Histopathology

Histopathology 2012, **60**, 531–546. DOI: 10.1111/j.1365-2559.2011.03854.x

REVIEW

Personalized medicine for lung cancer: new challenges for pathology

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(2012) *Histopathology* **60**, 531–546

Personalized medicine for lung cancer: new challenges for pathology

Recent advances in non-small-cell lung cancer (NSCLC) therapy mean the relatively simple discrimination between small-cell and 'non-small-cell' carcinoma is insufficient to determine the best treatment for individual patients. Safety, efficacy and prescribing requirements mandate more specific subtyping of NSCLC for several new drugs: practice made difficult by the tumour heterogeneity combined with the paucity of tissue in most diagnostic samples. Immunohistochemical approaches have emerged as accurate predictors of probable tumour histotype. P63 and/or cytokeratins 5 and 6 and thyroid transcription factor 1 (TTF1) are among the best predictors, respectively, of squamous and adenocarcinoma histology. Molecular characteristics may predict response to both newer molecular targeted agents and traditional cytotoxic agents. Specific mutations in the epidermal growth factor receptor (*EGFR*) gene as predictors of response to EGFR tyrosine kinase inhibitors (erlotinib, gefitinib) is the first example of markers which predict response to targeted agents. Actual drug targets [e.g. thymidilate synthase (TS) – pemetrexed] or markers of the tumour's ability to repair cytotoxic drug-induced damage [e.g. excision repair cross-complementation group 1 (ERCC1) – cisplatin] may well also complement NSCLC diagnosis. This extended diagnostic requirement from increasingly limited material provided by minimally invasive biopsy techniques poses major challenges for pathology.

Keywords: biomarkers, dihydrofolate reductase, diagnosis, epidermal growth factor receptor, EML4-ALK, immunohistochemistry, Kirsten rat sarcoma, non-small-cell lung cancer, p63, pathology, personalized therapy, targeted therapy, thymidilate synthase, thyroid transcription factor 1

Abbreviations: EGFR, epidermal growth factor receptor; ERCC1, excision repair cross-complementation group 1; GARFT, glycinamide ribonucleotide formyltransferase; GC, gemcitabine and cisplatin; IGFR, insulin growth factor receptor; NOS, not otherwise specified; NSCLC, non-small-cell lung cancer; PC, pemetrexed and cisplatin; SCLC, small-cell carcinoma; TKIs, tyrosine kinase inhibitors; TTF1, thyroid transcription factor 1

Introduction

In the last few years there have been remarkable advances in the treatment of non-small-cell lung cancers, with a clear departure from the historical management of advanced lung cancer based upon a relatively simple dichotomy of small-cell carcinoma (SCLC) or non-small-cell carcinoma (NSCLC). In the United Kingdom most oncologists use platinum-based chemotherapy in combination with etoposide for SCLC and a platinum doublet of their choice for NSCLC. Now, however, the concept of selective therapy has emerged as a major factor in optimal therapy of NSCLC. There is evidence, for some cytotoxic agents, of differential responses between histological subtypes of NSCLC. Newer, targeted anti-cancer agents (either small inhibitory molecules or monoclonal antibodies active against specific components of oncogenic cellular molecular pathways) show the greatest benefit in patients selected for such treatment on the basis of

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specific pathological characteristics of their tumour. We are now in an era where detailed pathological diagnosis is a key feature in determining the particular therapy a patient with NSCLC should, or equally importantly should not, receive.

Subtyping NSCLC: why does it matter?

There is evidence that cytotoxic chemotherapeutic agents are differentially effective in different forms of NSCLC. The strongest evidence for this concerns the anti-folate agent pemetrexed. This drug interferes with DNA synthesis by competitively binding a number of enzymes in the DNA synthetic pathway.[1] These enzymes include dihydrofolate reductase (DHFR), glycinamide ribonucleotide formyltransferase (GARFT) and thymidilate synthase (TS). TS is the major enzymatic target of pemetrexed, and levels of this enzyme are higher in SCLC and squamous cell carcinomas than in adenocarcinomas.[2] This is the most likely explanation for the improved antitumour activity of pemetrexed in adenocarcinomas and some other non-small-cell carcinomas, than in SCLC and squamous cell carcinomas, where the drug may fail to inhibit the higher levels of TS.[3] The differential clinical effect was demonstrated in a randomized study comparing first line chemotherapy with pemetrexed and cisplatin (PC) or gemcitabine and cisplatin (GC) in advanced NSCLC, where survival with PC in adenocarcinoma was superior to GC while squamous histology gave the opposite result.[4] A retrospective analysis of pemetrexed versus docetaxel in second-line treatment of NSCLC confirmed the superiority of response and survival using pemetrexed in the adenocarcinoma subgroup.[5–7] Similar histological variation has been demonstrated in the maintenance setting.[7] Pemetrexed is now licensed in Europe only for use in non-squamous NSCLC, while in the United Kingdom the licence stipulates its use only in adenocarcinoma and large cell carcinoma (http://www.ukmi.nhs.uk/applications/ndo/record_view_open.asp?newDrugID=4192, http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000564/human_med_000638.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125).

There is less strong evidence for differential efficacy by histology for other cytotoxic agents, although one meta-analysis has suggested that cisplatin may be more effective against adenocarcinoma while carboplatin is more effective against squamous cell carcinoma.[8] However, the poor quality of pathology data in most trials restricts the evaluability of other agents.[9]

Histological subtyping of NSCLC is also an important issue when considering the use of molecular targeted agents. Potentially fatal haemoptysis has been reported in patients with squamous cell carcinomas, but not with adenocarcinomas, treated with bevacizumab, a monoclonal antibody targeted against the vascular endothelial growth factor receptor (VEGFR).[10,11] Increased haemorrhagic complications were also reported in patients with squamous NSCLC treated with cytotoxic chemotherapy with or without the multitargeted drug sorafenib, which targets VEGF, Flt3, c-Kit and B-Raf.[12] The increased propensity of squamous cell carcinoma to haemorrhage may be explained partially by the fact that these tumours generally arise from the large bronchi, while adenocarcinomas usually take their origin from the peripheral lung epithelium. The potential of central tumours to invade major vessels, with blood supply from the bronchial arteries, could lead to large vessel haemorrhage at systemic pressure if treatment results in tumour necrosis. This hypothesis has, however, been questioned, and other as-yet undefined biological characteristics of squamous cell carcinomas are the subject of speculation. Whatever the exact reason, due to fatalities in early clinical trials, bevacizumab is not licensed for use in patients who have a diagnosis of squamous cell carcinoma.

