated great optimism for the treatment of cancers such as NSCLC, and drugs targeting the EGFR tyrosine kinase domain are now available.

Targeted therapies with EGFR inhibitors

Novel therapeutic agents, in particular those that specifically target members of the human epidermal growth factor receptor (HER (ErbB1)) pathway, have shown encouraging therapeutic efficacy. Initially, Trastuzumab (Herceptin) was approved for the treatment of HER-2 (ErbB-2)-positive breast cancer, and after its success, three EGFR (EGFR1/ErbB-1)-specific agents received regulatory approval. Cetuximab (Erbixux), a chimeric monoclonal G1 (IgG1) antibody that binds to the EGFR with high affinity, was licensed to treat metastatic colorectal cancer (mCRC) and squamous cell carcinoma of the head and neck (SCCHN).⁴ Cetuximab added to chemotherapy improved survival in the FLEX trial⁵ but failed to meet its primary endpoint of progression-free survival assessed by the independent radiological review committee in the BMS099 Phase III trial.⁶ This antibody blocks ligand binding and induces receptor internalisation and degradation resulting in the downregulation of surface EGFR expression. Gefitinib (Iressa) and erlotinib (Tarceva) were licensed to treat advanced or metastatic NSCLC, and erlotinib was licensed to treat advanced or metastatic pancreatic cancer. These two EGFR tyrosine kinase inhibitors (TKIs) reversibly and specifically inhibit EGFR downstream signalling by binding to the ATP site on the kinase domain of the receptor inducing apoptosis and inhibiting growth and cell proliferation.⁴

EGFR pathway deregulation in NSCLC

EGFR-TK signalling plays an essential role in the pathogenesis of many NSCLCs; EGFR protein expression is seen in up to 85% of NSCLC cases, although the prognostic relevance of EGFR in this disease remains equivocal.^{7–9} In general, the expression of a target protein is deemed sufficient to determine which tumours will respond to targeted cancer therapy.¹⁰ There have, however, been conflicting results regarding the value of EGFR protein expression in predicting survival benefit with TKIs.¹¹ Several studies have failed to demonstrate a role for increased EGFR expression in predicting response of NSCLC to oral EGFR TKIs.^{12–14}

In 2004, somatic activating mutations in the EGFR TK domain were described. Mutations were either small, in-frame deletions or amino acid substitutions clustered around the ATP-binding pocket of the TK domain. These mutations were mainly found in female, never smokers with adenocarcinomas, who were of Asian ethnicity.^{15 16} Cells harbouring these mutations become highly dependent on the constitutively active EGFR signalling pathway, a state referred to as 'oncogenic addiction'.^{17 18} A germline T790M EGFR mutation has been reported in a family with multiple cases of NSCLC.¹⁹ This T790M mutation has been described as an oncogenic mutation that confers a growth advantage to cancer cells.^{20 21} Initially, it was reported that the kinase activity of T790M mutant EGFR was similar to wild-type EGFR.^{22 23} However, Vikis and colleagues showed that T790M has enhanced kinase activity.²⁴ Mulloy and colleagues showed that mutant cells harbouring both the T790M and the L858R mutations or 'double mutants' demonstrated increased tyrosine phosphorylating activity.²⁵ The T790M mutation has also emerged as a secondary point mutation that is present in approximately half of lung cancer patients who develop resistance to EGFR TKIs (see below).²⁶

