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INVITED REVIEW

What's new in non-small cell lung cancer for pathologists: the importance of accurate subtyping, EGFR mutations and ALK rearrangements

WENDY A. COOPER*‡, SANDRA O'TOOLE*§||¶, MICHAEL BOYER†||, LISA HORVATH†|| AND ANNABELLE MAHAR*

**Tissue Pathology and Diagnostic Oncology, and †Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney, ‡Discipline of Pathology, School of Medicine, University of Western Sydney, Sydney, §Garvan Institute of Medical Research, Darlinghurst, ||Sydney Medical School, University of Sydney, Sydney, and ¶St Vincent's Clinical School, University of New South Wales, Sydney, Australia*

Summary

In the past, the only critical point of distinction in the pathological diagnosis of lung cancer was between small cell and non-small cell lung cancer (NSCLC). The emergence of new targeted therapies and clinical trials demonstrating differing efficacy and toxicity of treatments according to specific histological subtypes of NSCLC, has resulted in an increasing need for improvements in pathological diagnosis. Accurate distinction between adenocarcinoma and squamous cell carcinoma is now critical as histological subtyping has the potential to influence clinical decision making and impact on patient outcome. While morphological criteria remain the most important feature to distinguish NSCLC subtypes, use of mucin and immunohistochemical stains (TTF-1, p63 and CK5/6) can be of assistance in difficult small biopsy cases. With the emergence of selective kinase inhibitors targeting epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), there is a corresponding need to identify the subset of NSCLCs harbouring specific genetic mutations associated with sensitivity to these agents, almost all of which are found in adenocarcinomas. In this review, the importance of accurately subtyping NSCLC is discussed, along with a suggested approach for distinguishing histological subtypes in small biopsy specimens. The significance of EGFR and ALK mutations in NSCLC and the impact of these genotypes on pathology and clinical practice are also reviewed.

Key words: Adenocarcinoma, ALK, diagnosis, epidermal growth factor receptor, FISH, immunohistochemistry, non-small cell lung cancer, NSCLC subtype, protein-tyrosine kinases, small biopsy, squamous cell carcinoma.

Abbreviations: ALK, anaplastic lymphoma kinase; BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; FISH, fluorescence *in situ* hybridisation; NSCLC, non-small cell lung carcinoma; SCC, squamous cell carcinoma; TKI, tyrosine kinase inhibitor; TS, thymidylate synthetase; TTF-1, thyroid transcription factor-1; VEGF, vascular endothelial growth factor.

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INTRODUCTION

Lung cancer is the leading cause of cancer death in Australia and is the fifth most common registrable malignancy.[1] The majority of lung cancers are non-small cell lung carcinomas (NSCLC) and there is considerable evidence that different subtypes of NSCLC have distinct epidemiological, clinical, biological, pathological and molecular characteristics. In the past, these differences have been of minimal importance to pathologists and clinicians as patient management was similar for the different histological types of NSCLC. Histology of NSCLC is now of clinical relevance and predicts efficacy and toxicity of some treatments for advanced NSCLC as well as predicting likelihood of genotypic differences which may be important for treatment selection. This has resulted in a paradigm shift for both clinicians and pathologists in their approach to NSCLC[2] and there is now a greater obligation on pathologists to accurately subtype NSCLC in small biopsy and cytology samples. The increasing use of ancillary studies for assessment of predictive and prognostic molecular abnormalities also has implications for collection of material at the time of diagnosis.

IMPORTANCE OF DISTINGUISHING NSCLC SUBTYPES

Non-small cell lung carcinoma can be divided histologically into several subtypes, primarily adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma.[3] There are a number of genetic differences which underlie these morphological variations. In particular, mutations of specific genes such as *KRAS* and *epidermal growth factor receptor* (*EGFR*) are found almost exclusively in adenocarcinomas rather than SCCs.[4] Gene expression profiling studies also demonstrate different mRNA expression patterns in SCCs as opposed to adenocarcinomas.[5,6] Despite these variations, management of NSCLC patients has not depended on histological subtype in the past.

A number of recent clinical trials have completely altered pathologists' approach to lung cancer diagnosis, with distinction of NSCLC subtypes now carrying with it significant potential clinical importance. Briefly, these trials have shown:

1. Response rate and survival with the chemotherapeutic agent pemetrexed is significantly better in patients with non-squamous histology.[7–9]
2. Toxicity from life-threatening pulmonary haemorrhage with bevacizumab treatment of advanced lung cancer is associated with SCC histology.[10]

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3. NSCLC with EGFR mutations are more responsive to EGFR tyrosine kinase inhibitors (TKI) than wild-type tumours. As almost all EGFR mutant NSCLC are adenocarcinomas, histological subtyping is important in determining which cases are appropriate to undergo mutation testing.[11]
4. NSCLC harbouring EML4-ALK (echinoderm microtubule-associated protein-like 4 - anaplastic lymphoma kinase) translocations are sensitive to ALK kinase inhibitors *in vitro* and *in vivo* and are currently undergoing clinical trials.[12] EML4-ALK is found almost exclusively in adenocarcinomas.

To this we would add that there is a need to clarify pathological classification of NSCLC subtypes in a reproducible manner by using internationally accepted criteria so that data available for future clinical trials are more scientifically reliable, resulting in more accurate predictive and prognostic information for optimal clinical management of patients.

Pemetrexed in NSCLC

Pemetrexed is an antifolate anti-metabolite which targets thymidylate synthetase (TS) as well as several other intracellular enzymes.[13] Recently, a number of clinical trials of pemetrexed have identified differences in treatment efficacy and patient outcome based on NSCLC histology in Western and Asian populations (reviewed by Hirsch *et al.*[14]). Retrospective and prospective trials comparing pemetrexed and other chemotherapeutic agents in first and second line treatment of advanced NSCLC have shown significantly longer overall survival in patients with non-squamous histology (adenocarcinoma or large cell carcinoma) in analyses of overall survival by treatment and confirmed in treatment by histology interaction analyses.[15,16] In 2008, a prospective phase III study on treatment of advanced NSCLC showed for the first time survival differences in patients based on histological subtype of tumour.[9] These studies confirm a predictive role of NSCLC histological subtypes in determining efficacy of pemetrexed, whereas histology of NSCLC has not previously shown consistent differential sensitivity to chemotherapeutic treatments.[14]

The molecular basis underlying the differential efficacy of pemetrexed in non-squamous versus squamous subtypes of NSCLC is thought to relate to the enzyme thymidylate synthase which is involved in DNA synthesis and targeted by antimetabolites such as 5-fluorouracil and pemetrexed.[13] Lower levels of TS mRNA and protein expression are generally found in adenocarcinomas compared to squamous cell and small cell carcinomas[17] and similar differential expression of TS in cell cultures of differing histological subtypes of NSCLC correlates with sensitivity to pemetrexed.[18]

Bevacizumab in NSCLC

Bevacizumab is a humanised variant of a murine anti-vascular endothelial growth factor (VEGF) antibody that inhibits VEGF receptor mediated angiogenesis[19] and provides a survival advantage when used in combination with chemotherapy in the treatment of locally advanced or metastatic NSCLC.[20,21] While no differential efficacy has been found according to histological subtype of NSCLC, there is evidence of differential toxicity based on histological subtype.[10] In particular, life threatening pulmonary haemorrhage was found to be more frequent in SCC compared to adenocarcinomas and appeared to relate to centrally located tumours with cavitation, both features associated with SCC, although the overall number of patients experiencing these complications was small.[10] Subsequently, patients with SCC have been excluded from trials of bevacizumab in NSCLC.

