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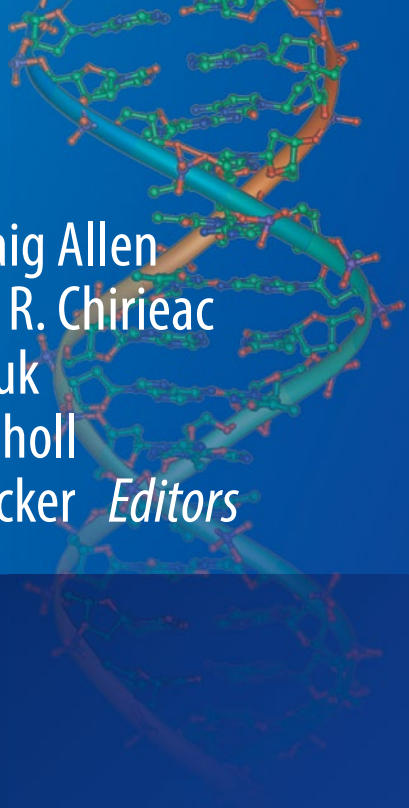

Philip T. Cagle · Timothy Craig Allen
Mary Beth Beasley · Lucian R. Chirieac
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Keith M. Kerr · Lynette M. Sholl
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Precision Molecular Pathology of Lung Cancer

Second Edition

Springer

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Philip T. Cagle
Houston, TX, USA

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ISSN 1935-987X ISSN 1935-9888 (electronic)
Molecular Pathology Library
ISBN 978-3-319-62940-7 ISBN 978-3-319-62941-4 (eBook)
https://doi.org/10.1007/978-3-319-62941-4

Library of Congress Control Number: 2017960212

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Part I
Background

Chapter 1
Precision Medicine in Lung Cancer

Keith M. Kerr and Gavin M. Laing

This textbook discusses in considerable detail the molecular landscape of lung cancer and how the molecular biology of the tumour is involved in the evolution, growth and development of the disease. Huge advances in our knowledge have been made in recent years, thanks in part to technology allowing whole exome and even whole genome sequencing. Apart from elucidating the molecular basis of this most fatal of malignant diseases, the molecular features of lung cancer can also be exploited therapeutically. Pathologists have known for a very long time about how different individual tumours can be from each other; to a large extent, we now understand that this morphological variation is a reflection of molecular heterogeneity. The development of so-called molecularly targeted drugs, and a realization that these drugs do not work for every patient, rapidly led to the need to select patients, often based on their molecular characteristics, to ensure a higher chance of therapy response. This idea of precision or personalized medicine is of course, not new. The concept of selective toxicity was pioneered decades ago [1] and has been a familiar practice in medicine, treating infections with antibiotics based upon sensitivity testing. In oncology, one of the first tumour types to have a precision medicine approach, selecting patients for therapy based upon pathological characteristics, was breast cancer. Oestrogen and progestogen receptor testing for tamoxifen therapy and, later, HER2 testing for trastuzumab therapy are well established in clinical practice. Precision medicine and personalized therapy in lung cancer is a more recent development, but this has developed into an extremely diverse and complex branch of oncology, bringing considerable benefits for groups of patients, multiple choices for oncologists and considerable demands on pathologists.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_1

Precision Medicine: Impact on Lung Cancer Therapy

In the 1980s cytotoxic agents were used in some patients with small cell lung cancer (SCLC). Systemic chemotherapy, different from that used in small cell carcinoma, was introduced into routine practice as a palliative measure in advanced non-small cell lung cancer (NSCLC) in the early 1990s. Thus, one could argue that the earliest selection of patients with lung cancer was based upon this paradigm of treating advanced small cell lung cancer in one way, and all other lung cancer by other means. Prior to the introduction of such systemic therapy, lung cancer treatment was based upon surgery and radiotherapy for localized disease and best supportive case (basically no active, cancer-directed treatment) for advanced disease. This rather crude discrimination actually spawned the concept of 'non-small cell carcinoma' as a so-called entity, a therapeutic grouping of convenience, which lumped together a group of pathologically and biologically very different diseases because they were all treated in the same way. Multiple cytotoxic chemotherapeutic regimens were developed which improved overall survival in advanced NSCLC to perhaps 6–8 months from diagnosis, but by around 2005, it was felt that a plateau had been reached; various cytotoxic therapy approaches all delivered more or less the same, limited efficacy [2].

Progress in treatment of SCLC has been very limited. Platinum-etoposide-based regimens have been the mainstay of treating this disease for many years. There have been very few signs of success in targeted therapy, and there are none in routine clinical use. The genetics of SCLC are described elsewhere in this book. Genomic studies of SCLC have failed to identify any promising drug targets [3]. One interesting recent development, however, is the exploitation of DLL3 expression on the surface of SCLC cells. An antibody against DLL3 is used as a means to selectively target SCLC cells expressing this marker and deliver an antibody-conjugated cytotoxic agent in patients who express high levels of DLL3 on their tumour, as assessed by a specific anti-DLL3 immunohistochemistry (IHC) assay [4].

Between 2004 and 2008, two therapeutic developments began a significant change towards precision medicine in advanced NSCLC. The anti-angiogenic agent bevacizumab demonstrated an increased risk of fatal haemorrhage in patients with squamous cell carcinoma, but not in adenocarcinoma where survival benefits were demonstrated in combination with platinum-doublet chemotherapy [5, 6]. The drug was approved only for patients with 'non-squamous' NSCLC. A pemetrexed-platinum doublet showed superior outcomes when compared to gemcitabine-platinum in adenocarcinoma and undifferentiated tumours; the drug label for pemetrexed required a diagnosis of adenocarcinoma or large cell carcinoma [7]. These events were the catalyst for the drive towards specific NSCLC subtyping in advanced disease small diagnostic samples and an attempt to eradicate the term NSCLC-NOS (not otherwise specified). This is discussed below and in Chap. 5.

Trials of inhibitors of the epidermal growth factor receptor (*EGFR*) tyrosine kinase (TKI) began to be reported around 2000, with mixed results, but with the observation that certain patients did spectacularly well on these therapies. These

patients tended to be younger, never-smoking females of East Asian ethnicity with advanced adenocarcinoma. It was discovered that these patients who responded particularly well to EGFR TKIs had tumours which bore mutations in the tyrosine kinase domain of the *EGFR* gene [8, 9] and there followed a series of successful trials demonstrating clear clinical benefit for EGFR TKIs in patients with a range of *EGFR* mutations in exons 18–21 [10–13]. This underpinned the need for *EGFR* mutation testing to select patients for EGFR TKI therapy, now a routine practice and standard of care.

The EGFR story highlighted the importance of identifying cancers driven by so-called addictive oncogenic changes [14]. Addictive oncogenes make excellent drug targets and provide biomarkers which are highly predictive of therapy response. The next to be discovered in NSCLC was a group of rearrangements involving the anaplastic lymphoma kinase (*ALK*) gene and a number of gene partners, leading to activation of the *ALK* gene tyrosine kinase [15]. The ALK TKI crizotinib rapidly proved its worth in treating patients with adenocarcinomas bearing *ALK* rearrangements [16–18]. There are now several other ALK TKIs at various stages in the trial regulatory process. Several other apparently addictive oncogenic changes have been discovered in lung adenocarcinomas which are variably associated with, but not exclusive to, the same patient demographic as for EGFR-mutated tumours. Essentially, this reflects an adenocarcinoma phenotype whose genesis is unknown; other than that tobacco carcinogens are not involved. ROS proto-oncogene 1 (*ROS1*) gene rearrangements are also associated with this adenocarcinoma phenotype, and crizotinib is now approved in many countries for the treatment of patients with such rearrangements [19–22]. RET proto-oncogene (*RET*) and neurotrophic receptor tyrosine kinase 1 (*NTRK1*) gene rearrangements and B-raf proto-oncogene (*BRAF V600E*) and erb-b2 receptor tyrosine kinase 2 (*HER2*) mutations account for small groups of adenocarcinoma patients with drugs undergoing trials [19–22]. *BRAF* inhibitors will probably be the first in this latter group to gain regulatory approval. MET proto-oncogene (*MET*) exon 14 skipping mutations are a promising target found in a number of NSCLC tumour types [23, 24].

The benefits to patients, of discovering therapeutically targetable molecular drivers in their tumours, and delivering the appropriate therapy, have been demonstrated [25]. The benefit is a real, treatment-related effect, rather than a prognostic effect related to the molecular alteration. The use of EGFR and ALK TKIs is now regarded as standard of care in those molecularly defined groups of patients, treatment for *ROS1* rearrangements is similarly regarded in many countries and as more drugs gain regulatory approval, so practice will change as newly introduced therapies are incorporated into treatment guidelines for advance stage NSCLC [26, 27]. Immunotherapy, specifically through the use of anti-PD1 and anti-PD-L1 immune checkpoint inhibitors, is rapidly becoming established in the treatment of advanced NSCLC, and with some of these drugs comes the need for biomarker-based patient selection. This matter is discussed in some detail in Chap. 20. All of these developments reflect the remarkable success or personalized, precision medicine for patients with lung cancer. Almost all of the recently approved treatments, which are largely responsible for overall survival for advanced NSCLC extending out to

beyond 12 months, are prescribed on the basis of a biomarker test. These advances have also transformed the diagnostic pathways for lung cancer, presenting exciting new opportunities and challenges in equal measure, for pathologists dealing with these cases.

Precision Medicine: Impact on Lung Cancer Pathology

Diagnostic Complexity

The development of lung cancer therapies specifically targeting pathologically and/or molecularly defined subsets of patients, as described above, has had an enormous impact on the diagnostic process required for cases of lung cancer, especially in the setting of advanced disease [28–31].

The diagnostic journey begins with the identification of carcinoma in the submitted sample. As discussed below, and in Chap. 5, most diagnostic samples from lung cancer patients provide only very limited amounts of tumour. Through dialogue with colleagues, discussion at the tumour board or multidisciplinary team (MDT) meeting and what should be regarded as mandatory clinical information provided with the samples sent for diagnosis, the pathologist should be aware of the likelihood of a diagnosis of primary lung cancer or any possibility of metastases to the lung. Separation of SCLC from other tumour types is followed by the subtyping of NSCLC cases as accurately as possible. IHC now plays a pivotal role in this process, as discussed in Chap. 5. It is imperative that IHC is carried out only when required. If the diagnosis of adenocarcinoma or squamous cell carcinoma can be made by morphology alone, usually so in 60–75% of cases in small sample diagnosis, IHC should not be carried out to confirm tumour subtype. IHC should only be used in those cases which the pathologist would morphologically classify as NSCLC-NOS. In a case of adenocarcinoma, clinical details should drive any IHC-based investigation of possible primary sites other than lung. There is evidence that pathologists overuse IHC in the small sample diagnosis of lung cancer and thus waste precious tissue, compromising the subsequent molecular testing in appropriate cases [32].

Current guidelines recommend that cases of possible, probable or definite adenocarcinoma should be submitted for molecular testing [33, 34], as these are the samples most likely to bear a targetable molecular alteration. Rare cases of squamous cell or small cell carcinoma in never or long-time ex-smokers should also be tested. As more molecular targets are defined, with approved drugs being made available, it may be justifiable to test all patients with NSCLC, using multiplex testing approaches such as next-generation sequencing (NGS) [35–37]. Currently, however, this broad approach is not financially justifiable, based on the limited number of drugs available in most health systems, and the very strong bias of current targets to an adenocarcinoma phenotype. In an academic setting, a more broad approach, such as testing for multiple targets to select

patients for clinical trials, is rapidly becoming common practice [36]; drug availability is the most important driver of testing practice, and this is highly variable from a global perspective.

Tissue Handling

Lung cancer patients mostly present with advanced, metastatic disease and are suitable for only palliative systemic therapy, if any treatment at all. A significant proportion of lung cancer patients are too unwell, either for investigation and tissue confirmation of their disease or systemic therapy. Practice varies, but 15–25% of patients with a clinical diagnosis of lung cancer are unfit for, or refuse, further investigation. If patients do have a tissue diagnosis of their tumour, this will usually be based upon a small tissue biopsy or a cytology sample taken from a site or sites deemed most easily accessible. As a consequence of limited accessibility due to disease location and patient comorbidities, lung cancer samples are almost universally small and contain relatively little tumour [38]. It is therefore essential that these samples are handled with great care, without waste and in ways that facilitate the possible extensive biomarker investigation that may be required once the complete histological diagnosis is achieved.

Tissue samples have to be fixed and processed before sections can be made for staining and examination. Although certain types of biomarker testing may be favoured by different fixation and processing methods, a sample can only be fixed and processed once, and that has to be suitable for all of the possible testing approaches that may be required. Thus, standard fixation using 10% neutral buffered formalin is recommended, and tissue should be fixed for between 6 and 72 h. Outside this window, DNA damage and protein epitope alterations may occur [39]. We have also learned that some IHC epitopes are not well preserved by alcohol fixation, and very short fixation times, which may help preserve DNA, can lead to poor IHC performance.

A conservative approach to the use of IHC in the initial diagnostic phase has already been emphasised. Biomarker testing in lung cancer is now pursued along two separate methodological lines. Some tissue from the formalin-fixed, paraffin-embedded tissue block is used for DNA and, perhaps, RNA extraction. Tissue sections are also required for morphology-based tests such as predictive IHC-based biomarker testing or biomarkers based upon in situ hybridization. For the latter, fluorescence methods (FISH) are more often used than bright field approaches such as chromogenic or silver-precipitant (CISH or SISH) methodology.

For samples where a possible lung cancer diagnosis is likely (various thoracic samples in a relevant clinical context), block cutting strategies can be employed to limit the number of times a block is (re)cut, as this wastes tissue on each occasion. Extra tissue sections taken up front, in anticipation of need, can be used as required for deeper sections, IHC, FISH, etc. It would be rare for laboratories to be able to

take sections for molecular analysis in this way, as those sections are normally made separately on a molecularly sterile instrument. These strategies certainly help maximize the use of very limited tissue resources [40].

Genes Versus Proteins

A central tenant in molecular biology is the transcription of DNA to produce mRNA message that is translated into protein. In cells, proteins are the active, effector molecules, encoded by genes. Proteins drive oncogenic events and are also the targets of drugs. Depending on the molecular abnormality being targeted by a drug, it may make more sense to use DNA, for example, for mutation testing, whilst some biomarker tests, for example, PD-L1, directly target the protein itself. Testing lung cancer samples for *ALK* gene rearrangements has been approached in many different ways [40]. The change in the DNA sequence signalling the rearrangement can be sought at the DNA level by FISH or next-generation sequencing approaches; unique mRNA transcripts can be looked for or elevated levels of ALK protein demonstrated by IHC. Each approach appears to predict for therapeutic response. It is likely that *ROS1* rearrangement testing will also develop in a similar way. Diagnostic practice is driven partly by evidence for the most efficacious approach, but also by perceptions about which is the easiest, quickest and cheapest method. Simple, low-cost, rapid testing is always attractive, but it may not necessarily provide the best answers, and pathologists need to be careful not to move their testing approaches too far away from what was validated in clinical drug trials; otherwise, patient selection may become inaccurate and inefficient.

Diagnostics and Quality Assurance

The rapid expansion in the number of possible biomarker tests required on a small lung cancer tissue sample poses the pathology community with some interesting challenges [28–31]. Some drugs are approved with a so-called companion diagnostic test. This is a specific test, carried out in a particular way, and is often the exact test used in clinical trials which provided the evidence of drug (and test) efficacy which underpins drug approval. An example would be the anti-PD-L1 IHC assay using the 22C3 clone marketed by Dako (Carpenteria, CA, USA). Prescription of pembrolizumab requires demonstration of PD-L1 expression in the tumour (see Chap. 20). Other commercially produced anti-PD-L1 IHC assays, based on clones 28–8 (Dako) or SP142 (Ventana, Tucson, AZ, USA), are marketed as complementary diagnostics for immunotherapy agents nivolumab and atezolizumab, respectively. Complementary diagnostics are not mandated for drug prescription but are regarded as an optional, though potentially informative, test [41]. This distinction is a relatively new concept in biomarker diagnostics. It remains to be seen how pathologists and oncologists navigate this matter.

Commercially manufactured kits for biomarker testing are generally high-quality, reliable products which have been manufactured using stringent quality controls. As mentioned above, they are frequently the tests that were used and validated in clinical drug trials. They are, however, generally relatively expensive and usually require specific equipment for their use. These latter factors often limit the adoption of commercial kit tests in some laboratories, where instead, pathologists prefer (or are obliged) to develop their own assays, so-called laboratory-developed tests (LDTs). Test cost is especially a factor when screening large numbers of patients for rare alterations [42]. Whilst these LDTs may be cheaper, they require rigorous validation. For some biomarker tests, it can be argued that the test methodology used is less critical, provided its characteristics are of adequate sensitivity and specificity for the biomarker assessed, in the clinical samples in use. How one demonstrates the presence of an EGFR mutation is less important provided test performance is adequate, and its use is regularly validated by external quality assurance. For IHC-based biomarker tests, the issue is less clear-cut. Subjective assessments, variable primary antibody performance and assay dependence on the detection system all introduce potential variation. This makes the use of LDTs, whose characteristics could vary markedly from a trial-validated 'gold standard', and for which, almost by definition, there are unlikely to be any clinical validating data, open to question. The College of American Pathologists has produced guidelines for LDT validation, but these are designed largely for diagnostic IHC tests, rather than predictive biomarker tests [43, 44].