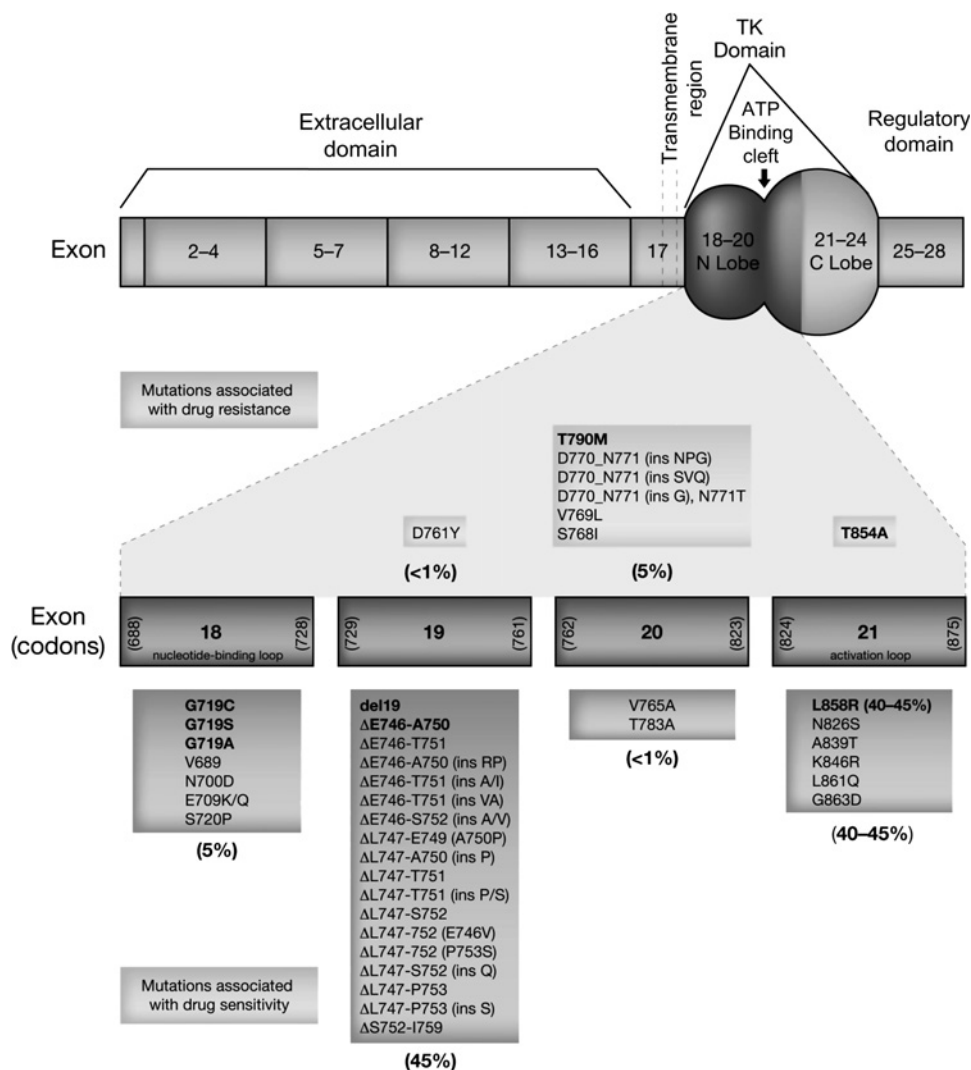
Pinter and colleagues have shown that there is little correlation between EGFR expression, as determined by standard immunohistochemistry, and EGFR-activating mutations, increased EGFR gene copy number or response to EGFR TKI.¹⁰

This suggests that the EGFR genotype, and not immunohistochemistry, is the best method to identify NSCLC tumours that are most likely to benefit from treatment with an EGFR TKI.²⁷

Response to EGFR TKIs

Although patients with specific EGFR gene-activating mutations are more likely to have amplified EGFR or high EGFR expression, it is the mutation status alone that predicts response to EGFR TKIs^{15 28 29} in first-line therapy.^{30–33} Many mutations in exons 18–21 of the kinase domain of the EGFR gene have been identified in tumours that respond to EGFR TKIs (figure 1); however, two mutations are most common—in-frame deletions in exon 19 (del19) and a substitution mutation in exon 21 (L858R). These two mutations account for 85–90% of the drug-sensitive EGFR mutations seen in NSCLC.²⁹ Two other sensitising mutations have been validated—substitutions in exons 18 (G719A/C) and 21 (L861Q).³⁶ In addition, activating mutations have been shown to demonstrate different sensitivities to EGFR TKIs, with exon 19 deletions being more likely to respond than those of exon 21.³⁷ Activating mutations in EGFR are associated with improvements in PFS and overall survival (OS).^{38–43} Conversely, some substitution mutations in exon 20 (T790M) and exon 21 (T854A) are known to confer resistance to some EGFR TKIs (figure 1);^{22 44 45} Tumours that initially respond to treatment with the EGFR TKIs erlotinib and gefitinib almost invariably develop acquired resistance to these drugs,^{22 46}