HISTOLOGICAL SUBTYPING OF NSCLC

WHO classification of NSCLC

The 2004 World Health Organization (WHO) classification is used for subtyping NSCLC in routine pathology practice.[3] The classification is largely based on assessment of standard H&E stained sections of resected tumours. Squamous differentiation is determined by identification of keratinisation and/or intercellular bridges, while adenocarcinomas are identified by acinar, papillary, bronchioloalveolar or solid growth patterns. For solid tumours, mucin must be identified with histochemical stains in at least five tumour cells in each of two high power fields.[3] Large cell carcinomas require exclusion of squamous, glandular or small cell differentiation; therefore, specific diagnosis can only reliably be made in resection specimens.[3] Interestingly, in gene expression profiling studies, large cell carcinomas tend to cluster either as adenocarcinoma or unique 'large cell clusters'.[6]

Accuracy of NSCLC histological subtyping

Based on the WHO 2004 classification, a study of interobserver variability found only moderate agreement in the distinction of squamous versus non-squamous carcinomas but results were slightly better for expert pulmonary pathologists (kappa values 0.55 and 0.64, respectively).[22] Studies based on the 1981 WHO criteria showed agreement between original diagnosis and expert review in only 83% of adenocarcinomas and 91% of SCCs.[23] In another study based on WHO 1981 criteria, of 257 cancers classified as SCC by regional pathologists, 19% were reclassified as adenocarcinomas by central pathologists.[24] These findings question how well defined the patient subsets are in clinical trials as well as the significance and consequence of clinical treatment decisions based on histological subtyping. Close scrutiny of most clinical trials reporting differential effects of treatment according to NSCLC histology reveal that the methodology for pathological subclassification is not clearly defined. One criticism has been that no centralised review of pathology was undertaken in the majority of these trials. In a phase III trial of maintenance pemetrexed, 14% of cases underwent masked central pathology review and there was agreement in the distinction of squamous from non-squamous NSCLC in 89% of cases.[7] In another phase III study of pemetrexed treatment in advanced NSCLC, patients were selected 'with histologically or cytologically confirmed NSCLC' with no further details regarding diagnostic criteria or whether or not centralised pathology review took place.[9] Interestingly, 33% of cases in this study (total of 577) were diagnosed cytologically, even though the WHO classification of lung cancer does not provide well defined criteria for subtyping NSCLC based on cytology and essentially precludes diagnosis of large cell carcinoma except in resection specimens.[3] Overall, there were 847 adenocarcinomas, 153 large cell carcinomas, 473 SCC and 252 NSCLC not otherwise specified (NOS).[9] No details were provided as to whether mucin or immunohistochemical stains were used to refine diagnosis and, if so, in what proportion of cases. Attention to greater precision

of histological classification is crucial for validating trial results and may improve clinical outcomes from use of these treatments.

Histologic subtyping of NSCLC in small biopsy and cytology specimens

Approximately 65–75% of patients with NSCLC present with advanced stage disease that is not amenable to surgery.[25] As such the diagnosis is frequently based on small biopsy and cytology specimens alone. Histological subtyping of NSCLC according to the WHO classification[3] is based on thorough histological assessment of resection specimens. Although cytological features of major tumour types are described, specific minimum criteria for distinction of NSCLC subtypes in small biopsies and cytological specimens are not provided. There are a number of limitations to classification of NSCLC subtypes in small biopsy specimens. While many well differentiated tumours pose no real diagnostic challenge, poorly differentiated tumours that require extensive sampling to identify their nature may appear undifferentiated in small samples. In small biopsy samples, one study found only 63% of NSCLC can be correctly subclassified in bronchial biopsies and 45% in cytology samples.[26] Others have shown only about 50% of small biopsies enable subtyping of NSCLC.[27]

Histological heterogeneity is not uncommon in lung cancers, with 5% of adenocarcinomas having a small squamous component and 15% of SCCs showing very focal glandular differentiation.[28] Tumour heterogeneity poses a potential confounding factor for both morphological classification and molecular characterisation of tumours in small biopsy specimens.

If after all attempts a NSCLC remains unclassifiable based on the limited amount of material available, then the term 'non-small cell lung carcinoma NOS' is recommended as the most appropriate diagnosis. This diagnosis enables communication regarding uncertainty of histological type to the treating clinician which can be factored into clinical decision making and, if appropriate, further biopsy could be attempted. Small biopsy or cytology specimens showing only undifferentiated carcinoma should not be labelled as large cell carcinoma as they may represent undifferentiated areas of another histological tumour type. Histological examination of resection specimens following biopsies diagnosed as NSCLC NOS show 55% of these tumours are adenocarcinoma with only a few true large cell carcinomas.[26]

Similarly, while bronchioloalveolar carcinoma (BAC) may be recognised or suspected in small biopsy or cytology specimens, an invasive component cannot be excluded and the diagnosis can only be made from thorough histological assessment of a resection specimen.[3,29] The distinction of BAC from invasive adenocarcinomas is important as small solitary non-mucinous BACs have an excellent prognosis.[29]

Use of ancillary tests to assist in distinguishing NSCLC subtypes

While light microscopic evaluation of morphology remains the mainstay of classifying NSCLC, a subset of cases cannot be differentiated light microscopically, particularly in small biopsy samples. There has been increasing interest in the literature regarding the utility of ancillary tests to improve pathological distinction of histological subtypes where clear cut morphological features are not present. A variety of different markers have shown differential expression in subtypes of NSCLC. Most pulmonary adenocarcinomas (Fig. 1) express cytokeratin 7 and thyroid transcription factor-1 (TTF-1), a few express cytokeratin 20[30–34] and about 30% express p63.[35] By contrast, SCCs (Fig. 2) frequently express CK5/6, p63 and 34βE12, but are usually negative for TTF-1 and CK7,[32,33,36] although most studies have been performed in resected tumours that were readily classified using morphology alone. TTF1 is expressed in about 70–85% of lung adenocarcinomas[37–40] with