Small molecular inhibitors of the internal tyrosine kinase (tyrosine kinase inhibitors: TKIs) of the epidermal growth factor receptor (EGFR), such as erlotinib and gefitinib, are most effective against tumours which have certain mutations in the EGFR gene.[13–15] The prevalence of EGFR mutation is negligible in some types of NSCLC,[16–18] and accurate subtyping of NSCLC is a useful strategy to select patients for EGFR mutation testing (see below). Other mutations which are likely to guide selection of particular therapies are also associated mainly with adenocarcinoma of the lung. In squamous cell carcinoma, some of the small molecular inhibitors of the insulin growth factor receptor (IGFR) have shown efficacy greater than in other histological subtypes, but toxicity has been a problem.[19]

In summary, prior to prescribing a number of important new drugs to treat NSCLC, knowledge of the specific NSCLC subtype is essential.

Subtyping NSCLC: the challenges for pathologists

The World Health Organization (WHO) classification of lung tumours has eight major categories; SCLC and seven groups of NSCLC.[20] This classification is intended for use in surgically resected tumours and assumes the opportunity to sample and examine all or a large part of the tumour. The presence and distribution of a characteristic somewhere in the lesion is diagnostic in

several categories; present in at least 10% of the tumour in adenosquamous and sarcomatoid carcinomas, present somewhere in atypical carcinoid, completely absent in large cell carcinomas and typical carcinoid tumours. The WHO classification is not applicable in full to small tissue biopsy or cytology samples, yet virtually all patients with a tissue diagnosis of lung cancer have their initial diagnosis made on just such small specimens. In the majority of cases, there is no subsequent surgical resection to allow full confirmatory diagnosis. The 80% of NSCLC patients with advanced disease receiving chemotherapy will have only small biopsy and/or cytology samples available for diagnosis.

Despite such small diagnostic samples, there is a high degree of diagnostic accuracy and interobserver consistency for determining whether a patient's carcinoma is, or is not, small-cell carcinoma. In one review of this topic the accuracy of diagnosis of small-cell carcinoma was around 90%, while for a diagnosis no more specific than saying the tumour was not small-cell the figure was 98%.[21,22] This does, however, mean that as much as one diagnosis of SCLC in 10 may be inaccurate. Diagnostic accuracy will be improved by an awareness of the numerous pitfalls that may lead to an erroneous diagnosis of SCLC [crush artefacts, lymphoid tissue, non-small-cell neuroendocrine (NE) malignancies, basaloid variants of squamous cell carcinoma, etc.] and the judicious use of immunohistochemistry.[23,24] Published Kappa scores of interobserver agreement at this level of tumour diagnosis are high, at around 0.86.[25,26]

Attempts to subtype NSCLC in small biopsy or cytology samples by morphology alone have shown disappointing results, with interobserver agreement generally poor (K = 0.25–0.39).[27,28] Diagnostic accuracy, as measured by comparing a preoperative tumour subtype with the 'gold standard' diagnosis of the tumour once resected, shows highly variable results. It is also worth remembering that diagnosis on surgically resected cases does not necessarily guarantee complete agreement.[29] Very few studies show high levels of accuracy (in the region of 80–85%) for both squamous cell and adenocarcinoma.[30–34] Most show relatively high levels of accuracy for one subtype (70–80%), usually at the expense of accuracy of the other. Two consistent factors are the poor accuracy for a diagnosis of large cell carcinoma, and for the subtype diagnosis of NSCLC by cytology rather than tissue biopsy samples. It is likely that there is an element of publication bias in this area, raising the possibility that the true situation may be even worse. As recommended in the WHO Blue Book, a diagnosis of large cell carcinoma, adenosquamous or sarcomatoid carcinomas should not be given on small tissue samples.[20] Many squamous cell or adenocarcinomas contain areas of tumour, sometimes accounting for most of the lesion, which lack either diagnostic features in individual tumour cells or architecture indicative of the particular subtype. In the WHO classification, a squamous cell carcinoma should be diagnosed only when individual cell keratinization and/or intercellular bridges are identified. Other nuclear or cytoplasmic features, intercellular gaps or a sheet or cord-like growth pattern of stratified cells which sometimes attract the description 'squamoid' are not diagnostic. Intercellular bridges are a feature which, in the author's opinion, is sometimes more imagined than real. For a diagnosis of adenocarcinoma, the identification of particular patterns is key; patterns which require a minimum area of tumour to be represented or, for the lepidic 'bronchioloalveolar' pattern, the correct anatomical context to be apparent. Most lung adenocarcinomas contain relatively few cells containing mucin, and many show none. The solid pattern of adenocarcinoma requires 'at least two high power fields each to show at least five mucin-containing cells' for diagnosis, recognizing that an occasional mucin containing cell is not uncommon in many tumour types and does not mandate a diagnosis of adenocarcinoma.[20]

Dealing with the morphological challenge: the NSCLC–NOS category

In recognizing the fact that the WHO classification remains unsuitable for application in full in the classification of lung cancer in small biopsy samples or in cytology, several authors recommended the pragmatic use of the diagnostic term 'non-small-cell carcinoma, not otherwise specified – NSCLC–NOS' when there are no diagnostic features to allow a specific NSCLC subtype diagnosis.[32,33] Pathologists should accept that there are occasions when a specific diagnosis is not possible because objective features are not present in the sample. This is an inevitable and unavoidable fact, given the morphology of most NSCLC and the relative paucity of tissue in samples submitted for diagnosis. By limiting the degree of diagnostic refinement to this level in those 'undifferentiated' cases, the accuracy of diagnosis of the remaining cases assigned a more specific category of squamous cell or adenocarcinoma increases.[34] The term NSCLC–NOS may be used differently by pathologists and oncologists. Pathologists refer to those cases in which a more specific diagnosis is not possible; oncologists have tended to consider all cases which are not SCLC in

the same category, regardless of whether or not the pathologist has given a specific diagnosis. While this may have been expedient in the past, driven by a relatively simple therapeutic decision, it is no longer suitable practice. NSCLC is not a single disease and references which imply otherwise are best avoided.

NSCLC–NOS: how many cases?

It is impossible to be prescriptive about the correct proportion of small sample diagnoses which should be NSCLC–NOS based on tumour morphology alone. None the less, it may be useful for pathologists and oncologists alike to consider what might be a reasonable figure. It is likely that both the experience and the bias of the pathologist (for the subjective identification of keratinization, intercellular bridges, acinar or papillary structures, etc.) play a part in the final call of pathological subtype.