The importance of biomarker tests in delivering precision medicine for lung cancer patients is clear. It is vital that the test performs in the required way, to guarantee the correct patient selection in order to ensure the predicted likelihood of therapy response. Following best laboratory practices and procedures, and an awareness of the pre-analytical factors that may influence test outcome, is complemented by participation and adequate performance in external quality assurance (EQA) schemes. These schemes generally drive up testing quality and help highlight issues, for example, the potential risks of using some LDTs [45].

Making Sense of It All

Biomarker testing is now a standard of care for patients with advanced NSCLC. This has made the pathological diagnostic process extremely complex, and this will only increase as more drugs, with their own biomarkers, are approved for use. The introduction of massively parallel sequencing technology (NGS) [35–37] allows the simultaneous screening of large panels of genes for mutations, rearrangements and, in some circumstances, gene copy number. Whilst these techniques are very powerful and allow assessment of many genes, in samples that might be insufficient to support multiple 'standalone' tests, they also generate a huge amount of additional data, on a large range of genes that may not be clinically useful. As mentioned above, in an academic setting, these data may be useful if they allow patients access to more drugs through clinical trials [36]. For routine practice, however, depending

on the health system environment involved, this data output can be extremely valuable or may be mostly un-actionable and can cause confusion [37]. Virtually all trials of targeted agents have involved selection using a single biomarker. Very little is known about the influence of coexisting mutations or other genetic changes that might alter treatment response to the primary target. Trials addressing this are underway, but they pose many challenges [46].

Molecular biology is a topic relatively unfamiliar to many tissue pathologists, but the growth of precision, personalized lung cancer medicine is requiring those working on lung cancer to learn fast! Collaboration with molecular pathologists is essential, to ensure that adequate and appropriate material is submitted to the molecular laboratory, consistent with the assay technology in use. The results of the assays need to be interpreted in the context of the samples used, and of any known issues with content, quality, processing and so on, as well as the actual tumour pathology. Much of this collaboration and dialogue is increasingly conducted in a molecular MDT or tumour board meeting, where experts in the significance of molecular findings can combine with tissue pathologists, oncologists and others to determine the best management plan for the patient.

Pathologists are increasingly using IHC as part of the biomarker testing plan for NSCLC samples. The interpretation of IHC-based biomarkers is often more complex than for IHC used as an adjunct to morphological diagnosis. For some assays, there may be a particular quantitative, as well as qualitative element to reading the IHC slides and signing out an opinion upon which clinical action should be taken. Training in reading some assays is often needed. An increased awareness of the assay details, how the dynamic range of staining looks and how that influences test results, and the staining artefacts and other characteristics that may lead to a false positive read [47], all take on great importance.

There is great interest in alternatives to tumour tissue samples, as sources of biomaterial for biomarker testing. The so-called liquid biopsy, using blood-borne, circulating cell-free DNA or tumour cells, or DNA from urine, appears to be a potentially effective alternative to tissue, especially for the detection of the *T790M EGFR* exon 20 resistance mutation [48, 49], but this approach is still evolving. Biomarker testing, in general, in the setting of the almost inevitable relapses suffered by patients after responses to many targeted TKI therapies, is also rapidly emerging as a significant problem, for patients, oncologists and laboratories. Resistance mechanisms are highly varied, in tumour regrowing after EGFR or ALK TKI therapy [50–54], and this creates another menu of biomarker tests that may have to be pursued, presuming new tumour samples can be accessed at the time of disease relapse, and that there are therapeutic interventions available, determined by those tests.

Conclusion

Precision, personalized medicine for patients with advanced NSCLC is now well established in routine clinical practice. Patients who have a targetable factor in their tumour, and who receive the targeted treatment, generally benefit, and to a greater

extent when compared to 'standard of care' unselected chemotherapy. Response rates to some targeted agents, when given to biomarker 'positive' patients, can be over 70%, when compared to response rates to chemotherapy of around 30%. The need for biomarker testing is clear and will increase as more drugs are approved. This continually poses challenges for pathology laboratories, dealing with limited sample resources. Diverse and increasingly sophisticated technology is needed to generate a wide range of biomarker data. We are still very early in the process of understanding the significance of interrelationships between various genetic changes which may be found in a patient's tumour. The molecular landscape in lung cancer is extremely complicated but is offering more and more opportunity for new treatments for our patients. In the following chapters are detailed discussions of the molecular pathology of lung cancer and its importance, not only in terms of tumour biology but also with regard to more effectively treating patients with these diseases.

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Chapter 2
Lung Cancer Epidemiology and Demographics

Ross A. Miller and Philip T. Cagle

The leading overall cause of cancer-related death in the United States [1] and the global population [2] continues to be lung cancer. Globally, it is the leading cause of cancer death in men and the second leading cause in women (second to breast cancer). However, in developed countries (including the United States), lung cancer mortality has surpassed breast cancer. Worldwide, nearly 1.6 million deaths were attributed to lung cancer in 2012 (1.1 million in men and nearly 500,000 in women) [2]. In the United States, 27% of cancer deaths in men and 26% in women are attributed to lung cancer [1], with 158,080 estimated deaths expected in 2016. This number surpasses the combined total estimate of cancer mortality for the next three most common causes of cancer death in men and women residing in the United States (men: prostate, colon and rectum, pancreas; women: breast, colon and rectum, pancreas) [1]. Lung cancer continues to be one of the most lethal forms of cancer with global 5-year survival rates ranging from around 10 to 20% [3] despite improvements in therapy and surgical technique. The net 5-year survival rate in the United States is around 19%, with only liver cancer having a worse 5-year survival [3].

Lung cancer incidence is tightly linked to tobacco use trends in a given region [4]. The differences in lung cancer rates seen between men and women correlate with historical differences in onset and cessation of tobacco use between the sexes. In relative terms, countries where tobacco use peaked early (such as the United States, the United Kingdom, and Denmark) have seen decreasing lung cancer rates in men; rates in women have leveled off [5, 6]. Countries where tobacco use peaked

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*, Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_2

later (e.g., Spain and Hungary) are now seeing a decrease in lung cancer among men but an increase in women [5]. In countries where tobacco use is currently on the rise or is at its peak (China, Indonesia, some African countries), lung cancer rates are expected to continue to rise [5, 7, 8].

Undoubtedly, the largest risk factor for lung cancer development is cigarette smoking [9]. Cigar and pipe tobacco increases risk as well. However, only around 10% of tobacco smokers develop lung cancer, implying other factors exist with regard to cancer development. In tobacco smokers, chronic obstructive pulmonary disease (COPD) is the greatest risk factor for lung cancer development; this may indicate activation of common signaling pathways by tobacco smoke for both diseases [10]. The latency period, total amount of exposure or "pack years" (pack years equals the number of packs smoked per day X number of years smoked), and enzymatic differences in the metabolism of tobacco smoke carcinogens and DNA repair are factors in cancer pathogenesis as well [11, 12]. The risk of developing lung cancer remains elevated in a former smoker for decades after smoking cessation. The risk does gradually decline compared to those smokers who do not quit [12–15].

Exposure to other agents can also increase one's risk for developing lung cancer. For example, exposure to radon gas (from soil and/or building materials) is thought to account for 8–15% of lung cancer cases in North America and Europe. Radon gas is the leading cause of lung cancer in certain regions after cigarette smoking [16]. Indoor air pollution, particularly from cooking fumes produced by burning solid fuels (like coal, which is fairly common in low-middle socioeconomic countries), is thought to account for 2% of lung cancer deaths in these particular regions [17]. A wide array of other agents and compounds increases one's risk; some of these include secondhand smoke, asbestos, various metals, organic chemicals, radiation, pollutants, dietary factors, and exposure to various other occupational-related compounds (particularly rubber manufacturing, paving, roofing, painting, and chimney sweeping) [18, 19]. Certain infections, for example, human immunodeficiency virus infection [20], human papillomavirus infection, and those with a history of tuberculosis [18], are also thought to be at increased risk for lung cancer as well.

An overrepresented demographic afflicted by lung cancer includes never-smoking young women, often of Chinese/Asian descent. This demographic has a particularly high incidence of lung adenocarcinoma that is often associated with particular molecular aberrations (e.g., epidermal growth factor receptor mutations, anaplastic lymphoma kinase fusion genes discussed in more detail in subsequent chapters) [7, 21].

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Chapter 3
Genetic Susceptibility to Lung Cancer

Timothy Craig Allen

Lung Cancer in Nonsmokers

Most lung cancers are attributable to tobacco smoking, which exposes airways and lung parenchyma to numerous carcinogens and procarcinogens including free radical species, aromatic amines, polycyclic aromatic hydrocarbons, and nitrosamines. Compared to cigarette smoking, other exposures implicated in lung cancer impact lung cancer risk much less [1, 2]. Approximately 15% of lung cancers in men, and 50% of lung cancers in women, are not related to tobacco smoking [3, 4]. Overall, approximately 25% of lung cancer patients are never smokers [3, 4]. Although passive inhalation of tobacco smoke, also termed environmental tobacco smoke, is believed to play a role in some percentage of cases of lung cancer in never smokers [3], these tumors are usually designated idiopathic; and their histologic types differ from the types found in cigarette smokers [5]. Many of these never smokers who develop lung cancer are young women who develop adenocarcinoma and who show an overall better prognosis than patients with smoking-related lung cancers [6, 7]. Nonsmoking-related lung cancers are being increasingly recognized; and the disease likely represents a disease process unrelated to smoking-related lung cancer. People are thought to have variable susceptibilities to cancer risk factors, including lung cancer risk factors [8–38]. A genetic basis for differing cancer risk factor susceptibilities has been proposed based on the observation that different susceptibilities appear to be inherited based on aggregation of cancers within families [39–66]. Inherited susceptibilities would help explain why some people develop lung cancer, such as individuals with minimal or no tobacco smoke exposure [30, 36, 67–75], frequently in association with family histories positive for

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_3

cancer [31, 75–79], or those who develop lung cancer from exposure at a significantly earlier-than-average age [80–87].

Complex gene-environment interactions occur, and genetic differences in susceptibility to tobacco smoke carcinogens exist; and lung cancers in both smokers and never smokers may have mutually common and distinct risk factors and gene-environment interactions [5]. Host susceptibility to lung cancer, individually or in synergy with smoking, is uncertain [88]; however, gene-environment interactions and genetic differences likely have a significant role in the development of lung cancer in never smokers [3, 86]. They also likely help explain why some heavy smokers do not develop lung cancer [3, 86, 89–92] and why some lung cancer patients have strong family histories of cancer [5, 93–96]. The human genome database and improving genotyping technology have substantially aided researchers in their search for and understanding of the genetic role of lung cancer development [3, 34, 86, 87, 91, 92, 97, 98].

Familial Clustering

Epidemiological studies suggest familial clustering of lung cancer occurs [3, 86, 87, 92]; the literature is in fact robust [60, 99–101]. Studies for which smoking exposure and occupational exposure were controlled have shown an increased risk of lung cancer in relatives of lung cancer patients [45, 49, 54, 59, 64]. Inherited polymorphisms in DNA repair genes and xenobiotic-metabolizing enzyme genes might account for the elevated risk, as might the genetic influence of substance dependence, including nicotine dependence [87, 102–107]. Multiple genetic loci may relate to nicotine dependence, including the promoter region of CHRNA5, a locus on chromosome 15 [108–110]. Studies of lung cancer patients' families who were nonsmokers or significantly younger than average have shown an increased familial risk of lung cancer, supporting the premise that genetic susceptibility is a factor in lung cancer development [33, 34, 38, 47, 49, 59, 67–71, 76, 77, 80–82, 84, 87, 92, 101, 111].

A 2012 study identified a 1.25-fold risk increase for family history of lung cancer in nonsmokers who developed lung cancer [3, 112]. It is important to understand, however, that familial aggregation of lung cancer alone does not in and of itself prove inheritance of genetic risk variants [3]. Clustering of close relatives with similar exposures to environmental risk factors may exhibit itself as a familial aggregation. However, there is evidence that some situations exhibiting familial aggregation are the result of genetic variants [3].

Gender

Gender affects lung cancer incidence [85]. Female lung cancer patients who are never smokers are influenced by familial history than by radon gas or environmental tobacco smoke exposure [85]. Research is conflicting as to whether women smokers

have an increased risk of developing cancer relative to men; however, some studies suggest women have an increased risk [113]. Some studies suggest that women smokers have an increased risk of developing lung cancer relative to men with the same smoking histories; however, other studies show women's risk to be equivalent to men's. Environmental factors, hormonal influences, and gender differences in xenobiotic-metabolizing enzymes are proposed reasons for reported differences in gender-associated lung cancer susceptibility.

Driver Genes

Epidermal growth factor receptor (EGFR), echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK), proto-oncogene B-Raf (BRAF), Kirsten rat sarcoma viral oncogene (KRAS), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) are driver genes that may be mutated in pulmonary adenocarcinomas [114]. Gefitinib and erlotinib are EGFR tyrosine kinase inhibitors (EGFR-TKIs) used for molecular therapy of pulmonary adenocarcinomas [115]. EGFR-TKI-responsive EFGR mutations are identified more frequently in pulmonary adenocarcinomas of nonsmoking Asian women than in other groups of patients and are thought to arise early [116]. For example, one study of lung cancer patients showed EGFR mutations in 44% of pulmonary adenocarcinomas, with 47.5% occurring in women and 15% occurring in men and 42% occurring in nonsmokers and 14% occurring in smokers [117]. Nonsmoking Asian women whose pulmonary adenocarcinoma contains the EFFR mutation have been shown to have improved survival, particularly when the tumor demonstrates a significant lepidic pattern, compared to men with lung cancers who have a smoking history and whose pulmonary adenocarcinomas do not contain a significant lepidic pattern [2, 87, 118]. Pulmonary adenocarcinoma is identified relatively more frequently in nonsmoking women, and a relationship between EGFR mutation and membranous ERα expression has been identified as an independent prognostic factor in patients with pulmonary adenocarcinoma [116, 119]. HER2, a proto-oncogene of the receptor tyrosine kinase superfamily, binds other members of the EGFR family in the activation of EGFR signaling; and HER2 gene polymorphisms have been shown to increase susceptibility to pulmonary adenocarcinoma in nonsmoking Korean women [120].

Genome-Wide Association Studies

Genome-wide association studies (GWASs) are population-level studies to identify genetic alleles associated with disease status or clinical phenotypes within the genome rather than in relation to a specific gene [85]. Lung cancer GWASs have shown several factors that correlate with lung cancer occurrence and progression [3, 85]. Several single-nucleotide polymorphisms (SNPs) in various genetic loci related

to lung cancer susceptibility have been found; however, the three major susceptible loci associated with lung cancer risk are loci 15q24–25, 5p15, and 6p21 [121–124]. Locus 15p24–25 has been associated with lung cancer risk in Caucasian populations only, while loci 5p15 and 6p21 have been associated not only with lung cancer in Caucasian populations but also with lung cancer in East Asian (Korean, Japanese, Chinese) populations [123]. Other less well-established loci, including 3q28–29, 13q12.12, 22q12.2, and 18p11.22, have been identified as being associated with lung cancer in Asian populations [125].

Specific Polymorphisms Associated with Lung Cancer Susceptibility

Polymorphisms of xenobiotic-metabolizing genes and DNA repair genes have found potential allelic variants associated with lung cancer risk [126, 127]. The concept of polymorphisms of xenobiotic-metabolizing enzymes and DNA repair enzymes is appealing; however, studies correlating single-locus alleles with lung cancer risk have generally produced conflicting results, probably due to a number of factors. In some studies, the number of cases might be too few to reliably gauge the effects on lung cancer risk. Also, the polymorphisms studied might vary. Further, different ethnic groups exhibit widely differing frequencies of some polymorphisms, effecting results according to the ethnic group studied. Finally, as the metabolism, detoxification, and repair processes involved with DNA adducts are complex, one single polymorphism most likely does not account for differences in DNA adduct levels. Studies examining several or many polymorphisms simultaneously in a single population are more likely to yield more comprehensive and consistent results; and newer technologies, permitting the study of SNPs and haplotypes, increase statistical sensitivity [85].

Xenobiotic-Metabolizing Enzymes

Xenobiotics are drugs, toxins, solvents, and poisons, which are metabolized xenobiotic-metabolizing enzymes. Xenobiotics often induce xenobiotic-metabolizing enzymes by various methods, including by acting as substrate ligands that bind receptors, by activating the xenobiotic enzymes by transcription, or by stabilizing the protein product. Phase I xenobiotic-metabolizing enzymes metabolize the xenobiotic chemicals into other compounds; but paradoxically can metabolically bioactivate xenobiotic substrates, transforming them into active or more potent toxins or carcinogens, so-called reactive intermediates. The cytochrome P450s or CYPs are important phase I xenobiotic-metabolizing enzymes. Phase II enzymes detoxify reactive intermediates and transform them into compounds that can be removed from the body; the glutathione-S-transferases (GSTs) are an

important class of phase II enzymes. Phase III transporters, including P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and organic anion transporting polypeptide 2 (OATP2) are associated with xenobiotic transport and excretion [128–131].