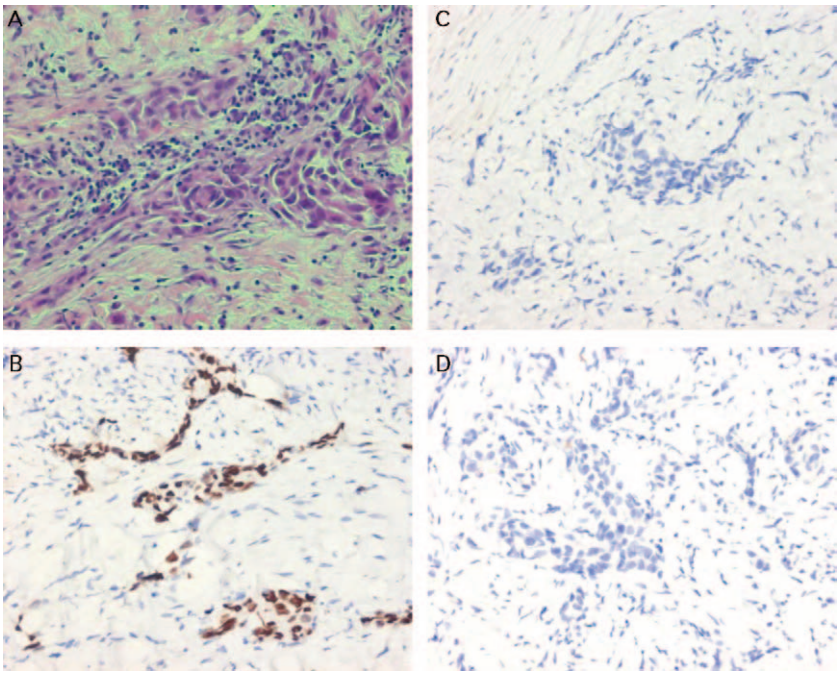

Fig. 1 Pleural biopsy from a patient with a lung mass. Undifferentiated carcinoma on H&E, with an immunoprofile favouring adenocarcinoma. (A) The tumour is a non-small cell carcinoma composed of islands and cords of neoplastic epithelial cells without definite glandular or squamous differentiation (H&E). (B) Neoplastic cells show positive nuclear staining for TTF-1. (C) Neoplastic cells show no staining for cytokeratin 5/6, or (D) p63.

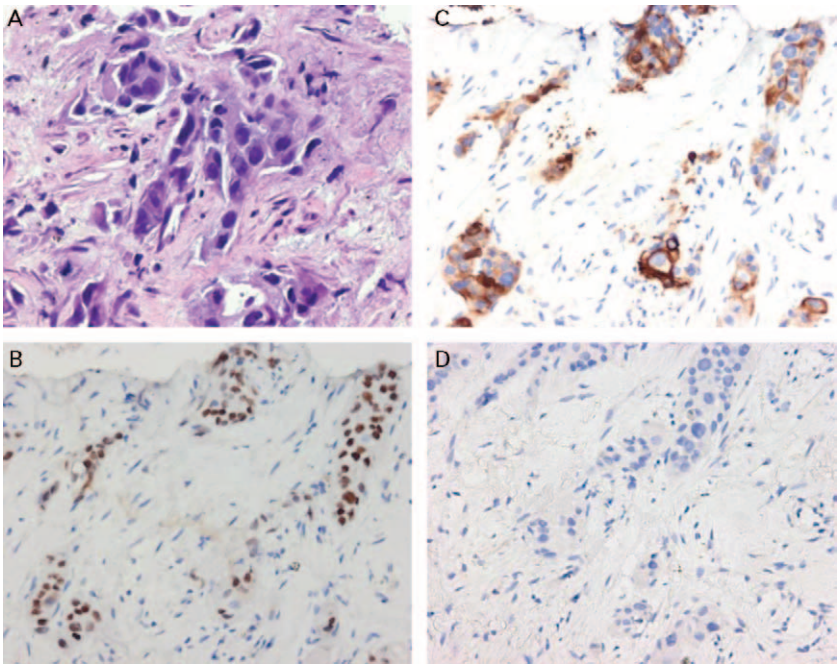

Fig. 2 Core biopsy of a lung mass, undifferentiated carcinoma on H&E, with an immunoprofile favouring squamous cell carcinoma. (A) The tumour is a non-small cell carcinoma (partly necrotic) formed by islands of neoplastic epitheial cells without definite glandular or squamous differentiation (H&E). (B) Neoplastic cells show positive nuclear staining for p63. (C) Neoplastic cells show positive cytoplasmic staining for cytokeratin 5/6. (D) A TTF-1 immunostain is negative in the neoplastic cells.

absence of staining typically observed in more centrally located non-terminal respiratory unit type adenocarcinomas and mucinous adenocarcinomas.[40] In some studies, TTF-1 is not expressed in pulmonary SCCs[37,39,41] but others have reported expression in 5–21% of SCCs.[34,38,42] TTF-1 also stains about 20–30% of resected undifferentiated large cell carcinomas and 50% of large cell neuroendocrine carcinomas, as well as some metastatic adenocarcinomas.[43]

There is evidence that a small panel of immunohistochemical stains together with mucin stains can improve diagnostic accuracy of subtyping NSCLC and an algorithm for such an approach is provided (Fig. 3). A number of studies have shown p63, CK5/6 and TTF-1 have fairly high sensitivity and specificity for distinguishing SCCs from adenocarcinomas, although these studies mostly included resected tumours that were readily classified based on morphological assessment alone.[44–46] Others have shown p63 immunohistochemistry used on cytological smears significantly increased sensitivity of detecting NSCLC with squamous differentiation.[47] In two recent studies, Loo *et al.*[41] and Nicholson *et al.*[27] proposed the most useful panel consists of TTF-1 and a mucin stain (DPAS or Alcian blue/PAS) for identification of adenocarcinomas, and p63 and CK5/6 for SCC. This approach lead to a more specific tumour subtype in 73% of bronchial biopsies[41] and 65% of small biopsy and cytology cases[27] classified as NSCLC NOS based on morphology alone, although a proportion of cases remained unclassified after taking this approach. The combination of TTF-1 and a mucin stain for predicting adenocarcinoma has moderate sensitivity (69%) and excellent specificity (97%).[41] Others have shown immunohistochemistry including TTF-1 and cytokeratin 7 can help subtype undifferentiated carcinomas in bronchial biopsies.[48] Importantly, no single marker or panel of markers is completely sensitive or specific for subclassification of NSCLC and results must be interpreted in the context of morphological and clinical features in each case.