It is clearly unsatisfactory for a majority of diagnoses offered by the pathologist to be no more specific than NSCLC. Possible explanations may be that the pathologist has a genuine problem in diagnosing NSCLC, or feels that there is no therapeutic imperative to be more specific. Equally incongruous is a situation where the NSCLC–NOS rate is close to zero; this is impossible. Around 10% of lung cancers are completely undifferentiated large cell or sarcomatoid carcinomas. Many more show large areas of undifferentiated tumour. No amount of skill or experience can discern diagnostic features which are absent, and there should be no pressure to do so.

What would be a reasonable rate for NSCLC–NOS diagnosis? In a 2009 study from the Californian Cancer Registry database on a range of tissue biopsy samples, the proportion of lung cancers diagnosed NSCLC–NOS between 2001 and 2006 was 25%, while for cytology sample diagnosis it was 40%.[35] Exactly the same figures were shown in the author's thoracic biopsy and cytology series published in 2000,[34] but comparisons are difficult, as the former data contain no detail of the use of immunohistochemistry (IHC) in tumour diagnosis and in the latter study IHC was not used. The Californian study observed a rise in the NSCLC–NOS rate over a number of years and speculated on whether this was due to an increase in the use of cytology as the only sample type used for diagnosis (EBUS, EUS, transthoracic FNA, etc.). It is also noticeable that the rise in NSCLC–NOS was mirrored by a fall in the rate of large-cell carcinoma diagnoses, probably as a result of improving diagnostic practice. The 2006 figures from National Lung Cancer Audit database for England (LUCADA) showed an overall NSCLC–NOS rate of 36%, varying between 8% and 59.5% across cancer networks (M. D. Peake, personal communication). In line with the NOS rates being generally higher on cytology samples, where a diagnosis of NSCLC–NOS is made on a bronchial biopsy sample, there is generally much less tumour present.[36] As expected, more limited samples will result in a less specific diagnosis.

NSCLC–NOS: the solution becomes the problem

While the rate of NSCLC-NOS may not have mattered in the past, progress in therapeutics means that increased specification is at least desirable if not essential. What type of NSCLC do patients actually have, when from a small sample an 'NOS' diagnosis is given? They do not all have large-cell carcinoma. In a study of all thoracic samples used to diagnose lung cancer, 64% of patients with the 'NOS' diagnosis proved to have adenocarcinoma after resection of their tumour,[34] a rate in excess of the background prevalence of adenocarcinoma in the resected population in that institution. In another study of bronchial biopsy samples only from the same institution a majority of the 'NOS' cases, when resected, proved to be squamous cell carcinoma, although adenocarcinomas were still overrepresented.[37] Thus, the majority of patients diagnosed with NSCLC–NOS on small samples do actually have differentiated tumours, which require more accurate diagnosis.

If NSCLC–NOS is a problem, what is the solution?

IHC is currently the most practical and best-validated solution to this problem. An IHC profile associated with a particular NSCLC cell type is, at best, suggestive or supportive of a diagnosis but does not confirm it. The WHO definitions of squamous cell and adenocarcinoma are predicated on tumour haematoxylin and eosin (H&E) morphology and mucin-staining and not IHC markers. Large cell neuroendocrine carcinoma is the only type of lung cancer which requires IHC (or ultrastructural) demonstration of, in this case, neuroendocrine differentiation. None of the markers in common use in NSCLC–NOS cases are unique to the tumour type they support. When used in the correct context, IHC markers are predictive of certain NSCLC cell types, but they are not diagnostic.

A number of markers have been associated with either squamous cell carcinoma [e.g. p63, cytokeratin (CK)5/6, high molecular weight cytokeratins (HMWCK: 34βE12), CK14, S100A7, desmocollin3] or

adenocarcinoma of the lung (e.g. TTF1, Napsin A, surfactant proteins, carcinoembryonic antigen: CEA, CK7).[37–48] Most of these markers were naturally identified by their preferential expression in differentiated squamous cell or adenocarcinomas, which were readily diagnosable on the H&E-stained sections of surgically resected cases. This does not prove their usefulness in the small-sample NSCLC–NOS setting. Relatively few studies have actually looked at the predictive value of markers in the context of NSCLC–NOS cases by comparing the IHC predicted diagnosis with the NSCLC subtype in a subsequent resection specimen.[37,47,48] Furthermore, many studies fail to define what is regarded a 'positive' or 'predictive' IHC stain. Loo *et al.* carefully defined the levels of staining for each antibody which gave the best discrimination and power to predict a squamous cell or adenocarcinoma subtype in NSCLC–NOS cases.[37]

For predicting a squamous cell subtype, p63 and CK5/6 appear to be among the best antibodies to use. Loo *et al.* demonstrated that these markers are predictive if one or other (usually both) show moderate or strong staining of over 10% (usually more than 50%) of the tumour cells in the sample, for the given technical staining conditions.[37] This is accurate in 83% of cases and has a positive predictive value of 82–84%. Up to 30% of adenocarcinomas may show some staining for p63,[41] negating acceptance of any p63 positivity as indicative of squamous cell carcinoma. An antibody to a possibly more specific p63 isoform, p40, may be better.[50] Desmocollin3 has emerged as a potentially useful predictive marker of squamous cell carcinoma in NSCLC–NOS cases[47] and deserves further study.

Thyroid transcription factor 1 (TTF1) is probably the most useful IHC marker for predicting an adenocarcinoma subtype in NSCLC–NOS cases. High positivity for TTF1 in tumour cells, at least in the context of bronchial biopsy samples, has predictive accuracy of 84% and a positive predictive value of 88%.[37] In practice, the author would accept, in the correct context, lesser levels of TTF1 staining as predictive. CK7 is advocated by many, but whether this, napsin A or the surfactant protein markers bring any additional benefit to the use of TTF1 remains to be shown. Conceivably, CK7 could be of use in helping identify those lung adenocarcinomas which are negative for TTF1 and mucin. Intuitively, those TTF1 negative tumours seem unlikely to express either napsin A or surfactant proteins. CK7 may also show focal positivity in squamous cell carcinoma.

Other less commonly used panels of IHC markers have been advocated in the context of small sample lung cancer diagnosis.[51] More complex approaches, predicated on the identification of mRNA, microRNA or other gene signatures have also been suggested.[52–54] Some of these techniques are currently very costly, complex and difficult to perform, and impractical for many smaller diagnostic histopathology laboratories. Most crucially, they are completely unproven in the context of the question which actually matters; those cases which cannot be classified by a competent pathologist using cheap, simple and ubiquitous H&E and mucin stains. Indeed, the accuracy of these 'molecular classifiers' is <100% in cases which are easily subtyped by simple morphology.