Phase I enzymes P450s or CYPs primarily catalyze xenobiotic oxidation; however, they also catalyze reduction reactions. Also, CYPs are involved in other processes such as biosynthesis of steroid hormones and prostaglandins [129, 132, 133]. These reactions generally occur in the liver but can occur in other tissues, including lung tissue. 237–240 CYP-dependent metabolism often produces intermediate compounds called reactive intermediates that may be more potent carcinogens than their parent compounds and that could covalently bind to DNA and form adducts. DNA adduct formation is an important step in carcinogenesis. These intermediate compounds are also converted to more soluble, inactive products that may be excreted or compartmentalized by phase II enzyme-dependent conjugation reactions. CYP metabolism therefore may be a double-edged sword, leading to production of reactive intermediates that are more carcinogenic than the original compounds, but also more readily detoxified and removed than the original compounds. Nearly 60 active human P450 genes, mostly polymorphic, have been identified. CYP enzymes and genes are designated by family number (an Arabic number), subfamily letter (A, B, C, etc.), and individual members of a subfamily (also an Arabic number). Class I polymorphic CYP enzymes, which include CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1, and CYP3A4, metabolize procarcinogens. CYP1A1 and CYP1B1 are particularly important for the metabolism of polycyclic aromatic hydrocarbons (PAHs) from tobacco smoke, and CYP2A6 and CYP2E1 are involved in the metabolism of nitrosamines from tobacco smoke [129, 131, 133, 134].

Many CYPs are induced by the aryl hydrocarbon receptor (AhR), which acts by dimerizing with the AhR nuclear translocator (Arnt) and inducing expression of CYP1A1 and CYP1B1. CYP1A1 and CYP1B1 encode aryl hydrocarbon hydroxylases as well as CYP1A2. Ligands for AhR include PAHs and other xenobiotics which are also substrates for the activated CYP enzymes. AhR shows either low affinity or high affinity for its ligands, producing low or high inducibility of CYP1 enzymes. AhR, after binding its ligand, translocates into the nucleus and dimerizes with Arnt protein. The AhR/Arnt dimer then binds to xenobiotic responsive elements (XREs) of the CYP1A1 gene and activates its transcription [135, 136].

Benzo(a)pyrene is an extensively studied PAH found in tobacco smoke. It binds to AhR in the lungs, causing the induction of CYP1A1 and CYP1B1. CYP enzymes metabolically activate benzo(a)pyrene to benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE). BPDE is a carcinogen that damages DNA by covalently bonding to the DNA, forming bulky chemical adducts, for example, by binding to guanine nucleobases in codons 157, 248, and 273 of p53—mutational "hotspots" in smoking-related lung cancers [137, 138]. Along with PAHs, tobacco smoke contains *N*-nitrosamines including 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-dimethylnitrosoamine (NDMA), *N*-diethylnitrosoamine (NDEA), *N*-nitrosophenylmethyl-amine (NMPhA), and *N*-nitrosonornicotine (NNN).

These *N*-nitrosamines are metabolically activated by CYP2A6 and CYP2E1 to compounds that form chemical adducts with DNA [139, 140].

The phase II enzymes GSTs act mainly to catalyze the conjugation of glutathione (GSH) to xenobiotics containing an electrophilic center, forming more soluble, nontoxic peptides that are excreted or compartmentalized by other enzymes, the phase III enzymes. The GST superfamily is made up of enzymes that catalyze the conjunction of glutathione to xenobiotics and is divided into three subfamilies, each composed of multigene families—the soluble or cytosolic (canonical) GSTs, microsomal or MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) GST, and plasmid-encoded bacterial fosfomycin-resistant GSTs. The cytosolic GSTs are polymorphic and make up seven classes—alpha, mu, and pi are regarded as specific and sigma, omega, theta, and zeta as common. Importantly, the cytosolic GSTs that assist in the metabolism of tobacco-derived carcinogens are GSTM1, GSTM3, and GSTP1 that detoxify reactive intermediates of PAHs such as benzo(a)pyrene and GSTT1 that detoxifies reactive oxidants such as ethylene oxide [141]. There are other phase II enzymes, including N-acetyltransferases (NAT), sulfotransferases (ST), UDP-glucuronosyltransferases (UGT), and NAD(P)H:quinone oxidoreductase (NQO1). Microsomal epoxide hydrolase (mEH) is a phase II enzyme which also acts as a phase I enzyme; it catalyzes the trans-addition of water to xenobiotics such as the PAH benzo(a)pyrene, producing dihydrodiol reactive intermediates involved in PAH-initiated carcinogenesis [141].

Xenobiotic-Metabolizing Genes

CYP Polymorphisms and Lung Cancer Susceptibility

Ayesh et al. suggested in 1984 that there was a relationship between lung cancer risk and a polymorphism of *CYP* (debrisoquine 4-hydroxylase or *CYP2D6*) [94]. Kawajiri et al. in 1990 proposed that *CYP1A1* polymorphisms may impact on lung cancer risk [142]. Further research into *CYP2D6* polymorphisms has produced mixed results [143–147]. Several *CYP1A1* alleles have been extensively studied. The *CYP1A1 m1* allele, also called *Msp*I, has a T to C transition in the 3′ noncoding flanking region. It has increased enzyme activity. In 1991, Hayashi et al. described a transition of adenine to guanine at position 2455 in exon 7 of *CYP1A1*, causing an isoleucine to valine amino acid substitution at codon 462 (Ile462Val) [148]. Similar to the *MspI* allele, the valine allele or *CYP1A1 m2* allele—also called *CYP1A1*2C*—has increased enzymatic activity (extensive metabolizer), thought to cause greater carcinogenic DNA adduct production and higher risk of tobacco smoke-related lung cancer. The *CYP1A1 m3* allele, with a mutation in intron 7, is thought to be specific to African-Americans. The *CYP1A1 m4* allele has a transition in exon 7 that causes a Thr for Asn substitution [148–154].

Several studies have explored the possible association between *CYP1A1* polymorphisms and lung cancer risk in various ethnic populations [155–162]. *CYP1A1*

m1 and *m2* polymorphisms strongly correlate with risk of lung cancer in several Japanese studies, especially with respect to tobacco smokers and squamous cell carcinoma of the lung [153, 163, 164]. Song et al. studied 217 Chinese lung cancer cases and 404 controls and identified an increased risk for pulmonary squamous cell carcinoma in patients with at least one *CYP1A1 m1* allele or at least one *CYP1A1 m2* allele [165]. Lin et al. has reported similar findings [166]. Persson et al. did not identify an association of lung cancer and *CYP1A1* polymorphisms and Chinese patients who were predominantly women with adenocarcinomas [167].

As the prevalence of the *CYP1A1 m1* and *m2* alleles is extremely low in Caucasians, studies have generally exhibited mixed results regarding these polymorphisms and Caucasian patient lung cancer risk [168–170]. Le Marchand et al., studying pooled data from Caucasians from 11 studies with a total of 1153 lung cancer cases and 1449 control patients, identified an increased lung cancer risk, predominantly squamous cell carcinoma, associated with the presence of the *CYP1A1 m2* allele [171]. Larsen et al., studying 1050 lung cancers and 581 controls, found an association between the *CYP1A1 m2* allele and lung cancer risk, particularly among women patients, younger patients, and patients with lesser smoking histories [158]. Studies of populations of Americans with mixed ethnicity have also identified an increased lung cancer risk associated with the *CYP1A1 m1* allele [169, 170]. Research from Brazil has also noted an increased risk of lung cancer associated with the *CYP1A1 m2* allele [172, 173]. An increased risk for pulmonary adenocarcinoma, but not for other types of lung cancer, associated with the *CYP1A1 m3* allele has been reported in African-Americans [174, 175].

New candidate genes have emerged in recent years as the percentage of pulmonary adenocarcinomas has increased, and the combination of CYP1A1*2B (Ile462Val) genotype and myeloperoxidase G/G (MPO G/G) genotype found to be associated with an increased risk of pulmonary adenocarcinoma. Increased risk in female never smokers has also been seen the slow genotype and the fast genotype among the polymorphisms of NAT2 [176]. Interestingly, as increased meat consumption may induce NAT2, lifestyle change may cause an increase in these xenobiotic gene activities [176, 177].

CYP2A6

CYP2A6 metabolically bioactivates *N*-nitrosamines in tobacco smoke [178]. Several alleles of *CYP2A6* have been identified, including *CYP2A6*4C*, *CYP2A6*7*, *CYP2A6*9*, and *CYP2A6*10*. The alleles have decreased the enzyme activity or decreased expression of *CYP2A6*. These variant alleles of CYP2A6 are associated with a decreased lung cancer risk, especially for squamous cell carcinoma and small cell carcinoma, and a decreased risk in heavy smokers compared to light smokers and never smokers, a finding consistent with the decreased metabolic bioactivation of *N*-nitrosamines [178, 179].

Genetic variations in the CYPA6 nicotine metabolic gene and the CHRNA5-A3-A4 nicotine gene cluster have been found to be associated with increased risk

of lung cancer. In addition to increased lung cancer risk, variation in CYP2A6 and CHRNA5-A3-A4 has been found to be associated with increased consumption of cigarettes and nicotine dependence [180]. Various studies have indicated that CYP2A6 deletions may be associated with increased risk of lung cancer [181–184].

GST and Lung Cancer Susceptibility

GST variants have been studied with respect to the risk of lung cancer, but the studies have yielded mixed results [185–190]. GST polymorphisms might also affect lung cancer cell type [191, 192]. These alleles occur in the *GSTM1*, *GSTT1*, *GSTP1*, and *GSTM3* genes and are associated with the reduced activity or deletion, with loss of all activity, of these phase II enzymes. These alleles include the *GSTM1**0 (*GSTM1* null) allele, a deletion of the *GSTM1* gene; the *GSTT1**0 (*GSTT1* null) allele, a deletion of the *GSTT1* gene; the *GSTP1* Ile105Val variant (I105V), caused by an A to G transition; the *GSTP1* Ala114Val variant (A114V), caused by a C to T transition; and the *GSTM3* intron 6 polymorphism, a three-base pair deletion in intron 6. Perera et al. found that adducts significant predicted lung cancer risk; that the combined *GSTM1* null/*GSTP1* Val genotype was associated with lung cancer generally, and especially in patients who were former smokers; and that adducts were significantly higher in patients who were current or former smokers with lung cancer who exhibited the *GSTM1* non-null/*GSTP1* Ile genotype [193]. In a meta-analysis of data from 130 studies containing 23,452 lung cancer cases and 30,397 controls, Ye et al. identified a weak association of the *GSTM1* null and *GSTT1* null polymorphisms with lung cancer risk and possibly weaker associations in studies of patients of European descent, whereas the *GSTP1*105V, *GSTP1*114V, and *GSTM3* intron 6 polymorphisms showed no significant overall associations with lung cancer [190]. Hosgood et al. found that GST genotype GSTM1 null genotype may be associated with increased risk of lung cancer [194]. Okazaki et al. showed a relationship between nicotine dependence, indicated by increased serum nicotine levels, and CYP2A6 and CYP2B6 genes encoding nicotine-metabolizing enzymes and also CHRNB3 and CHRNA6 genes encoding nicotinic acetylcholine receptor subunits [195].

Other Phase II Xenobiotic Enzymes

Studies of *NQO1* alleles and possible lung cancer risk have shown mixed results [196, 197]. Saldiver et al. noted that *NQO1* variant allele associated with reduced activity was associated with increased lung cancer risk in younger patients, in women, and in never smokers [197]. Other authors studying *NAT1* alleles and lung cancer risk have reached conflicting conclusions [198–200]. Habalova et al. identified a slow acetylation variant—**5B/*6*—to be associated with squamous cell carcinoma risk in younger patients, in nonsmokers, and in women, whereas Wang et al.

noted an increased risk of lung cancer in association with the *SULT1A1*2 allele* (*variant A allele*) which codes for a SULT1A1 sulfotransferase enzyme with decreased activity [200, 201].

Multiple Xenobiotic-Metabolizing Enzymes

Because xenobiotic metabolism is a complex process involving many enzymes, an accurate understanding of lung cancer susceptibility requires an understanding of the interactions of multiple genes and the effects of multiple enzymes. Several studies have examined the combined effects of two or more xenobiotic enzymes [202–205]. Hung et al., in a pooled analysis of data from 14 case-control studies that included 302 lung cancer cases and 1631 controls in Caucasian nonsmokers from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens, identified an increased lung cancer risk with the combined *CYP1A1 Ile462Val* variant and *GSTM1* null genotype relative to the *CYP1A1* wild type and *GSTM1* non-null genotype [203]. Raimondi et al. performed a meta-analysis of data from 21 case-control studies from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens that included 2764 Caucasians, 555 lung cancer cases and 2209 controls, and 383 Asians, 113 lung cancer cases and 270 controls, who had never smoked on a regular basis [205]. Raimondi et al., in their analysis of multiple xenobiotic-metabolizing enzymes, found a significant association between risk of lung cancer and *CYP1A1Ile462Val* polymorphism in Caucasians, found *GSTT1* deletion to be a lung cancer risk factor in Caucasian nonsmokers only, and found that the combination of *CYP1A1 wild type*, *GSTM1 null*, and *GSTT1 non-null* genotypes was associated with a lower risk of lung cancer. None of the polymorphisms examine studied by Raimondi et al. were associated with lung cancer in Asian nonsmokers [205].

New xenobiotic gene candidates have appeared as cases of pulmonary adenocarcinoma have increased. The CYP1A1*2B (Ile462Val) genotype with the myeloperoxidase G/G (MPO G/G) genotype displays a significantly increased pulmonary adenocarcinoma risk [159, 177]. Also, in nonsmoking women, the slow genotype and the fast genotype polymorphisms of NAT2 are related to lung adenocarcinoma risk; and lifestyle changes may increase these xenobiotic genes' activities [176, 177].

DNA Adducts

DNA adducts from metabolically activated intermediates of compounds found in tobacco smoke are mutagenic and carcinogenic [206]. Bulky DNA adducts can be identified with 32P-postlabeling of tumor tissues, peripheral blood lymphocytes and other tissues, immunoassays and immunohistochemistry, mass spectrometry, fluorescence, HPLC electrochemical detection, and phosphorescence

spectroscopy [207]. PAH-DNA adducts can be identified by BPDE-DNA immunoassays such as the BPDE-DNA chemiluminescence immunoassay (BPDE-DNA CIA); and elevated DNA adduct levels have been found in smokers' lung and other tissues. More DNA adducts are found in patients with smoking-related cancers than in patients without cancer [208]. A meta-analysis showed that smokers with smoking-related cancers had a statistically significant (83% higher) level of DNA adducts than controls [209]. Increased levels of DNA adducts have been in smokers' lungs relative to non- and never smokers' lungs [210]. Along with studies demonstrating the carcinogenicity of DNA adducts from tobacco smoke, these studies support a link between DNA adduct number and lung cancer development. However, it must be remembered that in retrospective case-control studies, the possibility that the levels of DNA adducts are the result of, rather than the cause of, the disease cannot be completely excluded. Nonetheless, that DNA adducts are causative is strongly supported by prospective studies where DNA adducts were measured in blood samples collected years before cancer onset. One study showed that disease-free current smokers with elevated levels of DNA adducts in blood leukocytes were three times more likely to be diagnosed with lung cancer 1–13 years later than current smokers with lower DNA adduct levels [211]. Peluso et al. noted that the levels of leukocyte DNA adducts in blood samples collected several years before the onset of cancer were associated with the subsequent risk of lung cancer [212]. The association with lung cancer was stronger in never smokers—whose sources would be environmental, such as secondhand tobacco smoke and air pollution—and in younger patients. These prospective studies strongly support a relationship between DNA adduct levels and lung cancer risk. The studies also suggest that individual patients have differing susceptibilities to carcinogen exposures, highlighted by the risks observed in those with fewer years of exposure, younger patients, and those with lesser levels of exposure, never smokers.

DNA Repair Gene Polymorphisms

Because tobacco smoke contains carcinogenic chemicals which damage DNA, lung cancer has been shown to involve DNA damage repair genes, including O6-alkylguanine DNA alkyltransferase (AGT), X-ray repair cross-complementing group 1 (XRCC1), NAD(P)H: quinoneoxidoreductase (NQO1), human 8-oxoguanine DNA glycosylase (hOGG1), cytosine DNA-methyltransferase-3B (DNMT3B), O6-methylguanine-DNA methyltransferase (MGMT), and several nucleotide excision repair (NER) genes [195]. These gene polymorphisms inhibit the repair of damaged DNA, increasing the risk of carcinogenesis caused by tobacco [195]. In cultured lymphocytes, DNA repair capacity (DRC) can be measured using the host-cell reactivation assay and a reporter gene damaged by the activated tobacco carcinogen BPDE. A fivefold variation in DRC has been found in the general

population. Also, decreased DRC has been associated with increased lung cancer risk [213–216]. Polymorphisms in DNA repair genes may be related to differences in efficiency of DNA repair; and decreased or increased ability to repair DNA damage is thought to impact the accumulation of significant genetic abnormalities required for cancer development.