Until further data are available regarding reliability and classification of NSCLC with use of immunohistochemical stains, it is recommended that undifferentiated NSCLCs in small biopsies are still reported as NSCLC with the qualification of 'favour SCC/adenocarcinoma' or 'probably SCC/adenocarcinoma' if the immunohistochemistry profile suggests a particular line of differentiation.

Whenever feasible, it is important that sufficient tissue is obtained to enable appropriate histological, histochemical and immunohistochemical assessment to be undertaken. Consideration is also required to preserve some tissue for possible genetic testing, so special stains need to be selected judiciously.

New approaches to the classification of adenocarcinomas

Using the WHO 2004 classification, adenocarcinomas are divided into acinar, papillary, solid, BAC, or mixed subtypes, along with several rarer variants.[3] However, the value of this approach has been questioned given that over 80% of cases are of mixed type[49] with any possible combination of the four patterns being observed.[50] Gene expression profiling studies suggest morphology, differentiation and gene expression are linked[6,49] and refinement of the current morphology-based classification with greater attention to clinically relevant features may be of benefit. A reproducible, well defined and internationally accepted morphological classification is an important foundation to enable correlation with molecular data and to enable meaningful comparison of results between different studies. Motoi *et al.*[49] have shown that determination of the major histological component of adenocarcinoma correlates with gene profile clusters and EGFR mutation status. They suggest further classifying mixed subtype adenocarcinomas according to major histological subtype and reporting the percentages of all histological subtypes. In a recent review of pulmonary adenocarcinomas, Kerr[43] supports the approach of providing quantitative information regarding adenocarcinoma subtypes in reports

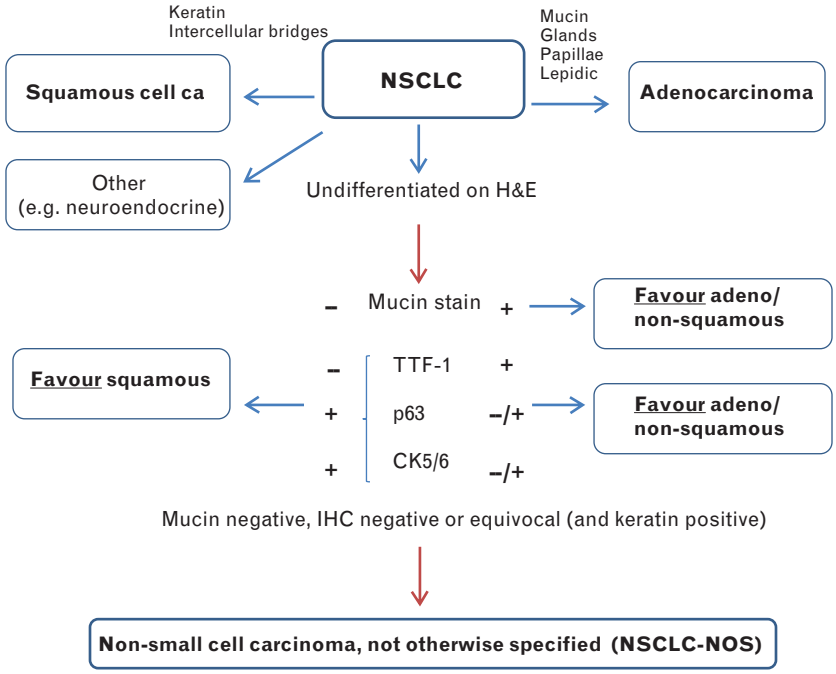

Fig. 3 Algorithm for approach to subtyping NSCLC in small biopsy specimens.

of resected tumours but acknowledges that the evidence of clinical significance for this new approach is only limited and still evolving. Tumours with a predominant BAC pattern of growth tend to have a better prognosis, while predominantly solid tumours tend to have the least favourable prognosis.[50,51] Some studies have found papillary predominant tumours,[52,53] or those with a micropapillary pattern in at least 5% of the tumour,[54] are also more aggressive. It seems reasonable to at least report the predominant adenocarcinoma pattern that is present in a mixed type adenocarcinoma.

New IASLC/ATS/ERS international multidisciplinary classification of lung cancer

A new International Multidisciplinary Lung Adenocarcinoma Classification is currently being developed under sponsorship of the International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) and is due to be published soon in the *Journal of Thoracic Oncology*.[2] This classification will address a number of clinically relevant issues regarding adenocarcinomas and is intended to be the basis for the next WHO classification.[55] Guidelines for diagnosis of histological NSCLC subtypes in small biopsies and cytology samples will be provided, including an algorithm for appropriate use of mucin and immunohistochemical stains. It is anticipated that other changes will include reclassification of BACs as adenocarcinoma *in situ*, addition of minimally invasive adenocarcinoma, mixed subtype adenocarcinomas requiring semiquantitative assessment of patterns (including micropapillary pattern) with determination of the dominant pattern and distinction of mucinous adenocarcinomas.[55]

EGFR MUTATIONS IN NSCLC

EGFR tyrosine kinase inhibitors

The EGFR TKIs erlotinib (Tarceva) and gefitinib (Iressa) are small molecules which selectively inhibit phosphorylation of the intracellular tyrosine kinase domain of EGFR.[56] This is an important new class of drugs capable of achieving a dramatic response in selected patients. In 2004, somatic mutations in the EGFR gene were identified in a subset of adenocarcinomas and were strongly associated with patient response to the EGFR TKIs erlotinib[56] and gefitinib.[57,58] Pooled data from these three studies showed a response rate of 81% in patients harbouring EGFR tyrosine kinase domain mutations[56] but <10% of patients with wild-type EGFR responded (reviewed by Riely *et al.*[59]). However, molecular studies were only possible on a minority of tumours in some studies due to the retrospective nature of the analyses.

Subsequently, numerous clinical trials have confirmed the strong association between EGFR tyrosine kinase domain mutations and response to EGFR TKI treatment in both Western and Asian populations,[60–62] and prospective trials have demonstrated that EGFR mutations predict a response rate of 65–90% (reviewed by Riely *et al.*[59] and van Zandwijk *et al.*[63]). By contrast, response rates to TKIs in wild-type EGFR tumours are very low, particularly when extensive mutation testing has been undertaken such as in the IRESSA Pan-Asia Study (IPASS study) where the response rate was only 1%.[11] There is also evidence that different mutations may have differential predictive value for sensitivity and resistance to TKIs but larger prospective studies are required to test this possibility.[64]

The IPASS study showed that selection of patients based on molecular characteristics of their tumour with EGFR tyrosine kinase domain mutation was superior to selection based on clinical criteria in predicting response to EGFR TKI treatment.[11] In this study, gefitinib was superior to combination chemotherapy as first-line treatment in EGFR mutant adenocarcinomas and the outcome was superior to clinical selection of patients based on Asian ethnicity, adenocarcinoma histology or light smoking history.[11] By contrast, patients with wild-type EGFR had a better outcome when treated with combination chemotherapy, highlighting the importance of accurate patient selection. Two other recent studies have also demonstrated superiority of EGFR TKIs to chemotherapy in patients with

EGFR mutations.[65,66] The results of these trials have helped establish EGFR mutation status as the most sensitive predictive marker for selecting patients most suitable for treatment with TKIs.