Implications of this diagnostic approach

The way in which IHC-predicted cases are reported is important. A predictive IHC profile does not confirm a diagnosis of squamous cell or adenocarcinoma. These cases should still be reported as 'NSCLC', but with the added caveat 'probably squamous cell' or 'probably adenocarcinoma'. This may appear a somewhat clumsy, even indecisive approach, but it is realistic and more accurate. It would also be appropriate for such diagnoses to appear in data sets and case record forms in clinical trials of advanced NSCLC. The list of diagnostic categories in the WHO classification is not applicable in full, and a more limited list of categories reflecting actual diagnostic practice would be more appropriate. The clinical trials which have demonstrated a need for discrimination of NSCLC subtypes, either between squamous or non-squamous cases, or the more specific diagnosis of adenocarcinoma, did not describe in detail the pathological diagnosis of cases. We therefore assume that, for example, an NOS tumour case classified predictively as squamous cell carcinoma using IHC will have the same pharmacobiology as a case diagnosable without IHC. Improved recording of pathological data in trials may help to answer this question.

It is noted above that the morphological subtyping of NSCLC, even when the undifferentiated cases are allocated to the NSCLC–NOS category, is not perfectly accurate. There is therefore a case to be made for the more widespread use of IHC, even in cases where the pathologist is more confident of the diagnosis on morphological grounds. There will obviously be variation between pathologists, as biopsy interpretation remains subjective, but a lower threshold for the use of IHC may be appropriate and improve diagnostic accuracy.

The use of IHC to augment morphological diagnosis of lung cancer on small tissue samples consumes much

more tissue than conventional morphological diagnosis alone. As well as helping subtype NSCLC, IHC is sometimes required to discriminate between primary and metastatic disease. Tissue should not, however, be wasted on unnecessary IHC tests, and full clinical details accompanying samples submitted for diagnosis will help limit the testing that is required. The routine preparation of cell blocks from fluid and needle aspiration cytology samples will also assist in providing extra diagnostic material for IHC and other tests. The importance of tissue preparation and preservation is emphasized by the emergence of predictive marker testing. Increasingly, this is the next diagnostic step in patients with confirmed advanced NSCLC.

Pathologists are well aware of the limited amount of tumour which may actually be present in diagnostic samples used for lung cancer diagnosis. A recent study demonstrated that the median percentage of tumour present in malignant bronchial biopsy samples was little more than 20% when squamous cell or adenocarcinoma was diagnosed, while this figure was only 10% in NSCLC–NOS cases.[36] There are no published data on other sample types, but the situation is unlikely to differ significantly. The same study showed that there is no tumour present in one-third to one-half of tissue fragments from a bronchoscopic examination that yields a malignant diagnosis. This highlights the folly of submitting such tissue samples for molecular diagnosis without prior pathological examination to confirm the presence of tumour.[36,55,56]

Molecular predictive markers in NSCLC

Biomarker testing is well established where required to guide treatment of breast and colorectal cancer, GISTs and some lymphomas. The emergence of selective, targeted therapies, directed towards a particular molecular characteristic of an individual patient's tumour, is now driving the need for biomarker identification and testing in NSCLC. These markers may be the mRNA or protein products of various genes, or gene mutation, amplification or translocation. Proteins may be located and quantified using IHC andmRNA and DNA may be extracted from tumour samples and assessed for quantity or mutation. *In-situ* hybridization (ISH) may be used to assess gene copy number or translocation in tumour cells. Currently, however, relatively few markers have reached 'prime time' use. In the United Kingdom the only biomarker which has an approved drug prescription predicated on a 'positive' test is the epidermal growth factor receptor (*EGFR*) gene mutation in the case of the EGFR tyrosine kinase inhibitor (TKI) gefitinib (http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001016/human_med_000857.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d124).[57] Several other markers are being investigated in clinical trials. Tables 1 and 2 list a number of possible markers and the drug(s) associated with each marker. These drugs fall into two broad groups: the newer molecular targeted agents, which tend to be either monoclonal

Table 1. Molecular targeted therapies and their potential biomarkers

Agents	Target	Potential biomarkers
Erlotinib, Gefitinib	EGFR internal TK	EGFR mutation EGFR gene copy number KRAS mutation MET amplification Not EGFR IHC
Cetuximab	EGFR extracelluar domain	EGFR IHC
Bevacizumab	VEGFR	None known
Crizotinib	Chimeric ALK TK	ALK1 protein EML4–ALK gene translocation (FISH)
IGFR1 inhibitors (CP751,871 etc.)	Insulin growth factor receptor TK	IGFR1 protein
Multi-targeted TKIs	EGFR, VEGFR, HER2 etc.	EGFR mutation

EGFR: epidermal growth factor receptor; KRAS: kirsten rat sarcoma; IHC: immunohistochemistry; VEGFR: vascular endothelial growth factor receptor; HER2: human epidermal growth factor receptor 2; TKI: tyrosine kinase inhibitors; EML4-ALK: echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase; FISH: fluorescence *in-situ* hybridization.

Table 2. Cytotoxic therapies and their potential biomarkers

Drug agent/class	Action	Potential biomarker
Platinum-based	Disrupt DNA synthesis Induce apoptosis	ERCC1 Serpin B3, p27, rap80, Abraxas BRCA1
Taxanes	Stabilize microtubules Disrupt cell division Induce apoptosis	BRCA1 Beta tubulin III
Vinca alkaloids	Prevent microtubule assembly Disrupt cell division Induce apoptosis	BRCA1 Beta tubulin III
Pemetrexed	Inhibits thymidylate synthase Disrupts DNA synthesis	Thymidylate synthase (TS)
Gemcitabine	Pyrimidine antimetabolite Disrupts DNA synthesis	Ribonucleotide reductase subunit M1 (RRM1)

ERCC1: excision repair cross-complementation group 1; BRCA1: breast cancer1, early onset.

antibodies or small molecular inhibitors of important enzymes in key oncogenic pathways, and more traditional cytotoxic agents. It is beyond the scope of this review to cover in detail the biology and clinical trial evidence for each of these markers.

The remainder of this review will make only general comments on some of these markers from a pathological perspective. Because *EGFR* mutation testing is now a practical reality in the United Kingdom and beyond, it is appropriate to expand discussion on this marker.