Prevalence of *XPD* alleles and genotypes varies greatly by ethnicity. Polymorphisms in codons 156, 312, 711, and 751 of the *XPD* gene are noted commonly, with an allele frequency greater than 20%. Polymorphisms of codon G23592A (Asp312Asn) of exon 10 and codon A35931C (Lys751Gln) of exon 23 cause amino acid changes in the XPD protein and have been studied with respect to lung cancer susceptibility [217–223]. Studies have examined the levels of DNA adducts associated with these polymorphisms as an indication of the efficiency of the different alleles at DNA repair. Most likely a higher level of adducts suggests that the allele has less efficiency at excising DNA adducts. With respect to codon 312 polymorphisms, most studies have found a higher level of DNA adducts in association with the *Asn* allele than with the *Asp* allele. The majority of studies have identified a higher level of DNA adducts in association with the *Gln* allele. As such, most studies indicate a difference in DNA repair efficiency between these specific *XPD* alleles [217, 221, 223, 224]. Hu et al., in a meta-analysis of data from nine case-control studies including 3725 lung cancer cases and 4152, found that patients with the *XPD 751CC* genotype have a 21% higher risk of lung cancer compared to patients with the *XPD 751AA* genotype and that patients with the *XPD 312AA* genotype have a 27% higher risk of lung cancer compared to the ones with the *XPD 312GG* genotype [224]. Performing a meta-analysis derived from the same studies as Hu et al., including 2886 lung cancer cases and 3085 controls for the *XPD*-312 polymorphism from 6 studies, and 3374 lung cancer cases and 3880 controls for the *XPD*-751 polymorphism from 7 studies, Benhamou and Sarasin were unable to conclude that one or the other of these polymorphisms was associated with an increased risk of lung cancer [224, 225]. After the conflicting meta-analyses, Hu et al. performed a case-control study that included 1010 lung cancer cases and 1011 age- and sex-matched cancer-free controls in a Chinese population [223]. Hu et al., studying eight SNPs/DIPs (deletion/insertion polymorphisms) of *XPD/ERCC2* and *XPB/ERCC3*, found that none of the eight polymorphisms was individually associated with lung cancer risk; however, the combination of genetic variants in *ERCC2* and *ERCC3* contributed to the risk of lung cancer in a dose-response manner.

In other studies, an increased lung cancer risk with combinations of XPD polymorphisms and polymorphisms of other DNA repair genes has been identified [226]. Zhou et al. identified a significantly increased lung cancer risk in patients with five or six variant alleles of *XPD Asp*312*Asn*, *XPD Lys*751*Gln*, and *XRCC1 Arg*399*Gln* polymorphisms versus patients with no variant alleles [227]. Chen et al. found that patients with variant alleles for both *XPD Lys*751*Gln* and *XRCC1 Arg*194*Trp* polymorphisms have a higher lung cancer risk than patients with only one variant allele in a Chinese population [228].

Other DNA Repair Genes

Other DNA repair gene polymorphisms have received some examination with respect to lung cancer susceptibility, generally with conflicting or unconfirmed results, including the *XPA* [229, 230], *XPC* [231], *XPG* [232], *XRCC1* [233, 234], *XRCC3* [235], *MMH/OGG1*, BER pathway [236, 237], and *MGMT* [238]. Studying *ATM* genotypes in 616 lung cancer patients and 616 cancer-free controls, Kim et al. found that the A allele at the site (IVS62+60G>A) was associated with a higher lung cancer risk than the G allele [239]. Patients with the ATTA haplotype showed significantly increased lung cancer risk versus patients with the common GCCA haplotype; and patients with the (NN)TA haplotype showed an increased lung cancer risk versus patients without the (NN)TA haplotype.

Multiple DNA Repair Genes

Zienolddiny et al. found that (1) for the NER pathway, *ERCC1* (Asn118Asn, C > T), *ERCC1* (C15310G), and *ERCC2* (Lys751Gln) variants were related to increased lung cancer risk and *XPA*, *G23A*, and *ERCC5/XPG* (His46His) variants were related to decreased lung cancer risk; (2) for the BER pathway, *OGG1* (Ser326Cys) and *PCNA* (A1876G) variants were associated with increased lung cancer risk and *APE1/APEX* (Ile64Val) variant was associated with decreased lung cancer risk and variant T allele of PCNA2352 SNP had a marginal effect on cancer risk; (3) for the DSB-R pathway, *XRCC2* (Arg188His) variant was related to increased lung cancer risk and *XRCC9* (Thr297Ile) and *ATR* (Thr211Met) variants were associated with decreased lung cancer risk; and (4) for the DR pathway, *MGMT/AGT* (Leu84Phe) variant in exon 3 exhibited a slight tendency toward a higher lung cancer risk [161, 240]. Kiyohara et al. found that XPA G23A, OGG1, and ERCC2 polymorphisms were associated with increased risk of lung cancer [241].

TERT Polymorphism

Telomerase reverse transcriptase (TERT) and cleft lip and palate transmembrane 1-like protein (CLPTM1L), both on chromosome 5p15.33, have been reproducibly associated with lung cancer risk [121]. TERT encodes a telomerase subunit involved in maintenance of telomere ends. GWASs have identified chromosome 5p15.33 as a region related to risk of pulmonary adenocarcinoma [121]. Lung cancer risk has also been associated with SNPs in the 5p15.33 region [242]. TERT and CLPTM1L are two candidate susceptibility genes found on chromosome 5p15.33. Because TERT encodes a telomerase subunit important in the maintenance of telomere ends, its overexpression causes cellular life span prolongation. CLPTM1L, also termed cisplatin resistance-related protein 9, provides apoptosis resistance [243].

The rs2361000 SNP in the TERT gene has shown a strong association with lung cancer risk in never smokers; and an increased frequency of rs2736100 (TERT) as the risky allele has been seen in pulmonary adenocarcinoma, with the most significant association of rs2736100 at 5p15.33 in pulmonary adenocarcinoma in nonsmokers [242, 244, 245]. Besides 5q15.33, studies have shown no association at three additional susceptible loci at 10q25.2, 6q22.2, and 6q21.32 [245–247].

Conclusion

Genetic factors have clearly been shown to be related to susceptibility to lung cancer, particularly pulmonary adenocarcinoma. Genetic polymorphisms have been identified that have the potential to increase lung cancer risk. These genetic polymorphisms involve genes that are associated primarily with the metabolism of tobacco smoke carcinogens and the suppression of mutations induced by those carcinogens. Tobacco-associated versus nontobacco-associated lung cancers, gender differences, and geographic differences continue to be confounding factors in the evaluation of the genetic factors involved in lung cancer risk. Future studies likely will focus increasingly on the genetic factors related to smoking behavior and the psychological processes involved, even as studies continue to address biological predisposition for the development and progression of lung cancer [248].

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Chapter 4
Lung Cancer Stem Cells

Timothy Craig Allen

Somatic, or adult, stem cells are another population of stem cells identified in human beings. Somatic stem cells have a limited capacity for self-renewal and play a role in tissue self-renewal [1–4]. Somatic stem cells are important for tissue repair and regeneration and have been identified in many tissues, including hematopoietic, neural, epidermal mammary, hepatic, mesenchymal, gastrointestinal, and pulmonary tissues. Adult stem cells include hematopoietic stem cells, typically found in the bone marrow, mesenchymal stem cells, and stem cells residing in specific organs, termed progenitor cells. Organ-specific progenitor cells are generally believed to aggregate in special tissue microenvironments, termed the stem cell niche [5–34]. These progenitor cell populations in the lung are thought to arise from differentiation of embryonic stem cells; however, these cells have also been considered to possibly arise from mesenchymal stem cells or hematopoietic stem cells [35–37].

With the study of cancer stem cells, the traditional "clonal evolution theory" [38] has given way to the "cancer stem cell theory" of carcinogenesis. The "clonal evolution theory" proposes that each neoplastic cell within a tumor has an equivalent carcinogenic potential. It is under the auspices of this theory that modern chemotherapy and radiotherapy, attempting to destroy all cancer cells with a high proliferation capacity, are based. Unfortunately, under this theory, cancer cures, particularly with solid organ neoplasms, remains elusive for many cancer patients.

The "cancer stem cell theory" proposes that uninhibited carcinogenic potential in malignant neoplasms is due to cancer stem cells, a rare subset of the overall tumor cell population with the ability to self-renew, differentiate into non-stem cancer cells, and produce new tumors via the formation of heterogeneous cell populations. Unlike tumor cells proposed under the "clonal evolution theory," these multipotent cancer stem cells are believed to produce intratumoral heterogeneity via aberrant capacity

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_4

for differentiation [39–44]. Cancer stem cells are able to resist chemotherapy and radiotherapy, with resultant tumor relapse and poor patient prognosis. Prevention of tumor relapse and resultant potential cure depends, under the "cancer stem cell theory," on the destruction of the cancer stem cell population. The theory posits asymmetric division of cancer stem cells, as is also a characteristic of normal stem cells, which results in a daughter cell that retains the characteristics of stemness such as indefinite self-renewal and in a daughter cell committed to differentiation, having lost the characteristics of stemness [45–47]. Microenvironmental pockets termed niches function as a protective habitat of stem cells and are regulated by a variety of factors, including pH, hypoxia, immune cells, and extracellular matrix [48, 49]. Within this niche, cancer stem cells are able to maintain themselves, and some cells may perhaps even have the ability to acquire stemness; however, the ability to reprogram for the acquisition of stemness has not been fully researched [40, 50].

Cancer Stem Cells

Cancer stem cells, supposedly rare cells that have the self-renewal properties, have the pluripotentiality and result in the production of a hierarchy of progenitor and differentiated cells as normal stem cells have been relatively well examined in hematopoietic malignancies. Leukemia stem cells have been found to be necessary and sufficient for leukemia maintenance. Cancer stem cells have been identified in a variety of leukemias, including acute myeloid leukemia and chronic myeloid leukemia [51–54]. Initial genetic hits have been identified in stem cells associated with hematopoietic tumors [55]. Once stem cells in the lung become malignant, these cells then proliferate, dividing to form one differentiated daughter cell and one daughter stem cell, maintaining the lung cancer stem cell population. Similar findings have been shown with some solid malignancies, including the brain and breast tumors [53, 56–58]. Disseminated or migrating cancer stem cells probably play a causative role in the development of metastatic disease [59]. Studies also show these cancer stem cells to exhibit resistance to chemotherapy and radiotherapy [58]. Studies also propose that the therapeutic stress of chemotherapy and radiotherapy may stimulate cellular plasticity, mediating the conversion of normal cancer cells to cancer stem cells [60, 61]. Lung cancers are often heterogeneous; but it is currently uncertain whether different cancer stem cell clones cause tumor heterogeneity or whether cancer stem cells have the pluripotentiality of normal stem cells [62].

Lung Stem Cells

The presence in adults of normal lung stem cells remains controversial, because lung epithelium is traditionally considered quiescent. Normal lung stem cells are thought to serve in the maintenance of normal lung architecture and reportedly lie

in functionally and anatomically distinct sites in the respiratory tract. It is hypothesized that these normal lung stem cells deregulate, leading to the development of disease. These lung stem cells exhibit specific characteristics; for example, proximal airway stem cells have been shown to have higher *Keratin 5* promoter activity, and bronchial basal cells have the ability to form heterogeneous spheres in vitro, evidence of self-renewal and multipotent potential [63, 64]. *Sox2* has been implicated in the experimental induction of pluripotency and has been linked to basal tracheal epithelium progenitor characteristics. Amplification of chromosomal segment 3q26.33, containing the *Sox2* locus, has been found to be associated with pulmonary squamous cell carcinoma, supporting the hypothesis that *Sox2* overexpression airway basal stem cells can change them into squamous cell carcinoma cancer stem cells [65, 66].

Lung Cancer Stem Cells

Although the understanding of lung cancer continues to progress, there are many areas for which there is not a complete understanding, but without which the development of successful therapies will remain elusive. As with other solid organ tumors, lung cancers can be hypothesized to contain hierarchically heterogeneous populations of transformed tumor cells with differing levels of differentiation and variably reduced potential for regeneration. Quiescent cells termed cancer stem cells allow the tumor to sustain itself and progress due to their abilities for self-renewal and asymmetric cell division. Cancer stem cells have the capacity for quiescence, indefinite self-renewal, the ability to produce differentiated progeny, and an intrinsic resistance to chemotherapy and radiotherapy. Cancer stem cells provide for tumor growth, resistance to therapy, tumor relapse, and tumor metastasis [42, 67]. Various hypotheses suggest cancer stem cells originate from normal somatic cells, somatic stem cells, and progenitor cells; however, the somatic stem cell hypothesis is most supported [68]. As cancer stem cells arise in a wide variety of tissues, they clearly exhibit uniform characteristics such as the ability to reestablish the primary tumor's phenotypic heterogeneity after serial transplantation in immunocompromised mice, and the ability to form mammospheres in culture in nonadherent conditions in vitro [51, 69]. There is substantial evidence of several stem cell populations in normal lung that are relatively specific to certain areas of the lung [22, 70–75].

Strongly CK5-immunopositive basal cells within submucosal gland ducts in the mouse trachea have been identified and are considered possible stem cells or progenitor cells involved in regeneration or repair of tracheal epithelium [70]. Club cell secretory protein-expressing cells, also termed CE cells, and basal cells that line mouse bronchi may be stem cells or progenitor cells [74, 76, 77]. The bronchioles in the mouse lung, CE cells, associated with pulmonary neuroendocrine cells, are arranged in small bodies of cells termed neuroepithelial bodies. The CE cells in the bronchioles are pollution resistant, most likely due to cellular deficiency of the drug

metabolizing enzyme CYP450 2F2 [75]. The bronchoalveolar duct junction in mice has been shown to contain pollution-resistant CE cells, not associated with neuroepithelial bodies, exhibiting both alveolar epithelia type II cell marker, surfactant protein C, and Club cell secretory protein. These cells probably play a reparative role for the terminal bronchioles, alveolar ducts, and alveoli [73]. These cells were identified as stem cells due to their expression of stem cell surface markers Sca-1 and CD34. Another stem cell niche of "variant" Club cells has been identified arising in the same location [72, 73, 78]. A third possible stem cell population has been identified, differing from the bronchioloalveolar stem cell population noted above by their CD34 immunonegativity and their immunopositivity with Oct-4 and SSEA-1, both embryonic stem cell markers related to self-renewal and pluripotency [79, 80]. Oct-4 positivity suggests the possibility that bronchioloalveolar stem cells arise from Oct-4 positive neonatal lung cells [78, 81]. Homeostatic regulation of bronchioloalveolar stem cell niches has been associated with expression of several tumor suppressor genes [78]. In mouse lung studies, stem cell niches have been identified that maintain epithelial differentiation within the airways. These niches are likely targets for lung cancer initiation and promotion [19, 73, 74]. It is thought that there may be as many as 40 different epithelial, mesenchymal, vascular, and lymphatic endothelial and immune cell lineages in the lung [17, 82]. It is important to remember that "lung cancer" is actually a variety of malignant pulmonary neoplasms that arise from cells that are phenotypically different [17, 19, 26].

Lung Cancer Stem Cell Regulation, Gene Expression, and Cell Surface Markers

There have been some common molecular pathways identified that are important in the development of cancer stem cells. Three embryonic patterning pathways, Notch, Hedgehog, and Wnt, are involved in early events leading to expansion and malignant transformation of normal stem cells in the lung [78, 83]. The Notch pathway is important for development and homeostasis in stem cells and helps stem cells maintain viability by asymmetric cell division. Notch signaling is required for lung development, and elevated Notch ligand and receptor levels have been shown in non-small cell lung cancer cell lines [78, 84]. The Hedgehog pathway is also important in early lung formation, and studies have shown it to be involved in epithelial-mesenchymal interactions controlling the branching of developing lung buds. In adult lungs, Hedgehog signaling is normally identified only at low levels in rare cells located in bronchial epithelium basal layers [78, 85–87]. Persistent activation of the Hedgehog pathway has been shown in small cell lung cancers, but uncommonly in non-small cell lung cancer cell lines [19, 88]. Wnt pathway signaling is also important in early lung development and lung disease [19, 89, 90]. Wnt pathway disruption has been found to be a factor in the development of non-small cell lung cancers [19, 91, 92]. These three pathways offer opportunities for future therapeutic intervention.

There are other genes involved with cancer stem cells, including Oct-4 (also termed Oct-3 and POU5F1), a gene regulated by the Wnt pathway that is involved in the maintenance of stem cell pluripotency. Interestingly, Oct-4 has been shown to be capable of reprogramming committed somatic cells and induce those cells to dedifferentiate and revert to an earlier, more developmentally potent state. Keratinocytes that overexpress Oct-4 have been shown to differentiate into other cell types [19, 93, 94].

There are various cell surface markers that may help identify cancer stem cells [19, 95]. These and future markers are important in helping to identify these stem cell niches and to identify mechanisms that transform normal stem cells into cancer stem cells. CD44, a transmembrane cell-surface adhesion glycoprotein involved in cell-cell interactions and cell-matrix interactions, linked to chemoresistance and poor prognosis in various malignant neoplasms, is increased in, and correlates with survival in, both non-small cell lung cancer and small cell lung cancer [19, 95–97]. CD133, also termed prominin-1, is a glycoprotein found in endothelial cells that has been identified in cancer-initiating stem cells of the brain, pancreas, and colon. As with CD44, CD133 is associated with chemoresistance [98]. Non-small cell lung cancers and small cell lung cancers have been found to have CD133-positive tumor cell subpopulations, showing similarities to a rare CD133-positive population of normal mouse lung cells that undergo significant expansion after naphthalene-induced lung injury [19, 95, 99]. Phosphatase and tensin homologue deleted on chromosome ten (*Pten*) inactivation in side population cells has been shown to result in spontaneous lung tumors; and studies have shown that activation of mammalian target of rapamycin (mTOR), involved in expression of CD133 in cancer cells, is related to upregulation of stem cells and progenitor cells in *Pten* conditional deletion models, suggesting that mTOR may be a potential therapeutic target [100–102].