In Australia, the clinical use of EGFR TKIs has until now been confined to the second line setting, after the failure of chemotherapy. However, recent studies[11,65,66] have demonstrated the efficacy of these agents as first line therapy in patients whose tumours harbour an activating EGFR mutation. Therefore, it seems likely that the use of these agents in the first line setting will increase in the future, provided the presence of mutations can be demonstrated.

EGFR mutations in NSCLC

The epidermal growth factor receptor is a transmembrane tyrosine kinase receptor which structurally consists of two globular structures called the N lobe and C lobe, encompassing an activation (A) loop, and a nucleotide triphosphate (e.g., ATP) binding domain, so called (P) loop.[67] When the receptor is inactive it has a low basal activity. In response to ligand binding (and there are at least 12 different ligands, of which most are growth factors) EGFR forms homodimers or heterodimers with other members of the EGFR family. This leads to phosphorylation of key tyrosine residues especially within the activation 'A' loop and results in rapid phosphorylation at other docking sites in the intracellular domain. The net effect is activation of the signalling cascade of key pathways involved in signalling and growth: Phosphoinositol 3 Kinase/AKT (PI3K/AKT) pathway, mitogen activated protein kinase (MAPK) pathway, phospholipase C (PLC), signal transducer and activator of transcription (STAT) pathway and SRC/FAK pathways. There is extensive cross talk between these pathways and when constitutively activated (e.g., via mutation or oncogene amplification) they promote cell growth and motility (reviewed by Laurent-Puig *et al.*[68]).

Aberrations in EGFR are common in a range of human cancers, however, the site of mutations appears to vary with the type of malignancy. One large study in 617 NSCLCs[69] from the USA, Japan, Taiwan and Australia performed initial sequencing of exons 18–24 which revealed all mutations were confined to exons 18–21, which encode the tyrosine kinase domain. They found no mutations in this region detected in neuroendocrine, colorectal, pancreatic, prostate, bladder, breast or gallbladder carcinomas. They also sequenced the KRAS gene in exons 12 and 13 and found 8% of NSCLC (mostly adenocarcinomas) had mutations, but that no tumours had both EGFR and KRAS mutations.

Within the NSCLC samples they identified 184 EGFR tyrosine kinase domain mutations among 130 tumours with three main categories of mutation observed: (1) in-frame deletions of a highly conserved region of exon 19 (within codons 747–749); (2) single nucleotide substitutions within exons 18, 20 and 21, of which a substitution of leucine for arginine (L858R) in exon 21 was the most common; and (3) in-frame duplication or insertions exon 20 (usually involving codons 770–776).

Current studies on mutations within EGFR in NSCLC include well over 3000 patients (reviewed by Riely *et al.*[59]) and data show that 90% occur within hotspots in exons 18–21 that code for the tyrosine kinase domain, confirming all clinically relevant mutations appear confined to the first four exons of the tyrosine kinase domain encompassing the N lobe (exons 18–20) and C lobe (exons 21–24) and the A and P loops. In-frame deletions in exon 19 account for 45–50% of all mutations, and L858R (which lies in activation loop A of EGFR receptor) for 35–45%.[69] These activating mutations lead to ligand-independent receptor activation, most likely by stabilising interactions between ATP and its competitive inhibitors[57,58,70] The remaining mutants are insertions in exon 20 (5%) and rare substitutions spanning exons 18–21 (e.g., Glycine 719 with serine, cysteine or alanine).

Cell culture studies and clinical studies both indicate that not all mutations are activating. Gruelich and colleagues[71] studied the differing affinity of the mutant receptor variants to TKIs. They reported that the common L858R mutant EGFR receptor binds gefitinib with a 20-fold greater affinity than the wild-type receptor, much more than the G719S mutation. In contrast, exon 20 insertion mutations were found to be highly resistant to EGFR inhibition and the authors suggested that new inhibitors need to be developed for this class of mutations. Kobayashi and colleagues[72] report the case of a NSCLC patient whose original diagnostic bronchial biopsy contained a deletion in exon 19 (delL747-S752) and who had previously shown a good response to gefitinib, but developed a relapse after 2 years of complete remission. The recurrent tumour was found to harbour a new mutation in exon 20, T790M (threonine substituted by methionine) in the catalytic cleft of the EGFR tyrosine kinase domain, as well as the original exon 19 deletion. Structural modelling suggested this mutation would interfere with the binding of gefitinib to the EGFR receptor in the ATP-binding pocket, and this resistance was confirmed in cell culture studies infected with mutant receptor and treated with a number of EGFR inhibitors. Shih and colleagues[73] described another case of a 55-year-old woman who harboured both the L858R EGFR mutation associated with sensitivity to gefitinib as well as the 'resistant' mutation T790M at diagnosis, suggesting this is not always an acquired drug resistance mutation, as this patient demonstrated primary resistance to gefitinib. Intriguingly, there is also a suggestion that particular mutations may be associated with site specific recurrence, for example a drug resistant mutation D761Y was reported in association with CNS recurrence,[74] while all T790M cases so far have presented with visceral metastases.

Secondary resistance to EGFR inhibitors may arise not only through specific EGFR mutations such as T790M, but also through amplification or overexpression of the MET gene which results in increased activity of the PI3K/AKT pathway.[75] Bean and colleagues[75] used array comparative genomic hybridisation (aCGH) to compare the genomic profiles of untreated patients with those from patients treated with acquired resistance. Increased copy number for MET gene (amplification) was noted in 21% of the resistant group, compared to only 3% of untreated tumours. Interestingly four of nine MET amplified patients also had the EGFR mutation T790M, associated with resistance. *In vitro* studies indicate that cell lines harbouring MET amplification as well as the EGFR T790M, mutation are resistant to erlotinib but sensitive to a multikinase inhibitor that inhibits MET. Intriguingly, a recent study by Turke *et al.*[76] has identified that many EGFR mutant tumours harbour a small clone of MET amplified cells which, when subject to selection pressure by EGFR inhibition, expand and confer acquired resistance by the tumour to treatment. These data suggest that regimens designed to prevent emergence of resistance clones (e.g., treatment with both an EGFR inhibitor and one targeting MET) would be a rational approach to improve treatment

responses in this subgroup of patients, although increased toxicity may be a barrier to this approach.