EGFR mutation testing

Mutations in exons 18–21 of the *EGFR* gene, coding for the receptor's internal TK domain, activate the receptor independently of extracellular ligand binding and are referred to as 'activating mutations'.[13,14] The consequent stimulation of several down-stream intracellular pathways, promoting cell cycle activity and cell division, and inhibiting apoptosis, is in NSCLC, an important oncogenic event. The term 'oncogenic addiction' has been used to describe tumours driven in this way by a single mutation.[58] Small molecular inhibitors of the internal tyrosine kinase domain of the EGFR (erlotinib, gefitinib) have been shown to be particularly effective in patients whose tumours have certain activating mutations (sensitizing mutations).[59,60] Approximately 85% of such mutations are deletions in exon 19 or the L858R substitution mutation in exon 21, with others found elsewhere in exons 18 and 21. Exon 20 is the locus of several much rarer mutations which confer resistance to erlotinib and gefitinib, the most important of which is a T790M substitution.[61,62]

EGFR protein expression demonstrated by IHC has not been a successful predictive biomarker in this setting. High *EGFR* gene copy number as measured by fluorescence *in-situ* hybridization (FISH) can predict response to these drugs, but this is probably largely a function of this change being associated with gene mutation.[13,14,63]

EGFR mutated tumours occur more frequently in East Asian patients and in females, but do occur – although less frequently – in Caucasians and in males. *EGFR* mutation is more likely to be found in a patient who is a never smoker, but also occur in former and even active smokers.[64] The vast majority of EGFR-mutated tumours are adenocarcinomas and, in Europe at least, the incidence of EGFR mutation is negligible in squamous cell carcinoma, large-cell neuroendocrine carcinomas and mucinous adenocarcinomas.[16–18] In Caucasian and European studies, the rate of EGFR mutation in adenocarcinomas is variably reported at around 10–15% of cases, whereas in East Asian cohorts it ranges from 40 to 60%. A strategy for selecting patients for EGFR mutation testing based on histology is logical. Performing unnecessary tests wastes time, tissue, money and laboratory capacity. Where a certain or IHC-supported diagnosis of those NSCLC subtypes mentioned above is secured, a no-test policy is reasonable. If there is doubt about subtype then testing should be performed.

Compelling data from a Phase III trial in Asia[65] demonstrated a 72% response rate and 6 months improvement in progression-free survival in patients

with stages IIIb and IV adenocarcinomas bearing a sensitizing mutation when treated with gefitinib as opposed to a standard platinum-based doublet chemotherapy. Crucially, this trial also showed that patients treated with gefitinib in the absence of EGFR mutation did much worse than with chemotherapy. As a result, in April 2009, gefitinib was granted a licence by the European Medicines Agency for use against NSCLC tumours bearing activating mutations. In the United Kingdom, the National Institute for Clinical Excellence (NICE) approved gefitinib in June 2010. These were key events in driving the development of EGFR mutation testing in the United Kingdom and Europe. Similar therapeutic responses have subsequently been demonstrated in trails in Japan.[66,67]

Pathologists play a crucial role in *EGFR* mutation testing, in diagnosis of the NSCLC subtype, selection of test material and result interpretation.[68] There are several different methods of mutation analysis, many of which are listed in Table 3, but details of these are beyond the scope of this review. The choice of particular methodology will depend upon the available technology or what is most appropriate for individual pathology departments or cancer networks to develop. Testing may be developed 'in-house' or in conjunction with existing local molecular pathology/genetics laboratories with the necessary expertise and technology, or may be outsourced to another laboratory/centre.

Two important issues should be considered when mutation methods are chosen (see Table 3). First, some methods are capable of detecting any mutation present in the exons examined. Others, so-called allele-specific methods, are capable of detecting only certain types of mutation (e.g. length analysis for deletions), or the particular set of mutations the testing methodology is designed to detect. Those involved in testing should be aware of the mutations potentially found, or missed, by their chosen methodology. The second issue is that of test sensitivity, in this context referring to the level at which the test can detect mutated genome when diluted by wild-type genome. A test sensitivity of 5% suggests a mutation could be detected at a dilution of 1:20.

Extracted tumour DNA will always include DNA, presumably with wild-type *EGFR*, from non-neoplastic cells in the sample. The pathologist must try to maximize the tumour content of material submitted for mutation analysis and minimize the amount of non-tumour tissue. Selection of material is based upon histological examination and, most often, macrodissection of glass-mounted unstained sections to select suitable tissue. Laser capture microdissection is more elegant but more time-consuming, less often available or practical and usually not necessary. Pathologists should strive to increase the tumour proportion in material for analysis well above the threshold of the testing method used, and should not rely upon the sensitivity of the test to compensate for poor specimens. The proportion of the sample that is tumour is more important than the absolute number of tumour cells used, but ideally at least 200 tumour cells should be present. Smaller numbers of tumour cells may provide a positive test if a mutation is present, but using fewer cells risks a false negative test and an outcome unrepresentative of the whole tumour, due to molecular heterogeneity. Using extremely sensitive methods and nested PCR is not recommended due to risks of false positive results. Detection of unknown/unreported mutations should always be confirmed, to avoid misinterpretation of fixation-induced artefacts, and checked against an EGFR mutation database.[70] Most testing laboratories report a technical failure rate of 3–8%, often due to polymerase chain reaction (PCR) failure due to poor quality DNA (this is due most probably to overfixation) or insufficient DNA to complete the testing procedure.

Given the relatively low prevalence of EGFR mutations in a UK patient population, a negative result (no sensitizing mutation detected) is the norm, so every effort should be made to avoid the possibility of false negative tests. The mutational analysis should be reported in conjunction with the pathology of the case, including details of any mutation detected, the method used, estimated test sensitivity and a comment on the nature and proportion of tumour in the sample tested. Testing laboratories should aim to provide a test result within 5–7 working days from receipt of test samples.