CD117, also termed c-Kit, is a stem cell factor in neuroendocrine lung tumors that is related to poor prognosis in early-stage non-small cell lung cancers; however, less than one third of patients exhibit CD117 tumor cell positivity [19, 95, 103]. While other solid organ cancers, such as brain and breast cancers, have demonstrated a variety of putative cancer stem cell markers, few other potential cancer stem cell markers have to date been identified in lung cancers. Unfortunately, two cell surface markers found in mouse bronchioloalveolar stem cells have given disappointing results in human studies. Sca-1 does not have a human counterpart, and CD34 does not correlate with putative human non-small cell lung cancer stem cells. Additional studies are necessary to identify and confirm whether each type of lung cancer arises from a single, normal lung stem cell, or whether there are multiple stem cell origins responsible for each cancer's cellularity [95, 104]. The cell surface markers urokinase plasminogen activator (uPA) and its receptor uPAR, also termed CD87, have been identified in small cell lung cancer cells that coexpress CD44; however, their contribution to the development and maintenance of a lung cancer stem cell population is not currently known [19, 105].

Lung Cancer Progression

Nonneoplastic tissue exhibits orderly development, with numerous cell types maintaining generations of differentiated progeny cells via epigenetic regulation; however, neoplastic cells exhibit disorganized cell programming, producing populations of heterogeneous tumor cells [106–109]. The niche provides a site of equilibrium between the cancer stem cells and the differentiated cells they produce, allowing for cancer development [110]. One study showed that for breast and prostate cancer, interleukin-6 has been identified as a factor in the transformative reprograming of non-cancer stem cells within the niche to cancer stem cells [110]. Epithelial-to-mesenchymal transition (EMT) has also been considered, along with its role in metastasis, as a method of cancer stem cell population maintenance within the niche [111].

Malignant neoplasms are relatively hypoxic due to their limited vasculature; and hypoxia with low pH reportedly maintains the niche's ability to maintain cancer stem cell self-renewal via the activation of genes associated with stemness [112]. Studies have shown that relatively acidic pH promotes stemness within the niche and correlates with increased tumor size [112, 113]. Hypoxia also is thought to support the dedifferentiation of cells to become cancer stem cells within the niche [40, 49, 114]. By stimulating the release of vascular endothelial growth factor (VEGF) and angiogenic factors, hypoxia also promotes angiogenesis, which is also promoted by the niche's low pH [112, 115].

Chemotherapeutic and Radiotherapeutic Resistance and Cancer Relapse

Lung cancer stem cells are quiescent and are therefore relatively resistant to conventional chemotherapy and radiotherapy; their quiescence provides for periods of cancer remission, where residual cancer is undetectable by current imaging methods. Frequently, periods of remission occur following a patient's receipt of prolonged chemotherapy or radiotherapy. Patient outcomes are frequently poor in this setting [116–118]. Pronounced chemoresistance and radioresistance in these patients, with subsequent tumor spread or tumor relapse, is believed to be a function of the neoplasms' cancer stem cells and likely promoted by the cancer stem cells' enhanced DNA damage response [117, 119–121]. Because radiation and common chemotherapies kill cancer cells via DNA damage, generally by the production of double-strand breaks, a cancer cell's inability to repair the DNA damage kills the cell [122, 123]. Rad51 is a repair gene associated with therapeutic resistance; it catalyzes the search for, and the invasion of, the homologous DNA strand and initiates repair via annealing [124]. Overexpression of Rad51 by cancer stem cells is thought to provide for therapeutic resistance [125]. Studies have shown that Rad51 inhibition promotes cancer stem cell therapeutic resensitization, supporting the hypothesis that cancer stem cells overcome DNA damage via their Rad51 expression-enhanced DNA repair ability [126, 127].

Niche Targeting

Due to lung cancer stem cells' enhanced therapeutic resistance properties, development of therapeutic approaches targeting these niche's cancer stem cells is vital. Because the niche enables and maintains stemness, limiting or ending cellular plasticity within the niche could destroy the niche environment supporting the cancer stem cell phenotype. Further, because reduced Rad51 expression within the niche may therapeutically resensitize cancer stem cells, research is focusing on finding therapeutic targets that can destroy cancer stem cells by inhibiting their self-renewal properties, by inducing toxicity to directly destroy them, and by resensitizing the cancer stem cells to conventional therapies [128, 129]. Niche targeting is particularly challenging because of niche plasticity and the ability of cancer cells to be reprogrammed into cancer stem cells. One concept is to maneuver cancer stem cells to differentiate and as such lose their stemness, using bone morphogenetic proteins which are known to initiate differentiation in cancer stem cells. Cancer stem cell differentiation might lead to cancer cells targetable with conventional therapies, thereby reducing or eliminating cancer growth and recurrence [129–131].

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Chapter 5
The 2015 World Health Organisation Classification of Lung Cancer

Gavin M Laing and Keith M Kerr

The 2015 World Health Organisation (WHO) classification of tumours of the lung, pleura, thymus and heart was published with several significant modifications from the previous 2004 iteration [1, 2]. With respect to the lung cancer classification, many of these changes are pertinent to this book, since they are driven by molecular data or practical requirements of testing lung cancer for molecular alterations. In the 11 years which elapse between these two editions of the classification, some very significant data emerged on the molecular characteristics of lung carcinomas, and adenocarcinomas in particular [3, 4]. Many of these findings have provided targets for molecularly targeted therapy which has transformed the landscape of lung cancer therapy [5, 6].

As for several previous editions of the published classification [2, 7], the pathology committee of the International Association for the Study of Lung Cancer (IASLC) formed the core of the pathology working group for the lung cancer components of this classification. This group was, once again, ably led by Dr. William Travis of Memorial Sloan Kettering Cancer Centre, New York. The classification is the results of several years of work by the IASLC committee, the adoption of proposals published in 2011 on adenocarcinoma classification [8] and, of course, many relevant studies published by the wider lung cancer community. For the 2015 classification, considerable emphasis was placed upon molecular aspects of these diseases, and in the final stages of the development of the classification, multidisciplinary

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*, Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_5

groups were formed to ensure the relevance of the classification for thoracic surgeons, radiologists, pulmonologists, oncologists and, most importantly, our patients with a lung cancer.

This chapter will briefly describe the major groupings of lung cancer and indicate the major new aspects of the classification. Details of the molecular pathology of many of the major tumour types will be presented in detail in subsequent chapters.

Adenocarcinoma

The new classification of adenocarcinoma presents one of the most significantly changed approaches to the diagnosis and reporting of what is now, for most parts of the world, the commonest histological type of lung cancer. There were several key factors and discoveries which drove the need for this revised classification. These were an understanding that (1) most resected adenocarcinomas were heterogeneous in their histology and this was not adequately acknowledged in the classification; (2) that this heterogeneity was a significant factor influencing post-operative survival in this disease; (3) that the term 'bronchioloalveolar carcinoma (BAC)' was a misused, misunderstood term which was causing great confusion; (4) that the microanatomy of primary lung adenocarcinomas is, to an extent, reflected in the high-resolution computed tomography (HRCT) imaging of the lesions and clinically important correlations were made; and (5) that the molecular landscape of adenocarcinomas had significance in terms of diagnosis and patient treatment. Most of the changes to the adenocarcinoma classification introduced in 2015 were adopted from proposals devised and published by a Joint Working Group brought together by the IASLC, in conjunction with the American Thoracic Society (ATS) and the European Respiratory Society (ERS) [8–10]. In brief, the most important aspects of the classification are as follows:

Preinvasive and Early Invasive Disease

Atypical adenomatous hyperplasia (AAH) retains its place as a precursor lesion, akin to dysplasia of the central bronchial epithelium (see below). Those lesions which fulfilled the criteria for BAC in the 1999 and 2004 classifications, and which were known to have 100% 5-year post-operative survival (5YS) [11–13], were correctly reclassified as adenocarcinoma in situ (AIS). These are lesions less than 30 mm in diameter which show a pure alveolar wall spreading growth pattern (lepidic growth), with no evidence of invasive disease. Similar lesions which do show evidence of invasion, but in foci limited to 5 mm diameter in extent, are minimally

invasive adenocarcinoma (MIA). This new category was introduced since these lesions, whilst having evidence of invasion, still appear to have no metastatic risk and 100% 5YS.

Invasive Adenocarcinoma

These tumours show a range of patterns of disease, and the invasive components exceed an extent of 5 mm in the lesion. It was recognised that the 2004 classification subtype of mixed adenocarcinoma was not very useful, and emerging data on the prognostic significance of particular patterns when dominant in the lesion [14–17] led to a change in approach to classification and reporting. Five major patterns of invasive adenocarcinoma are recognised: lepidic, acinar, papillary, micropapillary and solid (with mucin). Lepidic pattern shows tumour cells lining alveolar walls, as in AIS (Fig. 5.1). Acinar pattern represents neoplastic growth of

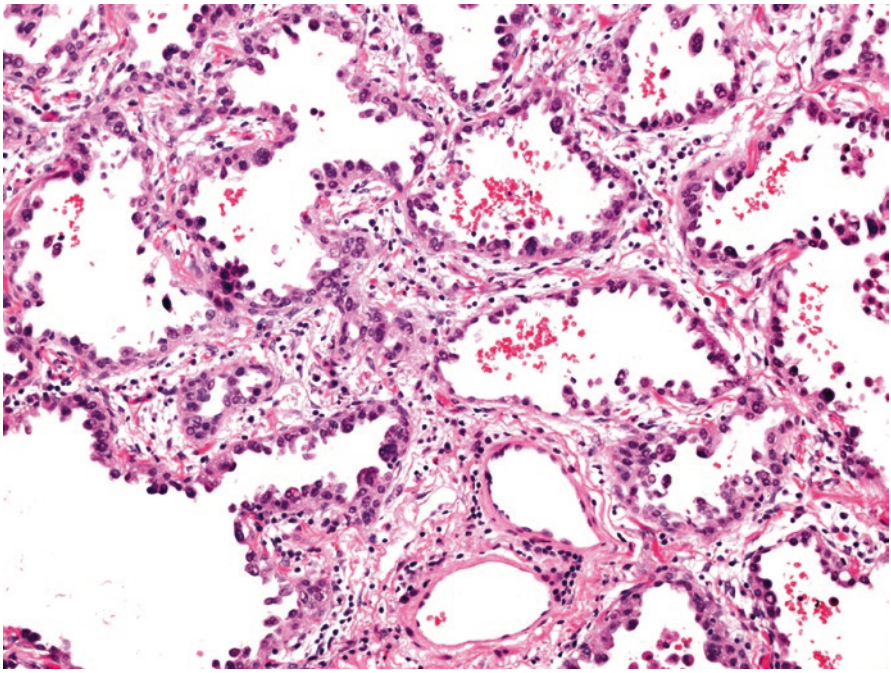

Fig. 5.1 Lepidic pattern adenocarcinoma. Tumour cells line intact, pre-existing alveolar walls. A lesion comprising only this pattern of tumour is adenocarcinoma in situ. This pattern is also common at the edges of invasive adenocarcinomas

invasive glands in a stroma showing a fibroblastic response (Fig. 5.2). Papillary growth shows tumour papillae with fibrovascular cores lined by adenocarcinoma cells, growing in a tissue-destructive manner (Fig. 5.3). Micropapillary pattern, a new introduction, comprises small, complex tumour tufts with secondary or tertiary branching, without fibrovascular cores (Fig. 5.4). Solid pattern adenocarcinoma is architecturally undifferentiated, but with at least five tumour cells in at least two 'high-power' microscopic fields showing cytoplasmic mucin vacuoles. In a significant addition to the classification, an alternative feature to the identification of mucin, for the designation of solid adenocarcinoma, is immunohistochemical (IHC) evidence of pneumocyte marker expression (in practice, TTF1) in such an undifferentiated case (Fig. 5.5). This will be discussed later in the large cell carcinoma section. It is recommended that a resected adenocarcinoma is reported, giving an estimated proportion of each of these patterns to the nearest 5–10%. The tumours are classified according to the predominant pattern, e.g. acinar-predominant adenocarcinoma, and so on. This is driven by abundant evidence that whilst lepidic-predominant tumours have a relatively good post-operative survival, those which are micropapillary or solid predominant have a poor 5YS [14–17].

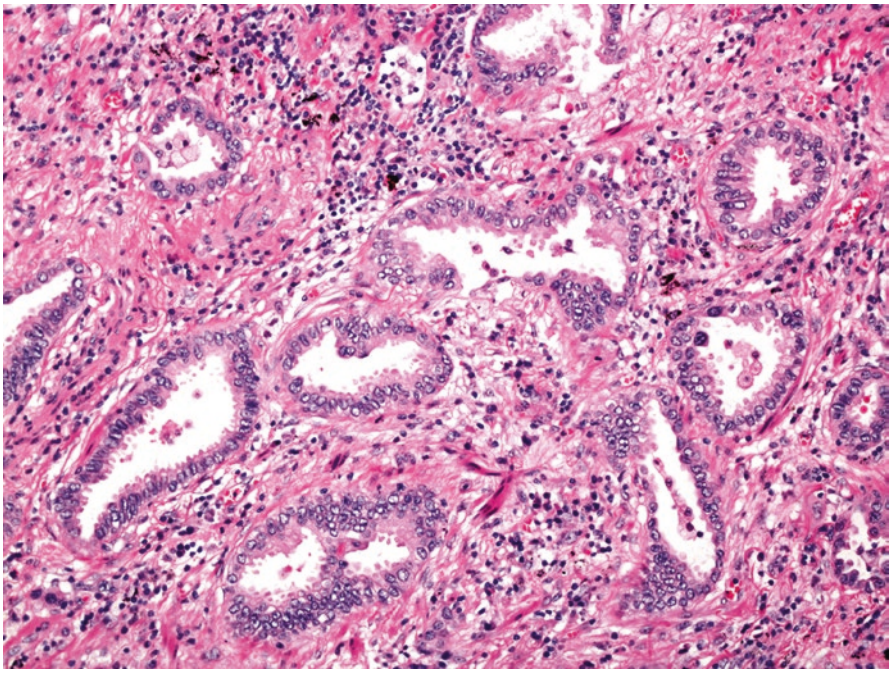

Fig. 5.2 Acinar pattern adenocarcinoma. Invasive glands embedded in a fibrous stroma. The presence of the fibroblastic proliferation helps recognise invasive disease

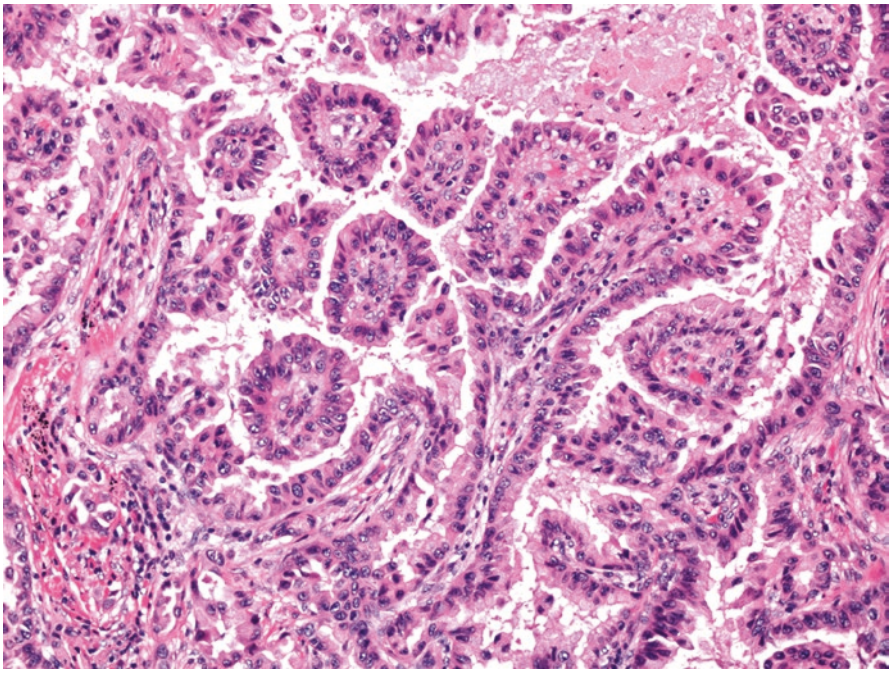

Fig. 5.3 Papillary pattern adenocarcinoma. Papillae are characterised by the presence of fibrovascular cores