Clinical features associated with EGFR mutation in NSCLC

From early on it was apparent that EGFR mutations mostly occurred in a relatively distinct subgroup of patients. The incidence of EGFR mutations in NSCLC varies according to ethnicity, being identified in approximately 10–15% of unselected Western patients[49,56,77–82] and 25–50% of unselected Asian patients with lung adenocarcinoma.[4,58,62,81,83,84] Interestingly, while EGFR mutations are associated with East Asian ethnicity[58,69,81,85] no association has been found with geography suggesting EGFR mutations are related to a genetic predisposition rather than environmental factors.[81]

EGFR mutations are significantly more frequent in females[61,62,69,77,79–84] and are mostly found in tumours from non-smokers. Lung tumours with EGFR mutation have been consistently associated with patients who have never smoked compared to former or current smokers in both Western and Asian populations.[4,56,61,62,69,77,79–88] The likelihood of EGFR mutation detection is inversely correlated to number of pack years smoked[62,87] and a history of never smoking or low smoking is the strongest predictor of EGFR mutation.[89] EGFR mutation status is generally not associated with tumour stage.[69,81,82]

Clinicopathological features associated with EGFR mutation can be used to help select which cases are most appropriate for testing, thereby increasing the rate of mutation detection. In one study, patients were selected for mutation analysis on the basis of at least one clinical characteristic associated with EGFR mutation (female sex, adenocarcinoma histology of any subtype, never-smoking history, or east Asian ethnicity) and 35% were found to have EGFR mutations.[90] Among adenocarcinomas from 'never smokers', the frequency of EGFR mutations was 47% in a study from a Western population.[56]

There is evidence that EGFR mutation status provides both prognostic and predictive information. EGFR mutations are associated with a superior survival in advanced NSCLC, irrespective of treatment,[59,78,85] and in patients treated with surgery but not TKIs.[91] Not all studies have found a survival difference, even when accounting for tumour stage,[69,81] but the sample size was relatively small in one of these studies.[81]

Histological features associated with EGFR mutation in NSCLC

EGFR mutations in lung cancer are very strongly associated with adenocarcinoma histology[4,61,62,69,80,81,84,85] and have only been reported in up to 3% of other NSCLC types.[69,82] Others have found no EGFR mutations in other NSCLC subtypes[61,81] including a study with large numbers of SCCs (454) and large cell carcinomas (31).[79] EGFR mutations are generally not found in fetal-type adenocarcinomas, neuroendocrine lung tumours or salivary gland-type lung carcinomas (mucoepidermoid and adenoid cystic carcinomas).[69,92] EGFR mutations (deletion exon 19) have been reported in both the adenocarcinoma and small cell carcinoma components of a mixed adenocarcinoma-small cell carcinoma of the lung.[93]

The only histological feature apart from adenocarcinoma subtype that has shown a consistent association with EGFR mutation is an absence of mucinous differentiation. No EGFR mutations have been found in mucinous invasive adenocarcinomas or mucinous BAC type tumours[4,92,94] but these tumours are strongly associated with KRAS mutations.[94] In one of these studies, cases underwent central pathology review and mucinous differentiation was defined as a 'morphology in which the individual tumour cells are tall and well differentiated, have basally located nuclei, and produce mucin'.[94] In keeping with these findings, EGFR mutations predominantly occur in tumours exhibiting differentiation towards type II pneumocyte or other elements of terminal airways consistent with so-called 'terminal reserve unit' features.[88] However, no association has been found between TTF-1 expression and EGFR mutations.[4]

There has also been evidence of possible associations between EGFR mutation and tumours with BAC or papillary features. Although a number of early studies emphasised presence of BAC type features in EGFR mutant adenocarcinomas, the results from many subsequent trials have been conflicting and no clear association has been identified.[49] In some studies, EGFR mutations were associated with pure non-mucinous BACs[79] or any degree of BAC pattern in a mixed-type adenocarcinoma.[4,56,57,77,83,91,95] In one study, adenocarcinoma with any BAC features was an independent predictor of response to gefitinib therapy.[96] By contrast, others have found no correlation between EGFR status and any BAC features[49,69,94] including BAC dominant pattern[49,81] or pure BAC.[69,81,88] EGFR mutations have been demonstrated in microdissected tumour from both lepidic BAC-type areas and invasive areas of the same tumour.[77] In one study, EGFR mutations were negatively correlated with solid type adenocarcinoma.[86] These discrepant results may be explained in part by differing criteria used to define histological subtypes of tumour as well as small numbers of cases in some studies, particularly of pure BAC. In addition, central pathology review was not undertaken in most of these studies.

Some studies have shown that adenocarcinomas of papillary subtype or with the major component being a papillary (or micropapillary) pattern are significantly more likely to harbour EGFR mutations.[49,86] Interestingly, a significant association has been found between patients with a predominant papillary pattern of adenocarcinoma and response to gefitinib treatment but EGFR mutation status was not reported in this study.[97] Tumours with a dominant micropapillary pattern, typically an aggressive variant of adenocarcinoma,[54] have also been associated with EGFR mutations.[49,95,98]

EGFR mutation testing

Testing for EGFR status in lung adenocarcinomas is an important clinical tool for predicting response to EGFR TKIs. EGFR mutation analysis is the currently accepted method for identifying patient response to EGFR TKIs[59,89] and direct DNA sequencing is the technique used in most studies. Following microdissection of the tumour, DNA is extracted and the exons of interest are amplified by PCR prior to DNA sequencing.[89] While some laboratories test only exons 19 and 21, others test exons 18–21.[89] The sensitivity of direct sequencing in part depends on the amount of viable tumour tissue and the proportion of tumour cells present in the sample. EGFR mutations may not be detected in cases with fewer than 25% tumour cells.[89] Other potential problems include lack of intact genomic DNA in tumour samples and formalin fixation causing artefacts in sequencing.[87] Rare mutations of the EGFR gene could be missed with use of mutation specific primers.[99] Standardised assay methodology and interpretation is essential for detecting

EGFR activating mutations and predicting response to EGFR TKIs. Cost-effectiveness, availability and turnaround time also need to be considered in integrating the testing into routine clinical practice. A variety of more sensitive mutation detection assays may be used[59] such as the Amplification Refractory Mutation System,[100] single-strand conformational polymorphism and denaturing high-performance liquid chromatography, but these require direct sequencing to confirm results.[89]