The issue of resistance to *EGFR* TKIs is increasingly important. The T790M mutation in exon 20 accounts for <5% of all *EGFR* mutations described in patients who are TKI-naive, but is found in 50% of those who relapse while on TKI therapy.[61] In TKI-naive patients the T790M mutation may be found using standard detection techniques, together with a sensitizing mutation, usually an exon 19 deletion in around 3–4% of mutated cases. If very high sensitivity methods are used, T790M mutations have been described in around 35% of cases with a sensitizing mutation,[71] implying the presence of minor secondary clones with a resistance mutation, which may be selected preferentially during TKI therapy. Around 20% of patients who relapse on TKI therapy show amplification of the *MET* gene. Increased MET activity confers resistance to EGFR blockade. Interestingly, minor clones of MET amplified cells have also been described in TKI-naive tumours, again suggesting the emergence of resistant clones

Table 3. Molecular biological methodology for epidermal growth factor receptor (EGFR) mutation analysis; modified from Ref. (69)

Technique	Limit of detection (% mutant DNA)	Mutations identified
Direct sequencing	10–20	All – known and new
*Taq*man PCR	10	Limited – specific known only
Loop-hybrid mobility shift assay	10	Limited – specific known only
dHPLC	10	All – known and new
Cycleave PCR	5	Limited – specific known only
Pyrosequencing	5	All – known and new
PCR-RFLP and length analysis	5	Limited – specific known only
MALDI-TOF MS-based genotyping	5	Limited – specific known only
PCR-SSCP	5	All – known and new
WAVE surveyor	3–5	All – known and new
High-resolution melting (HRM)	3–5	All – known and new
PNA-LNA PCR clamp	1	Limited – specific known only
Scorpion ARMS	1	Limited – specific known only
Single molecule sequencing	0.1	All – known and new
Mutant-enriched sequencing	0.1	Limited – specific known only
SMAP	0.1	Limited – specific known only

SMAP: smart amplification process; ARMS: amplification-refractory mutation system; PNA-LNA: peptide nucleic acid-locked nucleic acid; PCR: polymerase chain reaction; SSCP: single-strand conformation polymorphism; MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight mass spectrometry; RFLP: restriction fragment length polymorphism; dHPLC: denaturing high pressure liquid chromatography; MS: mass spectometry; WAVE® (SURVEYOR Nuclease and WAVE® HS DHPLC Platform, Transgenomics, Glasgow, UK).

during therapy.[72] It is not yet clear what role the search for these minor clones may play in our overall strategy of EGFR testing in NSCLC. *PIK3CA* and rare *HER2* mutations and *PTEN* loss, coexistent with EGFR mutation, may also account for TKI resistance.

Other mutations in adenocarcinomas

There is emerging evidence that, with very occasional exceptions, mutations in *EGFR*, *KRAS*, *BRAF* and *HER2* and the *EML4–ALK* translocation are all found mutually exclusively within lung adenocarcinomas, strongly suggesting an important oncogenic role for each.[73]

KRAS mutation is found in 30–40% of UK/European adenocarcinomas and is associated strongly with smoking and aggressive tumours with a poor prognosis which, together with the *de facto* absence of EGFR mutation, probably explains why Kirsten rat sarcoma (KRAS)-mutated tumours appear resistant to EGFR TKIs.[13,14,74] Despite this, the *KRAS* mutation has not been an effective selection factor for TKI therapy when tested in clinical trials.[75] None the less, it is the author's standard practice to look for *KRAS* mutations in conjunction with *EGFR* mutation testing. *KRAS* mutation testing is, in comparison to *EGFR* testing, relatively easy to perform. Mutations occur mainly in codon 12, with fewer in codon 13 and rare events in codon 61, and are quite robust even in poor quality samples. *KRAS* mutation is a useful finding in a small-volume or poor-quality test sample, removing what may otherwise be an understandable concern regarding a false negative *EGFR* mutation test. Therapies to target *KRAS*-mutated tumours, such as down-stream MEK inhibitors, are currently undergoing trials.

BRAF and *HER2* mutations are rare events in lung adenocarcinomas, described in approximately 3% and 2% of cases, respectively. There are several *BRAF* inhibitors undergoing trials. For example PLX-4032 has shown remarkable activity in malignant melanoma bearing the V600E *BRAF* mutation, but there are few data in lung cancer. Inhibitors of EGFR and human epidermal growth factor receptor 2 (HER2) are undergoing trials in non-small-cell lung cancer.[76]

After *EGFR* (and *KRAS)* mutation testing, the next issue likely to face pathologists in this context is testing for *EML4-ALK* translocation.[77,78] This is another rare (4%) oncogenic event in lung adenocarcinoma, but significant, as the drug crizotinib has shown strong activity in Phase II trails. The mechanism of action is through blocking the activated kinase, which results from the range of different translocations occurring in chromosome 2, placing segments of the *EML4* gene next to *ALK*. Testing strategies are not yet clearly developed. The range of different translocations has made even a multiplex PCR approach <100% reliable and FISH, using a break-apart probe, is the standard way to confirm the translocation.[79] This procedure is relatively time-consuming, even if testing were confined to *EGFR* and *KRAS* mutation-negative cases. The immunohistochemical detection of elevated levels of ALK1 protein seems to have a high positive predictive value for the translocation and probably has a role as a screening tool to select cases for FISH. There are, however, issues around the choice and availability of antibodies and the sensitivity of the IHC detection method employed.[80] Standard procedures used for detecting ALK1 in lymphomas appear to lack adequate sensitivity to detect the lower levels of ALK1 protein in lung adenocarcinomas bearing an echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4–ALK) translocation. The development of an antibody with high specificity and sensitivity for ALK fusion proteins would be very welcome.

Of the other drug–biomarker combinations mentioned in Table 1, bevacizumab is prescribed according to tumour histology (see above), but there is no currently known biomarker to assist patient selection. However, VEGFR may be a predictive biomarker for sorafenib. Cetuximab has shown activity in NSCLC and there are data to suggest that the IHC expression of EGFR protein may be a biomarker to predict response.[81] IGFR TKIs are at an early stage of trial development, and there have been issues around drug toxicity, but preliminary data suggest better activity in squamous cell carcinoma.[19] The role of IGFR1 protein expression as a biomarker is unknown.

Biomarkers and cytotoxic drugs

There are a number of potential biomarkers which may help predict the response of a NSCLC to a particular drug, and Table 2 lists those most studied. It should be emphasized that none of these markers is yet proven for clinical use although clinical trials are currently under way. In some instances there are retrospective data to suggest the marker has potential for selecting patients. These biomarkers have a possible role in selecting the most effective treatment either for advanced NSCLC or early disease in the adjuvant setting.

PLATINUM-BASED AGENTS

Platinum-based drugs such as cisplatin and carboplatin are central to the treatment of NSCLC, where they are used in a 'platinum doublet' in combination with another cytotoxic. These drugs form adducts by binding directly to DNA, preventing normal cell replication and triggering apoptosis. Possible ways in which a cancer cell may overcome this effect include an efficient DNA repair mechanism or an inefficient or compromised apoptosis pathway. There are many enzymes and other proteins involved in the execution of both these normal, physiological functions in cells, involving several different pathways. Some of these factors are beginning to emerge as possible predictive biomarkers.