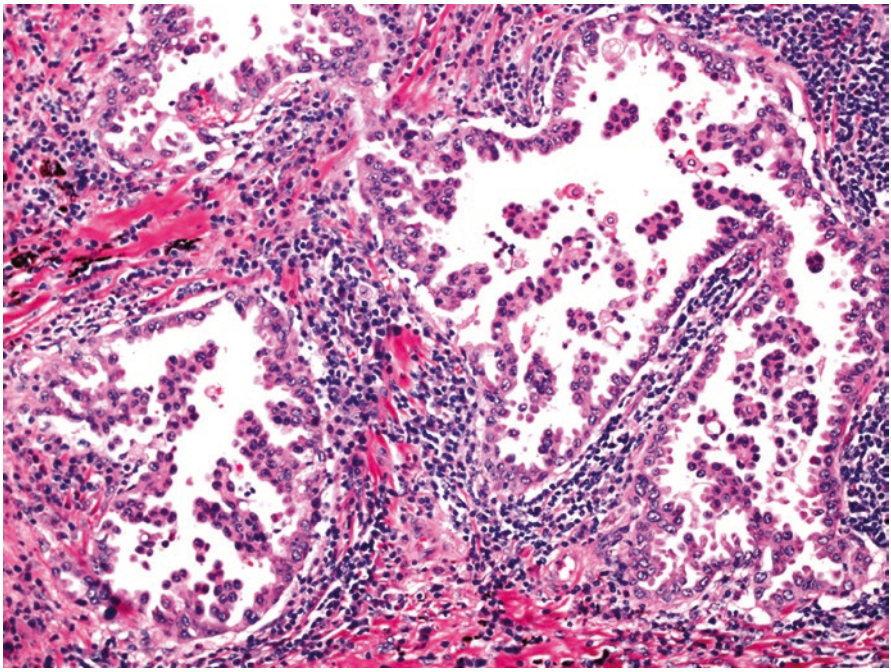

Fig. 5.4 Micropapillary pattern adenocarcinoma. Tufts and sprouts of adenocarcinoma cells often branch into complex patterns. These papillae lack fibrovascular cores

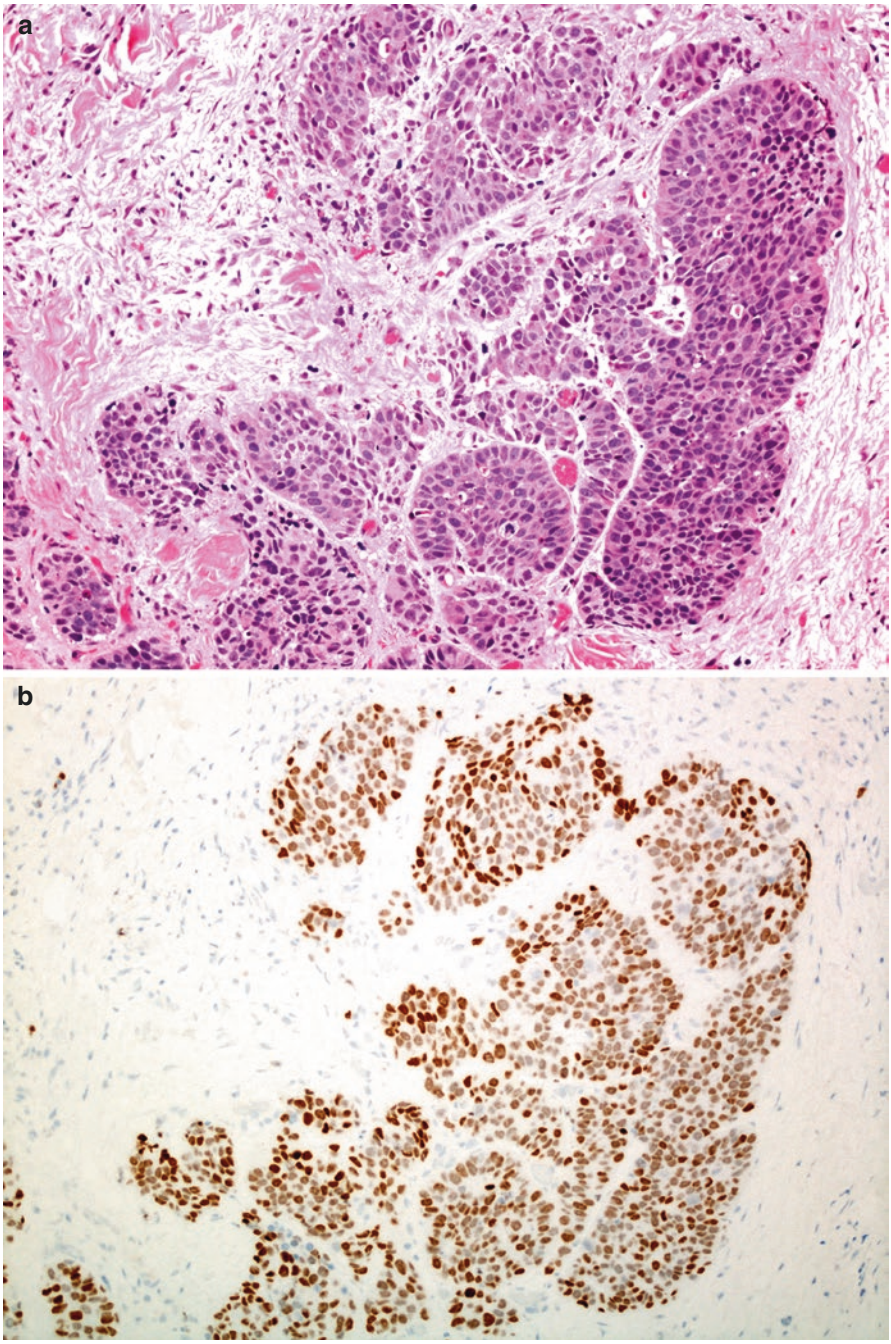

Fig. 5.5 Solid pattern adenocarcinoma. The least well differentiated pattern, lacking organised architecture (**a**); in some cases, undifferentiated carcinoma is defined as solid pattern adenocarcinoma when there is expression of thyroid transcription factor 1 (TTF1) (**b**).

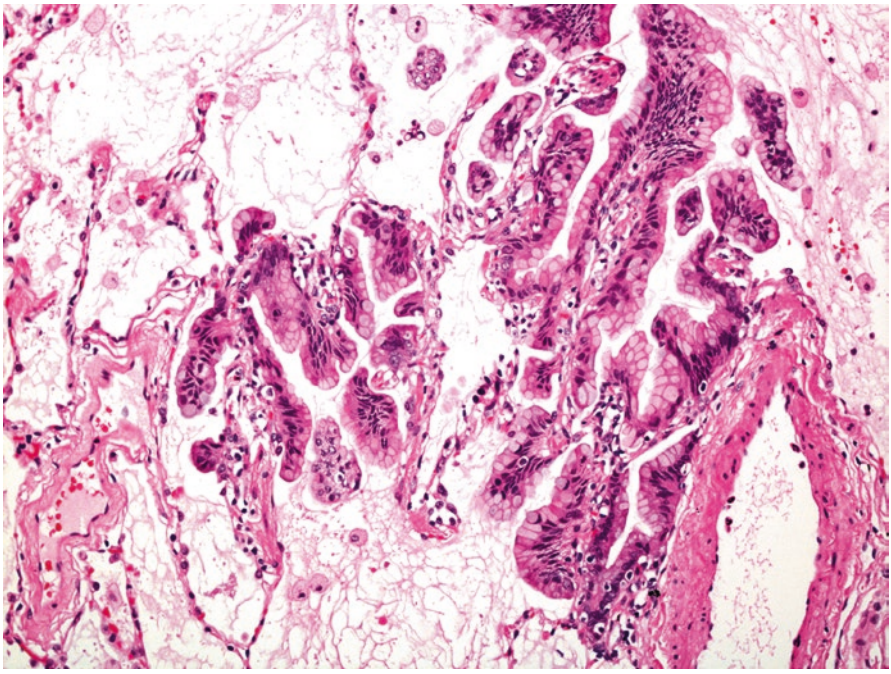

Fig. 5.6 Invasive mucinous adenocarcinoma. Whilst much of the tumour comprises mucigenic columnar tumour growing in a lepidic fashion as shown here, there are always foci of stromal invasion, somewhere in the lesion

Adenocarcinoma Variants

Clear cells or signet ring cells no longer define types of adenocarcinoma; they are now descriptive features only. The tumour formerly known as mucinous BAC is now referred to as invasive mucinous adenocarcinoma, acknowledging that whilst most of this lesion shows a particular pattern of lepidic growth and spread of mucigenic adenocarcinoma cells (Fig. 5.6), there are always foci of stromal invasion within the lesion. These lesions also have particular molecular features, described in Chap. 12. Colloid adenocarcinoma and foetal adenocarcinoma are retained as rare variants. An enteric pattern adenocarcinoma variant is also recognised, an important differential diagnosis of metastatic colorectal cancer.

Squamous Cell Carcinoma

In comparison to adenocarcinoma, less has changed with squamous cell carcinoma classification, although the changes are significant.

Preinvasive Lesions

The criteria for bronchial squamous dysplasia and squamous cell carcinoma in situ have not changed.

Invasive Squamous Cell Carcinoma

There are now three lesions recognised under this heading.

Keratinising squamous cell carcinoma is the classical lesion, recognised in all previous classifications and characterised by the defining features of morphological squamous differentiation, namely, individual cell keratinisation and the formation of intercellular bridges (Fig. 5.7). There are no criteria related to how much of either feature should be present in a tumour. The tumour cells are classically large, with abundant eosinophilic cytoplasm and large, pleomorphic nuclei showing granular chromatin. Like most things in lung cancer histopathology, however, squamous cell cancers can show a wide range of cytological detail.

A new category of non-keratinising squamous cell carcinoma was introduced. This is a significant development, since a squamous cell carcinoma diagnosis can now be

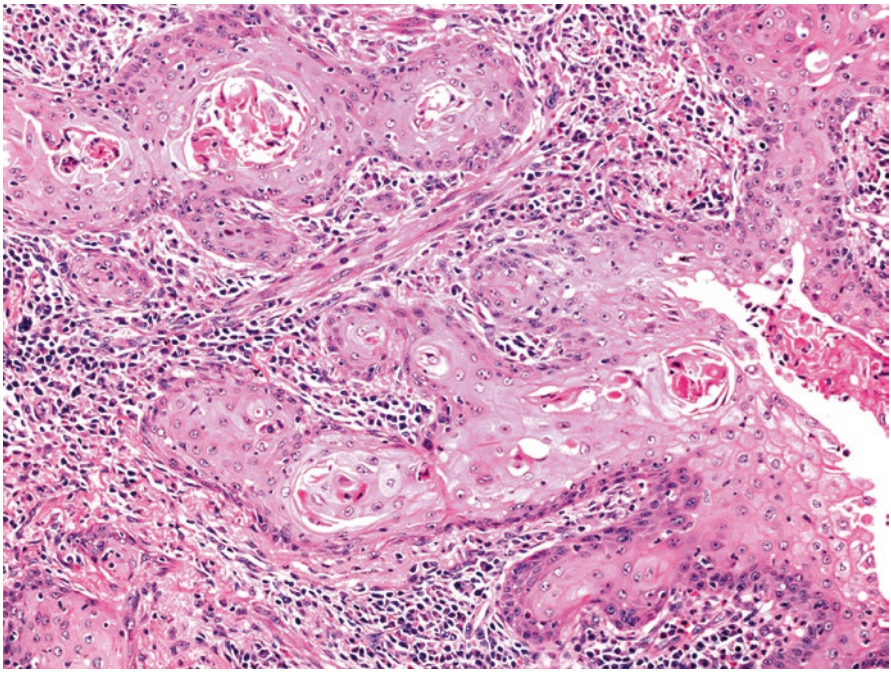

Fig. 5.7 Keratinising squamous cell carcinoma. In this example there is clear evidence of tumour cell keratinisation. Tumour islands are surrounded by an inflamed fibrous stroma

given to a resected tumour which lacks the very features which formerly defined a carcinoma as squamous cell type. Akin to IHC features being allowed to define solid adenocarcinoma, non-keratinising squamous cell carcinoma may be diagnosed in a morphologically undifferentiated tumour (lacking keratin or intercellular bridges) if there is 'strong and diffuse' expression of IHC markers which are associated with keratinising squamous cell carcinoma (p40, p63, cytokeratins 5 and 6) (Fig. 5.8).

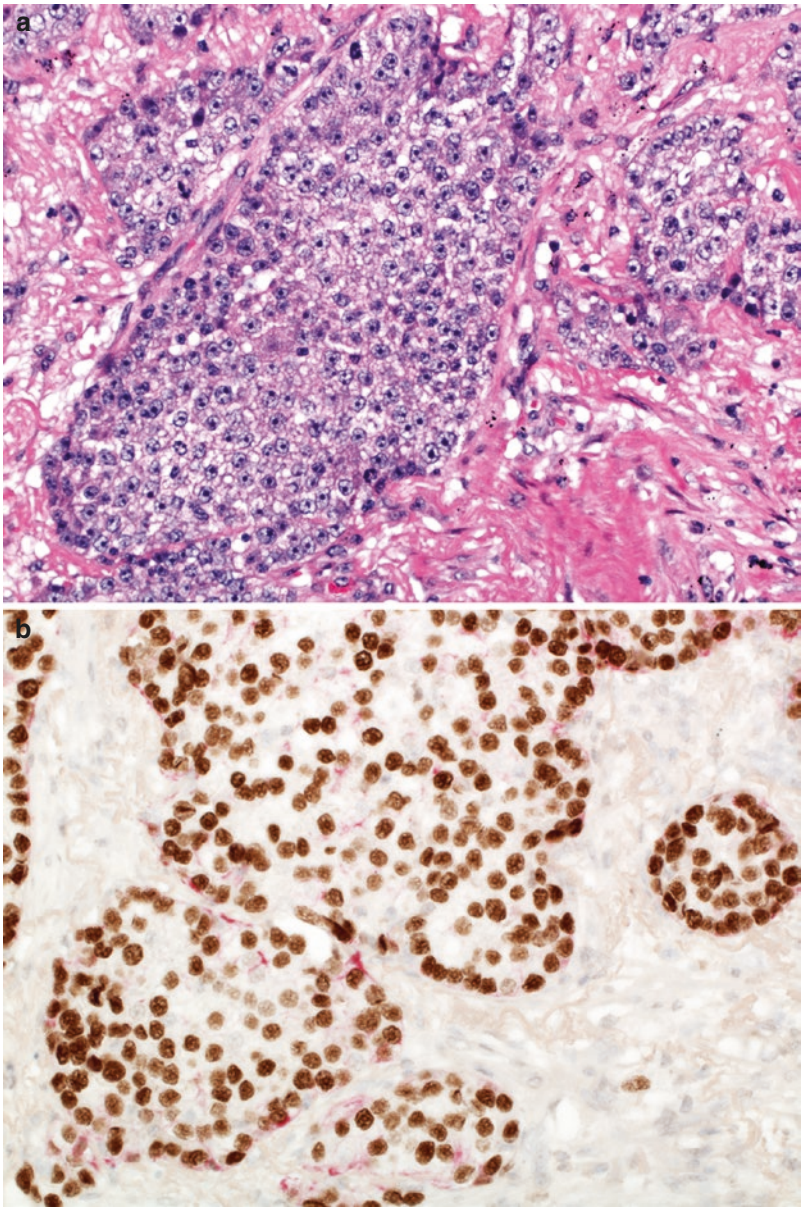

Fig. 5.8 Non-keratinising squamous cell carcinoma is defined as an undifferentiated non-small cell carcinoma (**a**) which expresses squamous-associated markers such as p40 (**b**)

Basaloid carcinoma, which was previously classified as a large cell undifferentiated carcinoma, is now subsumed into the squamous cell category. The histological criteria for this diagnosis have not changed. This move was made because all basaloid carcinomas have a strong 'squamous cell' IHC phenotype, described above (Fig. 5.9), and because they share molecular features with classical squamous cell carcinomas, yet also have unique features [18].

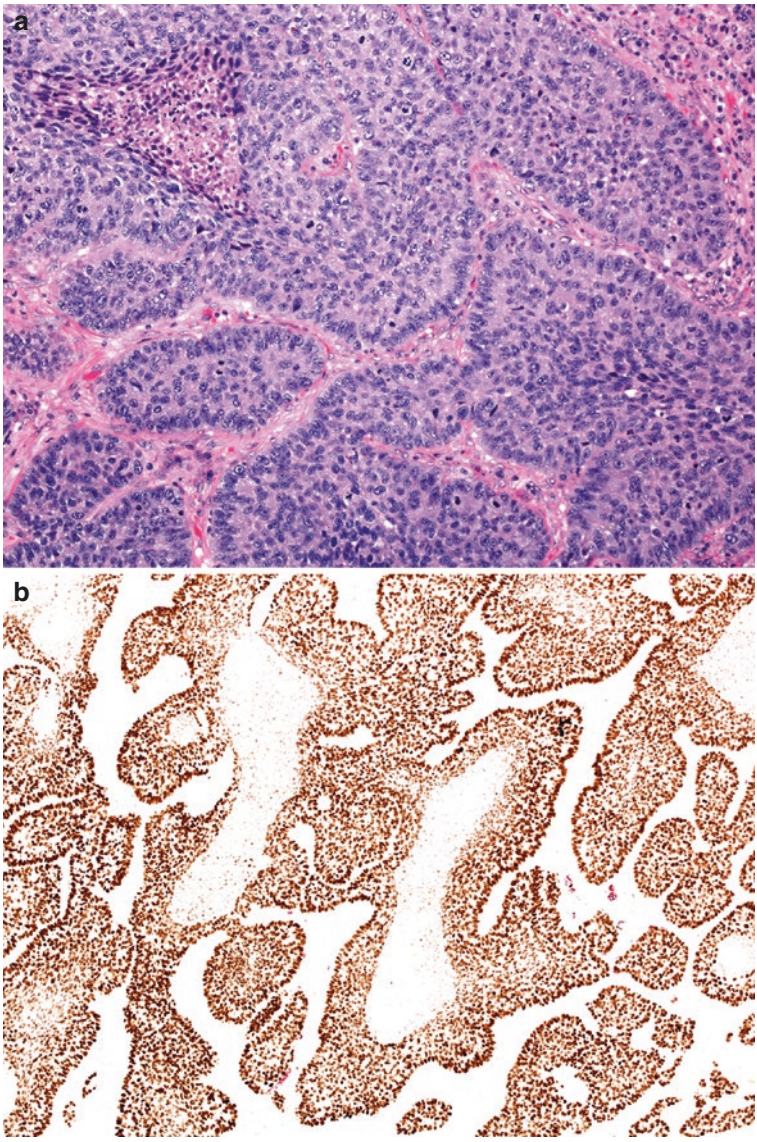

Fig. 5.9 Basaloid carcinoma shares morphological features with basaloid (basal cell) carcinomas encountered in many organs. Mitotically active tumours show discrete islands of relative small tumour cells with little cytoplasm (**a**). Tumour islands often show peripheral palisading and central comedo-type necrosis. These tumours strongly express squamous-associated markers such a p40 (**b**)

Neuroendocrine Tumours

In earlier editions of the WHO classification, tumours with neuroendocrine features were scattered in different subtype categories [2, 7]. Unified by neuroendocrine features, as evidenced by biochemical neuroendocrine differentiation shown by IHC and, in some, morphologic neuroendocrine features, it seemed practical to gather these lesions together into one category. IHC markers mostly used are CD56, synaptophysin and chromogranin. Molecular evidence that high-grade tumours, namely, small cell carcinoma and large cell neuroendocrine carcinoma, were in many ways similar [3, 19] and that both these tumours were radically different, in terms of morphology, behaviour and molecular features [20], from the low-grade carcinoid tumours was noted. Thus, these disparate neuroendocrine tumours are collected in a unified category, but the individual lesions are retained. Criteria for the diagnosis of each have not changed.