Virtually any routinely available pathological specimen can be used for EGFR mutation analysis including formalin fixed, paraffin embedded tissue from surgical resections, small tissue biopsies or cytological cell block preparations.[27,101] A considerable proportion of advanced stage NSCLC is diagnosed solely by cytology and several studies have demonstrated that cytological specimens including smears obtained from endoscopic ultrasound guided fine needle aspirations are adequate for EGFR mutation testing.[101–103] Successful mutation testing can be undertaken in at least 75% of cases[101–103] with insufficient material or low percentage of tumour cells in the sample accounting for most failures.[103] Others have suggested a higher false negative result may be found in fluid cytology specimens and it is currently recommended that tissue biopsy is preferable to cytological samples until reliability of cytology specimens is more fully established.[99,100] Although fresh unfixed tissue is superior to fixed tissue for PCR-based testing, with lower failure rates for amplification,[87,89] this is not feasible if the testing laboratory is not on site or nearby; in practice most samples are fixed. It is recommended that 10% buffered formalin be used for tissue fixation for optimal molecular preservation, with avoidance of Bouin or any fixative containing heavy metal.[89,100]

Other biomarkers of predictive value

Assessment of EGFR gene copy number by *in situ* hybridisation techniques (FISH or CISH), or protein expression by immunohistochemistry have also been proposed as candidate biomarkers for prediction of TKI response. In addition, the presence of KRAS mutations is associated with primary resistance to TKI treatment.[78] Although there has been considerable debate in the literature, gene copy number analysis appears to be inferior to mutation analysis. Comparative analysis of the biomarkers EGFR mutation, EGFR FISH and immunohistochemistry based on the IPASS results confirmed EGFR mutation analysis as the superior predictor.[11,99,104] EGFR gene copy number assessment is not currently used in routine clinical practice and currently seems unlikely to be of use as a predictive test on its own;[89] however, the relative significance of different EGFR alterations remains controversial. Studies comparing the predictive status of EGFR mutations and EGFR gene copy number have produced varying results that are difficult to interpret, partly due to technical and interpretative differences between the techniques in different studies.[89] While increased EGFR gene copy number correlates with mutations in EGFR and has been associated with response to TKIs, patients with increased EGFR copy number but no mutation have only a low response rate.[60,64]

Immunohistochemical assays have the advantage of being automated, cheaper and routinely available but EGFR immunohistochemical studies have produced contradictory results that have generally not correlated well with EGFR mutation status[60,77,81] or response to EGFR-TKI treatment[60] making this technique currently unsuitable for clinical practice.[89] Results of immunohistochemical assays vary greatly depending on antibody type, methodology, scoring methods and thresholds for determining positivity. The lack of an accepted standardised protocol has made comparison of different trials difficult. Mutation specific antibodies have recently been developed against the most common EGFR mutations in exon 19 (deletions E746-A750) and 21 (L858R point mutation) and have shown promising results with a reported sensitivity of at least 75% and specificity of >95%,[105,106] while others have found higher specificity but sensitivity of only 47%.[107] These results suggest mutation specific immunohistochemistry could be useful for mutation screening followed by mutation analysis in negative cases that would otherwise be suitable for TKI treatment.

Although EGFR mutation testing is currently the recommended technique for predicting sensitivity to TKI treatment, future clinical trials may help determine which predictive tests are of sufficient value for integration into routine clinical practice. It is possible that mutation specific immunohistochemistry, EGFR gene copy assessment or KRAS mutation testing could be of use in pre-screening algorithms in the future.[100]

EGFR genetic heterogeneity in tumours

EGFR mutation status is not necessarily homogenous within an individual tumour and heterogeneity has been demonstrated in morphologically different areas within mixed-type adenocarcinomas.[108] Others have found that a proportion of NSCLC cases that appear to lack EGFR mutations by ordinary PCR techniques actually harbour varying numbers of mutant and wild-type cells using more sensitive PCR techniques.[109] This could potentially explain the response of a small number of apparently wild-type EGFR tumours to TKI therapy. Importantly, cases with EGFR heterogeneity have significantly shorter overall survival and time to disease progression following gefitinib treatment compared to homogenous EGFR mutant cases.[109] An assessment of EGFR mutations using microdissection based cell cluster mutation analysis before and after TKI treatment found that in three of six tumours there was a mixture of mutant and wild-type EGFR tumour cells and TKI treatment led to selection of wild-type EGFR cells.[93]

EGFR mutation status can also differ between primary and secondary tumours with reported discordance rates of 12% between primary NSCLC and lymph node metastases[110] and 28% between primary tumours and non-nodal metastases.[111] Mutations may be found in primary tumours but not metastases or vice versa, and are found in cases without prior exposure to TKIs so cannot be completely explained by clonal selection.[111] These results may explain why some apparently wild-type EGFR tumours respond to TKI treatment. While such inconsistencies could have implications on selecting appropriate tumour sites for biopsy, until this is better understood, it is currently recommended that tissue biopsies are obtained from the most readily accessible site.[100]

Recommendations for EGFR mutation testing in NSCLC

A recent International Association for the Study of Lung Cancer workshop produced consensus recommendations for EGFR mutation testing in NSCLC.[100] Close multidisciplinary collaboration between pathologists, oncologists, molecular biologists and radiologists is required for implementation of EGFR mutation testing in routine clinical practice.[100] Selection

of patients for EGFR mutation testing is determined by the treating oncologist and would probably be considered in patients with adenocarcinoma (excluding mucinous BAC), cases of lung cancer with unclear histological subtype and NSCLC patients who are 'never smokers', regardless of tumour histology. It has been suggested that screening for EGFR mutations in NSCLC is appropriate in women who have never smoked and have tumours with a non-squamous histology[80] and non-mucinous features.[94] Although it is acknowledged that there may be discrepancy between EGFR status in primary and metastatic tumours, until this is better understood, it is currently recommended that tissue biopsies are obtained from the most readily accessible site.[100] Biopsy is preferable to cytology samples until reliability of cytology specimens is more fully established.[100] The ratio of tumour to normal cells within the sample is important and it is recommended that specimens contain at least 50% tumour cells for DNA sequencing, but a lower proportion is adequate if more sensitive techniques are being used.[100] The minimum number of malignant cells required for mutation assessment is not well established but larger samples are preferable, ideally with at least 200–400 malignant cells (21G fine needle aspiration ~100 cells, bronchial biopsy ~300, core biopsy ~500).[100] EGFR mutation analysis reports should include details of the biopsy sample as well as methodology used, exons tested, presence or absence of specific mutations and interpretation of the results, particularly if any mutations detected are associated with TKI sensitivity or resistance.[100]

Currently in Australia, EGFR mutation testing is not routinely undertaken on all NSCLCs or adenocarcinomas. Selection of appropriate cases for testing should be undertaken with consultation between oncologists and pathologists. The need for this close consultation provides a powerful rationale for pathologists to be considered part of the multidisciplinary team that manages lung cancer, and for their attendance at multidisciplinary team meetings. As most NSCLCs are diagnosed at advanced stage, usually the only tissue samples obtained are small biopsies or cytology specimens, so it is important that consideration is given to obtaining sufficient material for molecular testing as well as for diagnostic purposes. It remains to be determined how best to integrate new tests with traditional diagnostic techniques and ensure appropriate selection and timing of different molecular assays.