Excision repair cross-complementation group 1 (ERCC1) is one of the nucleotide excision repair pathway enzymes and is the best-known potential biomarker for predicting response to platinum-based drugs with high levels of ERCC1 predicting resistance.[82,83] Confirming findings *in vitro*, a retrospective study of the International Adjuvant Lung Trial (IALT) cases showed that patients with ERCC1-negative tumours gained benefit from platinum-based adjuvant chemotherapy, whereas those with ERCC1 positive tumours performed worse than controls.[84] Other data support the possible use of this marker in both the adjuvant and the advanced disease setting in NSCLC.[82]

Other markers have been proposed for selecting platinum-resistant patients. Cyclin-dependent kinase inhibitor 1B, also known as $p27^{Kip1}$, is a negative regulator of the cell cycle and may inhibit apoptosis through promotion of cell cycle arrest. Overexpression of p27 may allow repair of damaged DNA and prevent proliferation- induced apoptosis, indicating its potential use as a marker of platinum resistance. Data from the IALT trial support this hypothesis.[85] BRCA1 (breast cancer 1, early onset) is important in multiple DNA repair pathways and has, together with other components of these pathways such as RAP80 and Abraxas,

been suggested as a possible predictor of platinum response.[83,86,87] Serpin B3 is a cross-class lysosomal cysteine protease inhibitor whose targets include cathepsins L, K and S, conferring the function of a negative regulator of apoptosis. Overexpression of Serpin B3 mRNA identified in a gene expression array analysis predicted resistance to platinum in NSCLC, and Serpin B3 protein expression was verified subsequently as similarly predictive in a retrospectively studied patient cohort.[88]

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These drug classes act by stabilizing or preventing assembly of microtubules during mitosis, resulting in apoptosis. High BRCA1 levels appear to increase sensitivity to such antimicrotubule agents, at least *in vitro*.[83,89] Tubulin proteins are a key building block for microtubules in cells. High tumour levels of class III beta-tubulin (βTubIII) are known to predict resistance to antimicrotubule agents in patients with NSCLC.[82,90]

PEMETREXED

As discussed previously, the antifolate pemetrexed is a competitive inhibitor of a number of enzymes important in one-carbon metabolism in cells necessary for thymidine, and thus DNA synthesis and cell replication.[1] The most important target of pemetrexed is TS. Cells with high levels of TS may be relatively resistant to pemetrexed, as maximum achievable intracellular levels of the drug may be unable to inhibit TS function successfully. It remains to be seen whether or not measuring TS levels is a better way of selecting patients for pemetrexed, as opposed to the current practice of using non-squamous tumour morphology. There is some evidence that TS levels may predict response[91] but trials are ongoing to address this question.[92,93]

GEMCITABINE

Ribonucleotide reductase subunit M1 (RRM1) is important in DNA synthesis through deoxynucleotide production. This enzyme is the target of gemcitabine, which disrupts DNA synthesis and induces apoptosis by acting as a pyrimidine antimetabolite. There is evidence that tumour response to gemcitabine is greater when RRM1 levels in the tumour are lower.[91,94]

These are the best-known of the potential biomarkers for cytotoxic drug responses, but there are others that are yet to be clinically validated.[82,89]

Newer drug classes and biomarkers

Many novel compounds are in development or in early clinical trials, and these may yet prove effective in small groups of patients with NSCLC, defined by the particular biology of their tumour and identifiable using a biomarker. Most of these agents are small molecular inhibitors of gene function and targets include mTOR, heat shock proteins, poly(ADP-ribose) polymerase 1 (PARP1), MEK (mitogen-activated protein kinase kinase), MET (mesenchymal epithelial transition factor), sarcoma (SRC), histone deacetylation (HDAC) and hedgehog (Hh) signalling. If any or all of these agents emerge as effective treatments in NSCLC, there will be a requirement to test for the predictive biomarker.

Tumour vaccines may also offer a different approach to treating patients with NSCLC. The MAGRIT trial is a Phase III adjuvant study of MAGE-A3 cancer-testis antigen-specific immunotherapy.[95] Trial patients are selected on the basis of MAGE-A3 expression in their resected tumours.

Each predictive biomarker discussed so far concerns a single gene or gene product representing the drug target or a factor which enhances or detracts from drug efficacy. Global molecular profiles have also been proposed as predictive tools in NSCLC therapy selection. Gene expression array signatures have been proposed for response to cytotoxic agents and EGFR tyrosine kinase inhibitors,[96,97] but these have been superceded by the single markers mentioned earlier. There is no doubt, however, that such global gene expression screening methods will be used in the future in an attempt to identify predictive signatures. A serum proteomic classifier has also been demonstrated capable of identifying patients more likely to benefit from a combination of erlotinib and bevacizumab therapy.[98] It is difficult to say whether these approaches will have any advantage over the 'single marker' studies which dominate current thinking.

Delivering on this promise: challenges and opportunities for pathology

This review has concentrated exclusively on histological or molecular markers which might predict the probable response of an individual to a particular therapy for NSCLC. It has not addressed the important but equally complex issue of these same or similar factors being prognostic (influencing the outcome of the patient independent of therapy) as well as predictive (of therapeutic response) in NSCLC. The importance of the differentiation here rests on the fact that any apparent influence on patient outcome could be

confounded by a prognostic effect. None the less, there is currently not a single histological or molecular prognostic marker that can be considered mainstream in managing NSCLC patients, either with advanced disease or, possibly more importantly, in selecting for adjuvant therapy. Prognostic markers have been discussed in a number of recent publications.[97,99,100]

While there are plentiful data on the over- or underexpression of predictive biomarkers, there is less agreement on how these markers should be measured. Whether it should be IHC-determined protein levels or mRNA levels assessed by a quantitative polymerase chain reaction (qPCR) is obviously of crucial importance to the pathologist expected to perform and interpret the 'test'. Should gene copy number be assessed by *in situ* hybridization (ISH) or by qPCR? IHC has the advantage of being widely available, relatively easy to perform and, most crucially, retains morphological information, allowing confident assessment of the marker in the tumour cells (Figure 1). IHC is criticized as a subjective measure, interpreted by individual pathologists often with little published guidance about what is considered a 'positive' or 'negative', 'high' or 'low' score. The use of complex systems such as H-scores is a valid approach accounting for both the intensity and distribution of staining, but can be cumbersome to use. Whatever scoring method is used, consistency and clarity are essential to deliver reliable results. The vagaries of pre-analytical issues such as fixation, tissue processing and the IHC methods themselves also require careful attention to ensure consistency over time and between centres.[101,102] Automated image analysis platforms for IHC scoring exist but their use is not widespread and a healthy scepticism prevails over their value.