Preinvasive Lesions

Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) is a rare, but possibly under-recognised, widespread proliferation of bronchiolocentric neuroendocrine cells (NEC). As well as hyperplastic foci of NEC, this disease is associated with the development of multiple carcinoid tumourlets and, in most cases, carcinoid tumours. The latter are usually typical, but can be atypical—see below.

Invasive Neuroendocrine Tumours

Small cell carcinoma is the commonest type and is the most aggressive. Typically, these lesions comprise relatively small cells, averaging in diameter, less than that of three adjacent small resting lymphocytes (Fig. 5.10). It is recognised, however, that there may be variation in cell size, and some cases comprise variable numbers of larger cells. Cytoplasm is generally scant. Nuclear features are crucial to diagnosis; chromatin ranges from dense and featureless to finely stippled (so-called ‘salt and pepper’ chromatin) nuclei tend to be fusiform, and nuclear moulding is characteristic. Nuclear features like clumped, coarse chromatin and prominent nucleoli should question the diagnosis.

Large cell neuroendocrine carcinoma (LCNEC) is a rarer lesion. Cells are larger and have more cytoplasm, and nuclei lack ‘small cell’ features. Nuclei have open chromatin and prominent nucleoli, and moulding is not a feature. Organoid architecture with rosettes, trabeculae and peripheral palisading around tumour islands is characteristic, as is comedo-type necrosis (Fig. 5.11). Unlike small cell carcinoma, NEC differentiation must be demonstrated in this lesion, normally by IHC. A significant proportion of cases of LCNEC occur as a mixed lesion with invasive adenocarcinoma.

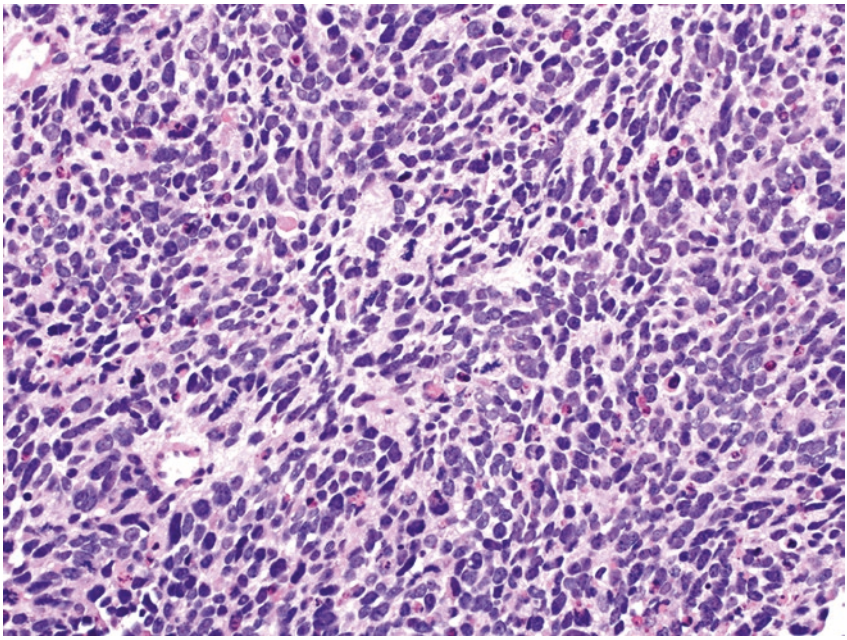

Fig. 5.10 Small cell carcinoma of the lung. This field shows relatively small tumour cells with featureless nuclei, little cytoplasm and nuclear moulding. Apoptosis and mitosis are evident

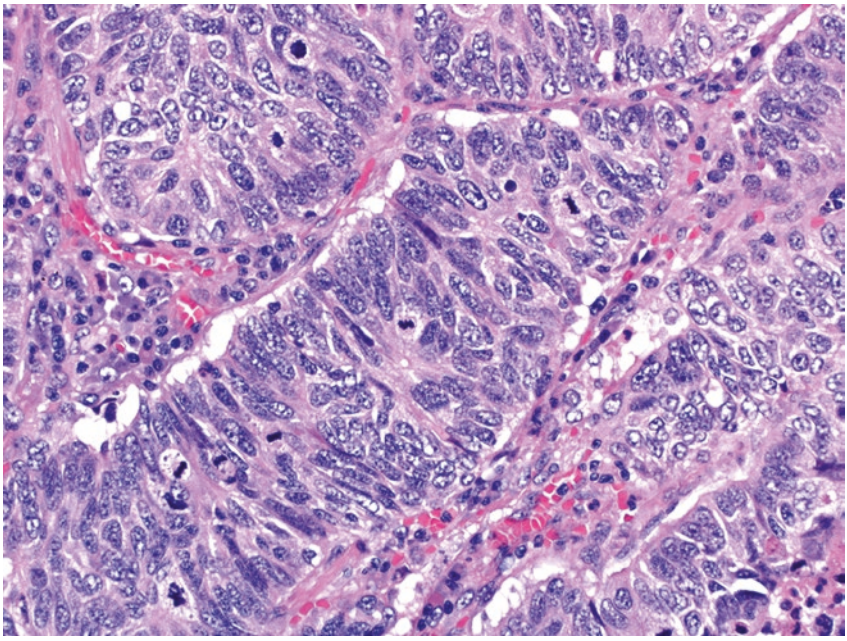

Fig. 5.11 Large cell neuroendocrine carcinoma (LCNEC). Taken at the same magnification as Fig. 5.10, this area shows trabecular architecture, a tumour rosette, abundant mitoses and nuclei with complex granular chromatin. This case was strongly positive for CD56, synaptophysin and TTF1 whilst chromogranin stained occasional cells

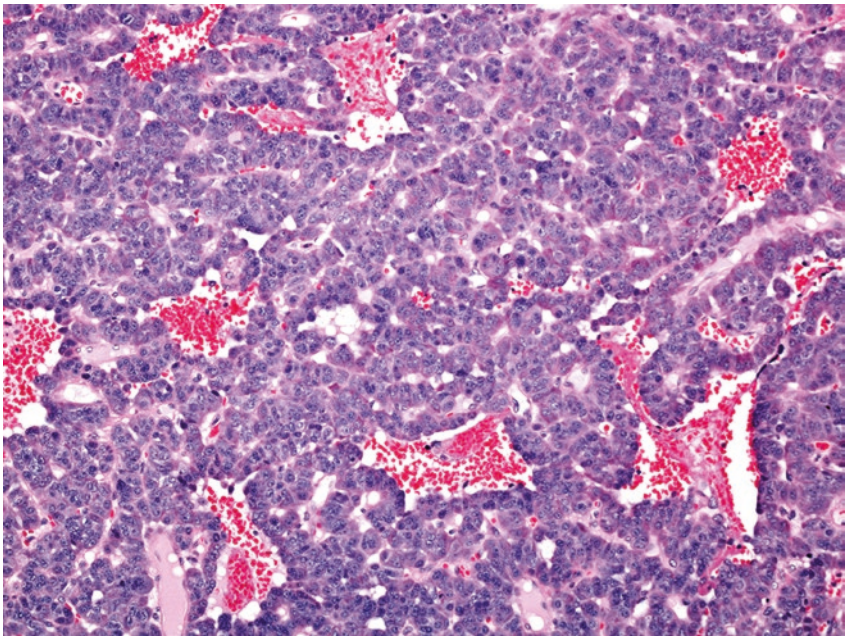

Fig. 5.12 Typical carcinoid tumour of the bronchus. In this example the trabecular and sinusoidal architecture is predominant, as is the vascularity. There was no necrosis and mitoses were very rare

Typical carcinoid tumour comprises nests, cords and occasionally acini, made up of regular, cuboidal cells with mostly regular nuclei (Fig. 5.12). It is the architecture and low-grade cytology which signals the diagnosis. Stromal features such as calcification, metaplastic bone and amyloid deposition can be found. These are invasive lesions, usually both endobronchial and confined to peribronchial tissues. Large, extensive lesions do occur, and all are associated with bronchial obstruction. Less often, a lesion comprises spindle neuroendocrine cells, usually in a peripherally located lesion. Assuming these architectural and cytological features, typical carcinoid tumour is defined by a mitotic count of no more than two mitoses per 2 mm^2 of tumour and a lack of necrosis. Cellular pleomorphism may be present and does not influence diagnosis. Carcinoid tumours usually strongly express chromogranin and synaptophysin.

Atypical carcinoid tumour shows similar histological features to its much commoner, typical relative, but is defined by the presence of a mitotic count exceeding two mitoses per 2 mm^2 of tumour or by the presence of often punctate tumour necrosis. IHC markers of cell cycle activity do not appear to provide any improved, or clinically more significant, definition or discrimination between the two carcinoid tumours and so are not included in diagnostic recommendations.

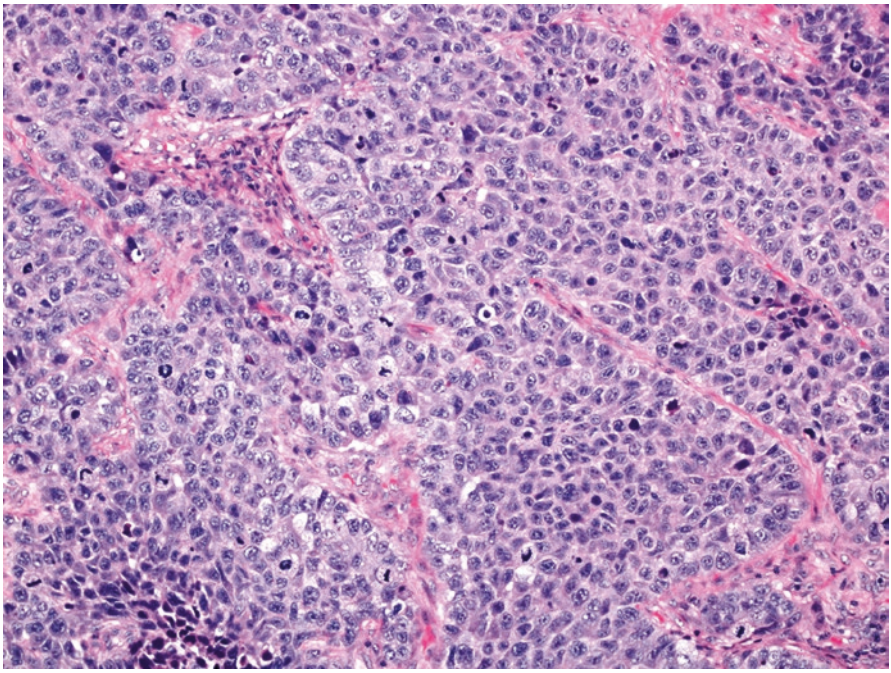

Fig. 5.13 This surgically resected primary lung cancer shows no morphological evidence of differentiation and no features of small cell carcinoma. Immunohistochemistry was 'null'; there was no staining with p40, cytokeratins 5 and 6 or TTF1. Mucin stains were also unhelpful. In this situation a diagnosis of large cell carcinoma is correct

Large Cell Carcinoma

Perhaps the most fundamental change in classification in the 2015 book is around the entity of large cell undifferentiated carcinoma. The changes were driven by two main factors. Firstly, the observation that a majority of cases morphologically classified as large cell carcinoma variably shared molecular features with other, differentiated tumour types [3], and secondly, that some large cell carcinomas, perhaps two thirds of cases, shared an IHC phenotype with either squamous cell or adenocarcinoma. Consequently, a large proportion of tumours which, in surgically resected specimens, would previously have been diagnosed as large cell carcinoma, are now allocated to either non-keratinising squamous cell carcinoma or solid adenocarcinoma, purely on the basis of the IHC phenotype (see above). A diagnosis of large cell carcinoma remains one solely for surgically resected specimens and is given when the IHC performed (a minimum of p40 and TTF1 is required) is either negative or inconclusive or cannot be performed (Fig. 5.13). When IHC is performed, about a third of former cases, based on morphology, remain in this category.

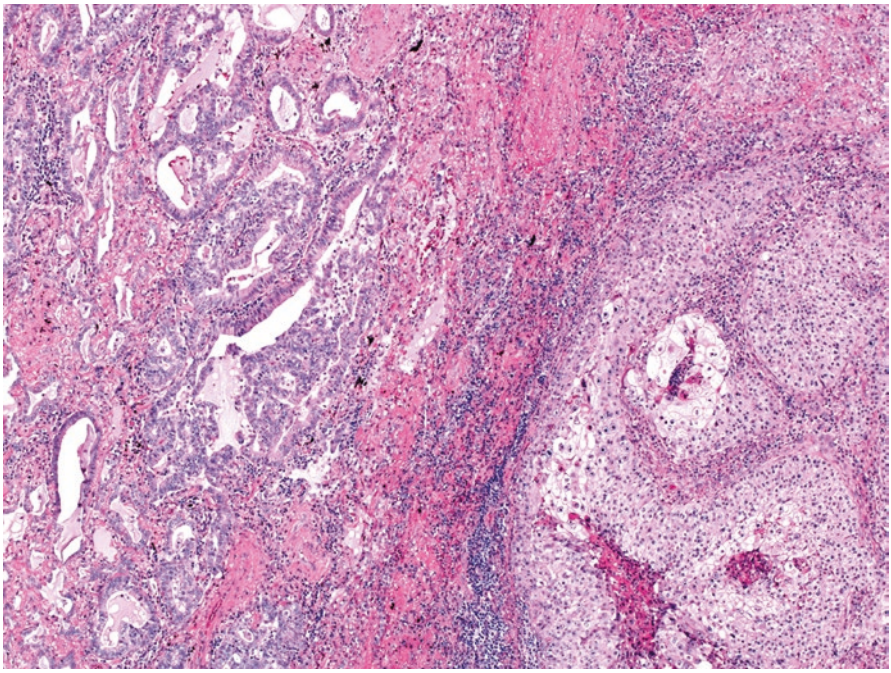

Fig. 5.14 Adenosquamous carcinoma. This surgically resected primary lung cancer shows both acinar adenocarcinoma (*left*) and poorly differentiated keratinising squamous cell carcinoma (*right*). The adenocarcinoma component of the tumour was in the minority but exceeded 10% of the lesion overall, permitting the diagnosis

Adenosquamous Carcinoma

This is a carcinoma showing a mixture of squamous cell and adenocarcinoma, each as defined and described above, where the overall lesion, when examined in toto in the context of a surgical resection, comprises at least 10% of the lesser component (Fig. 5.14). Following from the acceptance of IHC markers as *defining features* of squamous cell and adenocarcinoma, when a tumour is morphologically undifferentiated, these IHC markers may also be accepted in defining adenosquamous carcinoma, using the same rule as described above.

Pleomorphic (Sarcomatoid) Carcinomas

The only change in this category is in the preferred terminology—pleomorphic carcinoma—as opposed to sarcomatoid carcinoma. The latter is used as the collective term to include pleomorphic carcinomas and the ultrarare pulmonary blastomas and carcinosarcoma. Diagnostic criteria have not changed. Pleomorphic carcinomas are

diagnosed when at least 10% of the surgically resected tumour is examined in toto and comprises spindle cell, giant cell or extremely pleomorphic carcinoma. Lesions of such morphology in pure form are rare. Most cases show components of usual, differentiated squamous cell or adenocarcinoma.

Salivary Gland Tumours and Other Rare Lesions

The salivary gland tumours (mucoepidermoid carcinoma, adenoid cystic carcinoma and epithelial-myoepithelial carcinoma) remain in the classification unchanged. There are two new additions of note, from a molecular perspective. NUT carcinoma is a rare, often mediastinal, basaloid carcinoma of young patients, characterised and defined by the presence of a translocation involving the *NUT1* gene and overexpression of NUT protein shown by IHC. Also included is pulmonary myxoid sarcoma with *EWSR1-CREB1* translocation. Further details of the many rarer entities in the WHO classification are beyond the scope of this chapter.