Figure 1 Schematic of the epidermal growth factor receptor gene with locations of mutations in the tyrosine kinase (TK) domain. Adapted from Sharma *et al*³⁴ and Shigematsu *et al*.³⁵



which is conferred by the T790M mutation in 50% of cases.^{47–49} It was initially thought that the substitution of the larger methionine residue may cause steric hindrance to the binding of the drugs.^{22 23} A structurally similar reversible TKI is, however, able to overcome the T790M mutation.⁵⁰ The T790M mutation may result in increased affinity of EGFR for ATP compared with erlotinib or gefitinib and that this is the primary mechanism by which the mutation confers drug resistance.⁵¹ Thus, it should be possible to overcome the resistance by developing TKIs that have a higher affinity for the T790M kinase.^{44 52 53} New-generation EGFR TKIs that bind irreversibly to the EGFR-TK, forming covalent cross-links with EGFR, such as afatinib (BIBW 2992), have been shown to be active against tumours resistant to reversible EGFR TKIs^{22 52–56} and may offer an alternative therapy strategy. In addition, owing to their irreversible binding, new-generation TKIs may have a longer duration of action than reversible agents. In order to change the therapeutic agent at the optimal time to prevent tumour progression, it would be useful to know when the T790M mutation has developed. A recent publication found T790M in up to 38% of patients not previously treated with a TKI.⁵⁷ The existence of a T790M mutation in a few cancer cells at diagnosis confers a shorter time to tumour progression.⁵⁸ Such cells are subsequently ‘cloned out’ during the patient’s treatment with EGFR TKIs indicating that these patients may benefit from treatment with new-generation EGFR TKIs from the beginning. Alternatively screening for the emergence of the T790M mutation during treatment may allow early identification of acquired resistance to TKIs and treatment to be tailored as necessary.

In addition to these specific EGFR mutations, other factors such as amplification or activation of the insulin-like growth factor receptor⁵⁹ and MET amplification⁶⁰ have also been shown to confer resistance to EGFR TKIs. These nuances of the EGFR discussed above demonstrate that both preselection of patients most likely to respond to EGFR-targeted therapy and screening during therapy are crucial to determine the appropriate treatment regimen.

Selection of patient material for detection of EGFR mutations

The mutation status of *EGFR* has been determined in original tumour specimens obtained during surgical resection^{15 35 43 61–77} or at biopsy.^{10 15 61 62 65 66 70–72 75 77–80} These tumour specimens were stored as either paraffin-embedded tissues^{10 15 40 42 43 61–63 65–67 70–77 81–83} or snap-frozen samples,^{15 35 64 68 69 78 84 85} or analysed as fresh tissue.⁷⁹ Fine-needle aspirates (FNAs),^{86–88} bronchial brushings, serum and plasma, circulating tumour cells (CTCs) and pleural effusion samples have also been used to assess *EGFR* mutation status in patients receiving EGFR TKI therapy.

Kimura and colleagues assessed *EGFR* mutation status using DNA extracted from serum obtained 14 days after initiation of gefitinib therapy and found this approach to be a potentially convenient means of predicting sensitivity to EGFR TKI therapy.⁶⁵ Pao and colleagues assessed *EGFR* mutation status in three patients using DNA derived from biopsy samples and pleural effusions obtained after disease progression on EGFR TKI therapy.²³ The T790M mutation was identified in all samples from these patients. The authors suggest that rebiopsy of progressive disease should become a standard procedure, especially for patients in clinical trials of targeted agents. Kosaka and colleagues assessed *EGFR* mutation status in 14 tumour samples obtained at the time of progression after initial response to EGFR TKI therapy.⁸⁹ Seven tumours had the T790M mutation. In each of four patients in whom a T790M mutation was found

in pretreatment serum DNA, EGFR TKI therapy resulted in a poor outcome.⁹⁰

Whenever possible, tumour specimens should be obtained from the most easily accessible tumour tissue immediately preceding the treatment.⁹¹ In addition, close cooperation between clinicians, molecular biologists and pathologists is crucial.⁹¹

Sample quality and quantity

Although genotypic assessment may be useful in determining which patients are likely to respond to EGFR TKI therapy, problems are associated with obtaining suitable DNA for analysis. The quality of the samples, the quality of the extracted DNA and the quantity of DNA available using current methods may limit the routine use of genotypic assessment.