Many of these comments on IHC also apply to ISH. The retention of morphology is important, although can be difficult in fluorescence-based systems in lung cancer samples. Fluorescence ISH, in particular, is a more labour-intensive technique than IHC.

While a qPCR approach to measuring mRNA levels may appear more objective than IHC or ISH, there is reason to be cautious. Messenger RNA is rather labile, especially after formalin fixation. Some studies are predicated on examination of fresh frozen tumour material, something not often available in the 'real world'. As already mentioned, extreme care must be taken when cellular tissue is processed to extract the molecules of interest. No tumour is devoid of non-neoplastic tissue; such extracts will always contain material from non-neoplastic sources and some account must be taken of the content of the analysed sample if meaningful results are to be forthcoming (Figure 1).

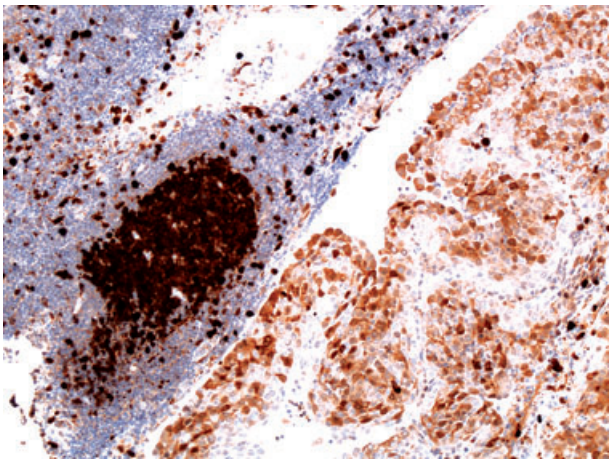

Figure 1. Poorly differentiated adenocarcinoma (bottom, right) stained with an antibody against thymidilate synthase (TS). Note that there is variability in the cytoplasmic staining of tumour cells. This tumour has a heavy lymphocytic infiltrate (top, left) and the proliferative germinal centre also shows strong TS expression. Some potential biomarkers may be altered physiologically in non-neoplastic tissue as well as pathologically in neoplasia. A morphological assessment of a biomarker may be more accurate than assessment made on molecular extraction from the total cellular content of a tumour sample.

Is there enough tumour tissue on which to perform these tests? There is no definitive answer to this question; how much is enough? Diagnostic samples from patients with NSCLC often contain very little tumour.[36] IHC can be performed and assessed on even a single tumour cell, but what does that mean? RNA and DNA may be extracted from a sample containing few tumour cells, but will be contaminated by nucleic acids from normal cells. How do we interpret the test result, especially if the molecular marker is not unique to the malignant process? Very few studies reporting biomarker data address these questions; some barely mention test methodology. Some biomarker data are based on study of fresh frozen or formalin-fixed, paraffin-embedded (FFPE) samples of surgically resected tumours. The use of such material is logical to establish a testing principle using abundant material, but such tests may not transfer to FFPE diagnostic biopsy or cytology samples in advanced NSCLC. The IPASS trial did report some relevant data:[65] for EGFR mutation testing, all cytology samples and biopsy samples containing <100 tumour cells were excluded from testing. However, in implementing the IPASS findings in clinical practice, treatment decisions are made regularly based on analysis of samples which would have been rejected in the IPASS trial, and these are therefore unvalidated.

Inextricably linked with test sample size is the crucial issue of tumour heterogeneity. Heterogeneity operates

at all levels in lung cancer cells; morphology, protein and mRNA expression, gene amplification and translocation and gene mutation. On average, the tumour in a malignant bronchial biopsy represents around one-billionth of the total cells in a primary lung cancer. There is also heterogeneity between the primary tumour and metastatic disease.[103,104] None the less, it will be necessary to perform these tests on whatever tissue is available, and most often this will be primary tumour, possibly obtained months or years before recurrent/metastatic disease presents. There are some data addressing the issue of small sample versus whole tumour and primary versus metastatic disease, but each is only valid for the individual marker tested.[103–112] These data do show variation. For some marker–method combinations, the small biopsy or the primary tumour is fairly representative, but for others, the majority, there is significant discordance and heterogeneity, be this IHC, ISH or mutational analysis. Although heterogeneity is described with EGFR mutation, it has been questioned as to whether this is a true biological phenomenon or a technical 'artefact'.

All these issues raise the most important question of all: does the test result obtained truly reflect the clinically relevant biomarker status of the patient's tumour burden?

Conclusion

We are now clearly entering an era of personalized medicine for non-small-cell lung cancers. Primarily, this is good news for patients as it should lead to more appropriate treatment and better outcomes. It is also a very positive development for pathology, ever more emphasizing the importance of a complete histopathological diagnosis in guiding the treatment of patients with cancer. It presents new roles and responsibilities for pathologists, and these should be welcomed. Apart from being good for the image of a speciality sometimes accused of being 'old-fashioned', it also emphasizes the key role of pathology in the cancer multidisciplinary team (MDT). A morphological perspective is also absolutely essential to ensure appropriate, accurate and adequate testing and interpretation. Molecular analysis is a logical extension or supplementation of our histological diagnoses. This is not diagnostic activity which can be executed by molecular biology laboratories in isolation from histopathology.

Obviously, a sense of perspective is necessary. Deploying testing in the context of a National Health Service (NHS)-funded service requires an evidence base to justify the test and excellent quality assurance to ensure that appropriate clinical decisions are taken. External quality assurance schemes will have an important role as predictive testing becomes widespread. Both pathologists and oncologists need to be very aware of the numerous limitations and pitfalls in various test scenarios, from the pre-analytical issues around sample collection, fixation and processing, the various methods used for marker detection and the subjectivity inherent in many diagnostic approaches. Many of these tests take time and tissue, both of which may be in very limited supply. Sophisticated testing methods need careful execution by skilled exponents; good laboratory practice often requires the safety of duplication of analysis; there may be no short cuts, yet there is a sick patient waiting on the 'result'. Diagnostic tissue samples from patients with advanced lung cancer are frequently extremely small. While technology may be able to overcome this to some extent, the heterogeneous nature of tumours will limit this compensation, if the test outcome is to remain valid and representative. New dialogue is needed on the topic of adequate, but safe and practical, tissue acquisition for complete diagnosis.

Acknowledgements

The author wishes to thank colleagues Dr M C Nicolson and Dr P S Loo for their critical reviews and comments on this paper.

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