ALK REARRANGEMENTS IN NSCLC

A newly defined uncommon molecular subtype of NSCLC characterised by rearrangements of the ALK gene has generated considerable interest due to the development of targeted ALK inhibitors. Genomic activation of ALK is a key feature of anaplastic large cell lymphomas and is also found in inflammatory myofibroblastic tumours and a small proportion of neuroblastomas.[112,113]

Genetic features of ALK rearrangements in NSCLC

The novel fusion gene EML4-ALK identified in NSCLC results from a small inversion in chromosome 2p leading to constitutive expression of a fusion protein with ALK kinase activity.[114] Subsequent aberrant activation of downstream growth stimulating pathways has an oncogenic effect that has been demonstrated *in vitro* and in mouse models, with tumours demonstrating sensitivity to ALK TKIs.[114–116] Variations in the break and fusion points of EML4 result in various isoforms of the fusion gene but the complete intracellular portion of ALK including the kinase domain is preserved in all cases,[12] and the significance of these different isoforms, if any, is unknown.

Methods of detecting ALK rearrangements

A variety of methods can be used to detect ALK gene rearrangements in NSCLC including reverse-transcriptase polymerase chain reaction (RT-PCR) of complementary DNA (cDNA), FISH and immunohistochemistry.[117] While immunohistochemical detection of the ALK protein is useful in anaplastic large cell lymphoma, it is not as sensitive in NSCLC as the protein is only expressed at low levels in ALK-rearranged tumours[118] and routine immunohistochemistry for ALK has given variable results.[119–121] Techniques to amplify the signal or the use of novel antibodies have shown some success, suggesting immunohistochemistry could potentially be used as a screening device for ALK rearrangements in NSCLC.[118,122–124] FISH can be used to detect the translocation but can be difficult to interpret due to the small size of the inversion.[122] The optimal method for identifying EML4-ALK fusion NSCLC is yet to be determined.

Clinicopathological features of NSCLC with ALK rearrangements

A review of reported incidence of ALK rearrangements in NSCLC found an overall incidence of 3.8% in a total of 2835 tested cancers.[117] In largely unselected Western populations the incidence is 3.4% and in Asian populations 4.2%,[117] but at present it is unclear whether there are any significant racial or geographical differences in mutation frequency. The similar frequency of ALK rearrangements in different populations suggests ethnic differences may not be as important as for EGFR. EML4-ALK fusion appears to be mutually exclusive of EGFR and KRAS mutations[117,119,120,122,125–127] although a single case of coexistent ALK rearrangement and EGFR mutation has been reported in NSCLC.[115]

Associations with clinical and pathological characteristics are not well established as study sizes have been relatively small but some of the features appear to overlap with those found in EGFR mutant NSCLC. Rearrangements of ALK are generally associated with never smokers or light smokers[115,122,123,125–127] and in some studies there is an association with younger patient age[122,123,125,127] and male gender.[125] No survival differences have been identified based on EML-ALK fusion;[120,125] however, the small numbers of cases with ALK translocations has made statistical assessment difficult in most studies.

EML4-ALK is found almost exclusively in adenocarcinomas and no cases have been identified in pure SCC;[115,117,120,126,127] however, ALK translocations have been reported in adenosquamous carcinomas including a low grade mucoepidermoid carcinoma.[116,125,127] Some studies have found associations with predominant acinar patterns of adenocarcinoma,[120,126] papillary[126] or solid patterns with signet ring cells[122,125] but sample sizes in all studies are relatively small and confirmatory studies are needed. In one study EML4-ALK fusion was associated with poorly differentiated tumours.[126]

Selection of patients based on clinical criteria such as smoking status can considerably increase the likelihood of identifying EML4-ALK and EGFR mutations. In one study of a clinically enriched patient population, EML4-ALK and EGFR mutations were found in 22% and 32% of patients,

respectively. Among EGFR wild-type tumours, 33% had ALK rearrangements[125] suggesting the yield for ALK testing may be greater in appropriately selected cases.

ALK inhibitors in the treatment of NSCLC

ALK kinase inhibitors have demonstrated marked activity against most NSCLC cell lines harbouring ALK translocation[112,114,115] and in murine tumours with ALK.[116] ALK kinase inhibitors are not presently available as clinically approved treatments but there are several currently being evaluated in a preclinical or early clinical setting including a dual MET/ALK inhibitor.[12] The most advanced of these agents is crizotinib, which is currently in phase III studies.

ALK rearrangements are a potential therapeutic target and although only present in a small proportion of NSCLC cases, this could result in clinical benefit for considerable numbers of patients given that NSCLC is such a common disease. The exact clinical implications and most appropriate means of detecting EML4-ALK in NSCLC have yet to be clarified but it is hoped that it may be a useful molecular target in selected patients and results of clinical trials are awaited with interest.

Role of pathologists in NSCLC management

Although NSCLC has previously been treated as a single entity, there is considerable variation in clinical, histological and genetic features and recognition of these differences can improve patient management and further our understanding of NSCLC. Recent clinical trials have demonstrated differing efficacy and toxicity of particular treatment regimes based on histological subtype of NSCLC, placing an obligation on pathologists to accurately distinguish NSCLC subtypes on small biopsy samples where possible. Adenocarcinomas can be categorised into different subsets based on distinct pathogenic genomic changes, some of which determine sensitivity to targeted therapeutic agents, and pathologists are being increasingly required to provide more detailed information to assist in selection of appropriate patients for genetic testing. Genotype guided treatment decisions are now important and impact on therapeutic choice and in the future possibly management of drug resistance. Pathologists now provide not only diagnostic information but also prognostic and predictive information and accurate distinction between adenocarcinoma and SCC is critical in this role. While morphological criteria remain the most important feature for pathologists, use of histochemical stains and immunohistochemistry are recommended where appropriate.

The role of pathologists and pathology laboratories in NSCLC management now includes:

1. Diagnosis of malignancy.
2. Accurate histological subtyping, where possible.
3. Selection of tissue for molecular testing (following request by the treating oncologist).
4. Genetic testing, e.g., EGFR or ALK mutation assessment.

It remains to be determined how best to integrate new tests with traditional diagnostic techniques as well as appropriate selection and timing of different molecular tests. A multidisciplinary approach with close communication between pathologists, oncologists, respiratory physicians and radiologists is required for ensuring sufficient biopsy material is obtained and maximal pathological information is provided to assist in optimal patient management.

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Address for correspondence: Associate Professor W. Cooper, Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Missenden Rd, Camperdown, NSW 2050, Australia. E-mail: wendy.cooper@sswahs.nsw.gov.au

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