As described above, cancer cells from various sources have been used for genotyping. These include archived surgically resected tissue from patients who subsequently develop recurrent or progressive disease,^{15 35 43 61–77} biopsy tissue,^{10 15 61 62 65 66 71 72 75 77–80} cytology specimens from lavage,⁸⁰ pleural effusion,^{23 72 77 80} an unspecified source,^{74 75} FNA cytology^{70 72 77–80} (CTCs), tumour DNA in serum⁶⁶ and tumour DNA in plasma⁹⁰ or blood.^{57 92}

Each of the sources described above has merits and drawbacks. The mutation status of *EGFR* can be determined in tumour specimens obtained during curative surgery or at biopsy. Surgery offers the best chance of high-quality and high-volume tumour tissue samples, but only 20–25% of lung cancers are suitable for curative surgery,⁹³ and EGFR TKI therapy is only licensed for use in patients with advanced disease. There is generally only a small amount of tumour in routine diagnostic biopsy samples from patients with advanced disease,⁹⁴ often only sufficient to distinguish, and possibly subtype, NSCLC from small-cell-lung cancer (SCLC) and adenocarcinoma from squamous-cell cancer. It is unclear what percentage and absolute number of tumour cells need to be present in a sample for reliable mutation detection. With the exception of laser micro-dissection, <100 malignant cells may be inadequate for accurate detection, and such low numbers may not be representative of a heterogeneous solid tumour. The ratio of malignant to normal cells may, however, be a more important factor, provided a minimum number are present, and a sufficiently sensitive technique is employed for mutation detection. In adenocarcinoma-bearing bronchial biopsies, the median tumour proportion is around 23%; for NSCLC (not otherwise specified) cases, it is 10%.⁹⁴ Manual micro-dissection is recommended for tumour enrichment in small biopsy samples.⁹¹ The following are the minimum recommended amounts of material necessary with a good probability of providing a sufficient amount of tumour for mutational analysis: at least two cylinders of tissue from a CT-guided biopsy, eight FNA passes/samples per cell block and eight smears from a brush biopsy.

Lim and colleagues were able to obtain sufficient genomic DNA for genotypic assessment from more than 80% of their 24 low-volume samples (needle or forceps biopsy or fine-needle aspiration).⁷⁹ Of the 139 patients studied by Shih and colleagues,⁷² only two had insufficient DNA for analysis, whereas Savic and colleagues⁸⁰ successfully sequenced the DNA from 93% of their 84 cytological NSCLC specimens. Nakajima and colleagues⁷⁰ determined *EGFR* mutation status in all 43 patients in their study, and Yoshida and colleagues⁷⁷ determined *EGFR* mutation status in all 35 fine-needle aspiration/biopsy samples in their study. Thus, it is possible to obtain DNA from cytology-type samples, but it is questionable as to how much of

this DNA is tumour-derived and how representative of the patient's tumour such small samples are. What is of crucial importance is that any tested sample is checked for adequacy in terms of the number and percentage of tumour cells. Two recently published studies reporting successful EGFR mutation detection from FNA cytology samples found EGFR mutation rates significantly lower than those reported from the same laboratories using tissue biopsies.^{95 96}

Follow-up biopsy to determine T790M mutation status may be unnecessary if further research supports the initial finding that DNA sufficient for genotypic assessment can be isolated from blood samples.^{65 83 90} Until recently, it has not been possible to obtain 'pure' CTCs, only a CTC-enriched fraction.⁹⁷ However, a new method developed by Maheswaran and colleagues allows the isolation of CTCs at high purity from almost all samples tested.⁵⁷ This method provided sufficient DNA for EGFR mutation analysis in 11 out of 12 patients. Although some groups have seen a 92% detection rate in CTCs, these cells are difficult to detect in NSCLC, and further validation of these techniques is necessary before implementation into routine diagnostics.

Genetic analysis

The majority of DNA analysed has been obtained from paraffin-embedded tissue,^{10 15 40 42 43 61–63 65–67 70–77 81–83} although some authors used frozen samples,^{15 35 64 68 69 78 84 85} and Lim and colleagues used fresh tissue.⁷⁹ Various methods and kits have been used to extract genomic DNA. Small samples may be inadequate for genotypic assessment, as this requires significant amounts of tumour cell DNA to avoid contamination with wild-type DNA from normal cells,⁹³ and mutation artefacts can be observed following PCR amplifications of very small amounts of DNA isolated from paraffin-embedded tissues owing to post-mortem deamination of the DNA.⁹⁸ This means that PCR amplification and genotypic assessment need to be repeated several times to exclude artefacts.

Although two mutations (del19 and L858R) are most commonly associated with response to EGFR TKI therapy, and one mutation with resistance (T790M), many other mutations linked to response have also been described. Genotyping by direct DNA sequencing has been used to determine EGFR mutation status and will identify any mutation, whether common or novel. Pyrosequencing, a real-time sequencing technology, using luminometric detection, permits mutation characterisation as well as the quantification of the percentage of mutated alleles in a sample.⁹⁹ A recent comparative study by Querings *et al* demonstrated that dideoxy 'Sanger' sequencing is less sensitive than pyrosequencing.¹⁰⁰ Mutations have been identified in DNA provided by both PCR amplification and real-time quantitative PCR (qPCR) and also in genomic DNA.^{79 81} Both PCR amplification^{10 15 35 40 42 43 61–68 71–73 76 78–80 82–84 89 101–103} and qPCR^{40 69 83 89 104} limit the detection of mutations to those within the amplified region.

Detection by PCR is more amenable to routine use but can only be used to identify specific mutations. Several methods have been used. These include loop-hybrid PCR assay^{70 105}; allele-specific PCR combined with qPCR^{15 16}; peptide nucleic acid (PNA)-locked nucleic acid (LNA) PCR clamp^{74 75 102}; TaqMan assay^{78 106}; restriction fragment length polymorphism (RFLP)¹⁰⁷; common fragment analysis^{44 77}; qPCR using the Cycleave PCR core kit and specific probes^{77 89}; heteroduplex detection using SURVEYOR (a DNA endonuclease-digesting heteroduplex PCR product), and high-performance liquid chromatography¹⁰⁸; and mutant-enriched PCR analysis using

a restriction enzyme to specifically digest wild-type DNA.¹⁰⁹ The EGFR gene copy number has been determined using fluorescent *in situ* hybridisation FISH,^{10 40 80 85} chromogenic *in situ* hybridisation CISH^{63 78 84} and real-time quantitative PCR.⁸¹

Kim and colleagues have compared methods for detecting mutations.¹¹⁰ They found that direct sequencing analysis has a relatively low sensitivity. A number of methods have been investigated to detect low-abundance mutations and increase the sensitivity of the assay. These include single-strand conformation polymorphism followed by sequencing,⁶⁸ heteroduplex detection using SURVEYOR and high-performance liquid chromatography,¹⁰⁸ and high-resolution melting analysis.^{111 112} These methods allow the rapid screening of large numbers of samples with high sensitivity but still need direct sequencing to confirm mutations. Single-strand conformation polymorphism analysis can detect mutations that are undetectable by direct sequencing, and heteroduplex PCR using SURVEYOR and high-performance liquid chromatography has successfully detected mutations without false negativity in formalin-fixed, paraffin-embedded tissues. The scorpion amplification refractory mutation system⁶⁶ and microfluidics digital PCR¹¹³ are other sensitive methods that can also be used to rapidly detect specific EGFR mutations using qPCR.

Many methods have been used to detect the 'hot-spot' mutations del19 and L858R. For clinical use, appropriate methods include fluorescence-labelled length, restriction-fragment-length polymorphism and mutant-enriched PCR analysis, which are simple and readily applicable. However, these are limited to detection of del19 and L858R mutations. Techniques using specific probes (PNA-LNA PCR clamp, qPCR) can be modified and applied to other mutations once those mutations have been identified by sequencing methods. DNA obtained from pretreatment serum cannot be used to detect all the EGFR mutations found in tumour samples, although it is a valuable option for patients without tumour samples for mutational analysis.⁶⁶

At present, none of the mutational analysis techniques described above can detect all possible mutations at the necessary sensitivity. Therefore, a small percentage of mutation positive patients, who may benefit from EGFR TKIs, may be missed. However, a recent development in Taqman PCR technology has enabled the detection of EGFR DNA mutations, even within abundant backgrounds of normal material. These assays, based on competitive allele-specific TaqMan PCR or CastPCR, are sensitive enough to detect single mutant molecules in a background of 1 million normal copies.¹¹⁴ This is at least a 10-fold greater sensitivity than currently available mutation-detection assays. CastPCR combines allele-specific TaqMan primers and allele-specific minor groove binder blockers in order to suppress non-specific amplification from wild-type alleles. These assays are currently being validated by us and other early-access customers, and the results are eagerly awaited.

Logistics and infrastructure

Since the licensing of gefitinib in 2009, the NHS in the UK and the INCa/Ministry of Health in France have set up national programmes for EGFR gene-mutation screening in patients with locally advanced or metastatic NSCLC. The identification of patients who are most likely to benefit from EGFR TKIs has resulted in substantial savings to healthcare systems worldwide. As the treatment of NSCLC moves from an empirical approach to molecular-based and personalised therapies, similar screening systems may be implemented for other biomarkers in the future.

It is crucial for each cancer centre/hospital to implement a strict policy for EGFR mutation screening for NSCLC

patients.⁹¹ The two main issues to be considered are (1) which samples to test and (2) where the testing should be carried out. Both the pathologist and the oncologist play a role in deciding which patients should be tested. Where a certain diagnosis of large cell neuroendocrine carcinoma (LCNEC), mucinous adenocarcinoma or carcinoid tumour is made, testing is not necessary. The incidence of EGFR mutations in squamous cell cancer is also low, and as a result, routine testing is not performed in many centres. Nonetheless, if a squamous carcinoma occurs in a non-smoker or where there is any doubt about tumour type, testing is reasonable. Samples suitable for testing are those that have been fixed immediately after procurement and have an adequate number of tumour cells present (see above). Manual micro-dissection to enrich samples for tumour may be necessary.

Ideally, testing would be carried out in the same centre, where the patient has been pathologically diagnosed. Testing should be carried out in an accredited laboratory following standard operating procedures, including pathological assessment of the sample prior to testing. These measures considerably reduce the risk of contamination and enable greater accuracy and reproducibility of results. On-site testing is only viable in centres where there is an adequate throughput of patients to be tested, to ensure assay efficiency, and where the appropriate equipment and technical/pathological expertise are available. Depending on the assay used for screening, either a first- or second-generation sequencer or a real-time PCR system is necessary. Where any of these criteria are not fulfilled, it may be more cost-effective to outsource the testing to another centre. The main difficulties for outsourcing testing are the logistics and delays involved in getting the samples delivered.

There is a real need for a rapid, sensitive and low-cost assay for EGFR screening. The majority of groups are currently using either direct sequencing or the Scorpion ARMs assay for mutation screening. Depending on the assay used for EGFR mutation screening, the turnaround time can vary—a realistic time is 5–7 working days. Direct sequencing is thought to be less sensitive and more time-consuming than the Scorpion ARMs assay. The Scorpion ARMs assay requires batching of samples, which may cause delays in the turnaround time of the assay. Newer tests, such as EGFR mutation-specific antibodies, are promising.^{115 116} They might help to conserve tissue, which is of importance, given the emergence of other predictive biomarkers. Immunohistochemical analysis is also quick, cost-effective and routinely carried out in all pathology laboratories.

Translation to the clinic

In order to successfully treat NSCLC with TKIs, it is important to identify those patients who are likely to respond to treatment. Mutations in *EGFR* have been identified that are sensitising, or which confer resistance to treatment. Knowledge of such mutations may enable the physician to create individual treatment regimens based on their knowledge of their patients' genotypes. This review has presented the methods used for genotypic assessment of tumour-associated DNA to identify such *EGFR* mutations, each of which has advantages and disadvantages. The major problem with this type of analysis has been obtaining suitable DNA, in terms of both quality and quantity. A new method that allows isolation of CTCs from the blood of patients with lung cancer could potentially provide enough DNA for molecular analysis of mutations.⁵⁷ This method could be a step forward and may enable patients' treatment regimens to be adjusted over the course of their disease by monitoring changes in their tumour genotypes during treatment.

Take-home messages

The identification of patients most likely to respond to EGFR-TKI therapy is essential to optimise the benefit of these agents for the treatment of NSCLC. Tumour-associated activating mutations in EGFR increase the sensitivity of a tumour to EGFR-TKI therapy; however, the presence of additional mutations confer resistance to reversible drugs. The identification of molecular markers of response is therefore of scientific and clinical interest. Various methodologies are used to identify such molecular markers and observe how a tumour genotype can change over time; these technological developments may enable patients' treatment regimens to be tailored during the course of their disease, potentially leading to improved patient outcomes.

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