Diagnosis on Small Diagnostic Samples

One of the recurrent and justified criticisms of previous WHO lung cancer classifications was that the classification and its rules were designed for use in the surgically resected tumour setting. Criteria required, as they still do, extensive examination of the tumour to exclude certain components or features, to determine the percentage of the lesion showing a feature or the quantification of a feature in a minimum area of tumour. The classification in full was not applicable to small biopsy samples or cytology-type preparations. Yet pathologists still attempted to apply the classification to such samples, and the result was error and misclassification, mostly for adenocarcinoma and, especially, large cell carcinoma. The latter is a diagnosis impossible to make, and never intended for, small diagnostic samples. Consequently, and in order to persuade pathologists not to guess the subtype when morphological features were absent, the term non-small cell carcinoma, not otherwise specified (NSCLC-NOS), was recommended [21, 22]. In oncology practice, a precise subclassification of lung cancer other than small cell carcinoma was not required, until relatively recently. When, however, treatment of patients with advanced NSCLC became specifically determined by NSCLC subtype [23, 24] and adenocarcinomas or tumours other than squamous cell carcinoma became selected for molecular profiling [25–27], the NSCLC-NOS diagnosis became a problem. It is worth remembering that most small samples classified as NSCLC-NOS due to a lack of morphological differentiation derive from differentiated carcinomas which have been inadequately sampled [28]. Recognising that about 75% of lung adenocarcinomas express TTF1 and all squamous cell carcinomas express p40, p63 or cytokeratins 5 and 6, a practice to refine the diagnosis of NSCLC-NOS by IHC was developed

[10, 29–35]. For the first time, the 2015 WHO classification describes the use of IHC to predict the likely NSCLC subtype in NOS cases. Thus we have a recommended nomenclature for small sample cases classified in this way: NSCLC, probably (or favour) squamous cell carcinoma—when p40, p63 or cytokeratin 5 and 6 staining is moderate to strong and diffuse—and NSCLC, probably (or favour) adenocarcinoma, when TTF1 expression is found. This change in practice has transformed pathological diagnosis of small diagnostic samples in lung cancer patients. Most patients present with advanced disease and never have their tumour surgically resected. Yet their treatment is intimately dependent upon an accurate and specific diagnosis, which includes molecular characterisation. This process has been hugely facilitated by this IHC-based approach to diagnosis, but only when required. Most cases can be adequately diagnosed without this IHC. It is imperative not to waste tissue on unnecessary IHC, as this can leave insufficient tissue for molecular analysis in some patients.

Conclusion

The 2015 WHO classification incorporates a subcategorisation of lung tumours with detailed criteria, based on robust historical observation, clinical experience and morphological examination, but complimented by immunohistochemistry. The classification in its current form is more user-friendly for pathologists, although more complicated and work intense for some diagnoses. It is more relevant for thoracic radiologists, surgeons, oncologists and lung cancer patients. The development of this classification was, at least in part, driven by our rapidly expanding knowledge of the molecular features of lung tumours and how they complement, but sometimes conflicted with, morphological diagnosis. Details of these molecular features in a range of lung cancer types are found in later chapters in this book.

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Part II
Testing Methods

Chapter 6
The Molecular Pathology of Lung Cancer: Pre-analytic Considerations

Lauren Ritterhouse and Lynette M. Sholl

Pre-analytic testing considerations are critical to the successful implementation of genomic testing in lung cancer. These considerations include the type and quality of diagnostic specimen that was obtained, how the specimen was handled, as well as how the specimen was processed at the grossing bench and in the histology laboratory. Additionally, great care should be taken to make the most judicious use of specimens in order to obtain all of the ancillary diagnostic information that is now being demanded for use in clinical care. Assuring that adequate diagnostic material is obtained for molecular testing requires multidisciplinary communication and coordination among pathology, oncology, radiology, surgery, and the molecular genetic laboratory. This chapter will review the criteria that determine specimen adequacy for molecular diagnostics and the myriad pre-analytic factors that can impact molecular test results, as well as strategies to streamline the implementation of such testing into routine pathology practices.

Specimen Adequacy

In the current management of lung cancer, there are increasing demands for pathogenetic characterization, including the histomorphologic subtyping of lung cancer, predictive immunohistochemistry (ALK, ROS1, PD-L1), and fluorescence in situ hybridization (FISH) studies, as well as molecular genotyping to identify driver mutations and potential targetable alterations. In addition, all of these growing demands are simultaneously occurring in the setting of minimally invasive

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_6

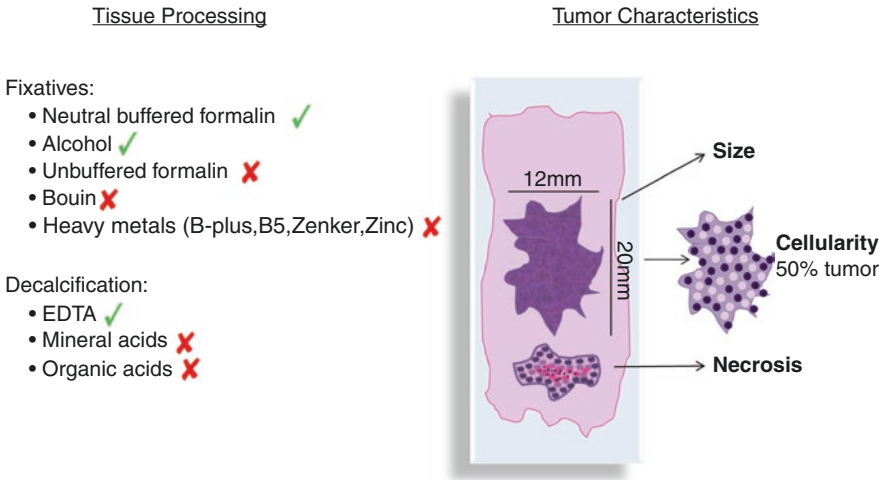

Fig. 6.1 Tissue processing and tumor characteristics involved in specimen adequacy assessment. Tissue processing fixatives and decalcification methods that are suitable for molecular testing are designated with a *green check*, and those that are contraindicated for use are designated with a *red* "*X*". Tumor characteristics important in adequacy assessment include two-dimensional size, % tumor cellularity in the area sampled (%, tumor nuclear content/total cellular nuclear content including inflammatory and stromal cells), and the presence of absence of necrosis

procedures yielding decreasing amounts of tumor tissue on which all of these studies must be performed. In this current scenario, the demands fall principally to the pathologist to identify and preserve tissue for the many ancillary studies that will guide the clinical care of the patient.

Multiple pre-analytic factors contribute to determining the adequacy of a particular specimen for molecular testing (Fig. 6.1). The principal factor is the volume of tumor that is present in the specimen, including the two dimensions present on the slide, as well as the third dimension (i.e., how much tissue remains in the paraffin block, how many unstained slides or scrolls are available). The amount of tissue present influences the amount of DNA that will be isolated from the specimen. Depending on the particular molecular platform that is being used, some minimum amount of input DNA will be required for testing.

The second and often more challenging factor to consider is the percent tumor cellularity present in the sample. When determining cellularity, it is important to remember that it is the nuclear content that should be assessed, not merely the area on the slide that the tumor cells are occupying. For example, inflammatory cells are much smaller than most tumor cells and can be present in a high density, which can contribute to a large percentage of the total DNA content present. Additionally, there are many instances in which a smaller specimen with a higher tumor cellularity (e.g., cytologic specimen) is preferable to a larger specimen with a very low tumor cellularity (Fig. 6.2). This is due to the fact that "contaminating" non-tumor DNA will dilute the mutant allele fraction, which could then fall below the analytic

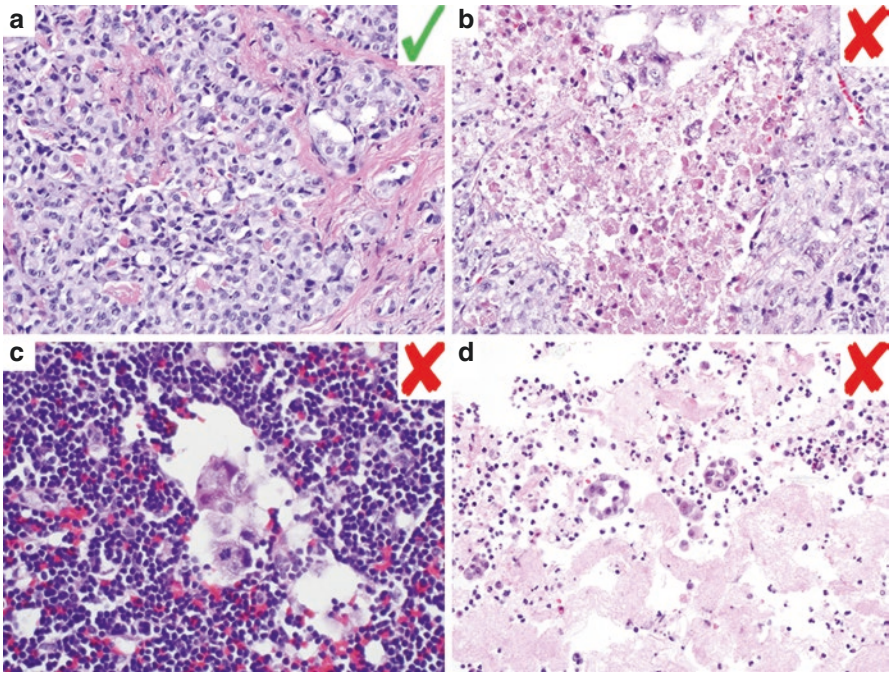

Fig. 6.2 Histologic examples of adequate and inadequate lung cancer specimens for molecular testing. (**a**) H&E histologic section from a lung adenocarcinoma specimen that is adequate for molecular testing with high tumor cellularity (minimal stromal and inflammatory cells) and no necrosis. (**b**) H&E histologic specimen from a lung adenocarcinoma specimen that is inadequate for molecular testing due to abundant necrosis. (**c**) H&E histologic section from a lymph node metastasis that is inadequate for molecular testing due to very low tumor cellularity. (**d**) H&E cytology cell block specimen that is inadequate for molecular testing due to scarcity of tumor cells and low overall tumor cellularity

sensitivity and limit of detection present in the molecular testing platform. However, estimating the true tumor cellularity by eye can be difficult, and studies have shown poor interobserver variability [1].

Necrosis is often encountered in pathology specimens and can significantly alter the quality of the DNA isolated (Fig. 6.2), as necrotic cells undergo rapid nonspecific digestion of nucleic acids. However, the effect of necrosis on molecular testing is variable and depends on the percentage of the cells that are necrotic. While there are no strict guidelines dictating percent necrosis acceptable for molecular testing, studies across multiple tumor types have chosen <20% as the cutoff [2–4]. Thus, if possible, it is best to select specimens that have minimal tumor necrosis present or perform a tumor enrichment strategy such as microdissection to avoid necrotic regions. Although degraded DNA samples are not suitable for most molecular techniques, optimization strategies exist for DNA fragments <100 bp, many of which have been developed for use in forensic pathology. Such strategies include ligase detection coupled with PCR [5], as well as the use of short amplicons in multiplex PCR assays [6].

Finally, there are multiple factors related to sample handling that can significantly influence the suitability of the specimen for molecular testing (Fig. 6.1). The most commonly used fixative that has been shown to be well suited for the use in molecular testing is neutral buffered formalin [7, 8], which is the most widely used and preferred fixative for molecular testing [9]. In contrast, any fixatives that contain strong acids or heavy metals are generally not suitable for molecular diagnostics.

Importantly, the adequacy of a particular specimen must also be interpreted in the specific context of the molecular assay that is to be performed, as the requirements will vary based on each test.

Tissue Processing and Fixatives

The most commonly used fixative suited for use in molecular testing is neutral buffered formalin (NBF). Several types of DNA damage have been identified in formalin-fixed tissues, which can serve as sources of sequence artifacts. Formaldehyde, the main component of formalin, is highly reactive with DNA bases and proteins and generates various cross-links between protein, DNA, and histones. Formaldehyde cross-links DNA by reacting with imino groups involved in base pair hydrogen bonds, thereby weakening the bonding strength of double-stranded DNA [10]. Additionally, formaldehyde cross-links DNA bases with histones, which results in a conformational change, also reducing the stability and causing partial denaturation of double-stranded DNA [11]. Deamination of cytosine bases is also seen in formalin-fixed tissues [12–14]. On average, molecular testing on formalin-fixed tissue requiring DNA segments less than 300 base pairs is successful, while those requiring longer stretches of DNA are less successful [15]. Formalin fixation also causes random nucleotide base substitutions, which can lead to false-positive results, and is particularly problematic in the setting of low DNA concentrations, in detection of rare variants, or in ultrasensitive assay designs [16–18].

In contrast, unbuffered formalin oxidizes into formic acid over time, which subsequently causes degradation of nucleic acids [19]. Similarly, any fixatives containing acids are also not suitable for molecular testing, including Bouin's fixative, which contains picric and acetic acid, as acidic solutions fragment DNA significantly [20, 21].

When the specimen of interest is a bony metastasis, the decalcification process is a common problem for molecular laboratories. Most commercial formulations of decalcification solution contain acid, either mineral or organic acids, which are used to rapidly decalcify bone specimens. The best solution to decalcify tissues needed for molecular testing is to use non-acid-containing chelating solutions, such as EDTA, as it leaves the nucleic acids relatively intact [21, 22]. However, the slow rate of EDTA-based decalcification is generally incompatible with most surgical

pathology workflows given expected turnaround times. Several commercial solutions and other supplemental methodologies are available to speed the process of decalcification when using EDTA, such as the addition of heat and agitation. Due to increasing requests for molecular testing on many specimen types, including core biopsies from bony metastases, there will likely be future demands to process bony specimens in such a way that preserves suitability for molecular testing.

Alcohol, in the form of 70% ethanol, is another fixative that is suitable for molecular testing, as it has been shown to preserve DNA as well as, or even superior to, neutral buffered formalin [23–25]. Alcohol is not a favored fixative for tissue specimens in routine histology laboratories for a multitude of reasons, including cost and compatibility with other FDA-approved ancillary studies; however, it is commonly used in cytology preparations. Therefore, many cytology specimens are indeed suitable for DNA-based molecular testing.

In addition to acid, any fixatives containing heavy metals (mercury, zinc, etc.), such as B plus, B5, and Zenker, will interfere with molecular testing. While the DNA itself may be intact, the heavy metals in these solutions compete with the cofactor magnesium that is required for most DNA polymerases and other enzymatic reactions [26–28].

Determining Assay Requirements

Selection of a molecular diagnostic testing platform depends on the clinical question that is being asked, the timing of test requests relative to clinical management, as well as the specimens that are available for testing. Additionally, the testing platform that is employed will influence what mutant allele fraction can be detected as well as how much DNA input will be required (Table 6.1). Before the widespread implementation of massively parallel sequencing, most of the molecular diagnostic

Table 6.1 Molecular diagnostic testing platform comparison

Technique	Analytic sensitivity (% mutant allele)	DNA requirement	References
Sanger sequencing	15–20%	100 ng	[29, 30]
PCR-RFLP	1–5%	50 ng	[34–37]
Real-time PCR	0.5–5%	15–20 ng	[35, 38, 39]
Digital PCR	0.1%	10–50 ng	[40]
Pyrosequencing	5–10%	25–100 ng	[41–43]
Single nucleotide base extension and size separation	5–12.5%	200 ng	[44–46]
Massively parallel sequencing			
Hybrid capture	3–5%	50–500 ng	[31]
Amplicon	3–5%	10–50 ng	[32]

assays available were single gene assays. While Sanger sequencing has long been considered a gold standard for sequencing methodologies, significant disadvantages to this technique include the limitation of analyzing a single amplicon (such as a single gene exon) at a time and low analytic sensitivity. As the analytic sensitivity of Sanger sequencing is approximately 25% mutant allele fraction, this requires a tumor specimen with at least 50% tumor cellularity, which would exclude a significant number of specimens from testing [29, 30].

Currently, in the realm of less expensive sequencing technology, multi-gene massively parallel sequencing assays are becoming more commonly used as a first-line test choice. While the large multi-gene panels are more comprehensive in their approach, they may not be as sensitive as other more targeted approaches, such as specific PCR-based assays. For example, if one is interested in identifying a subclonal event, such as a resistance mutation present in a small fraction of cells, a more tailored assay is better suited to answer that question. While both PCR- and NGS-based assays have their strengths and weaknesses, they can also be tailored to address specific needs and requirements.

PCR is a technique used to amplify a few copies of target DNA to several orders of magnitude, generating thousands to millions of copies of a particular sequence. Multiple PCR-based testing methods have been used routinely in molecular diagnostics, including quantitative PCR (real-time or QPCR), allele-specific PCR, and digital PCR (dPCR). PCR-based assays provide highly sensitive mutation detection (as little as 0.5% allele fraction), but only a single target can be interrogated in a single reaction and that target must be known a priori.

In contrast, massively parallel sequencing examines many different targets at once, with analytic sensitivity depending of assay design including breadth of coverage and depth of sequencing, but optimally down to 3–5% mutant allele frequency. Several different methodologies exist for library preparation prior to massively parallel sequencing, including hybrid capture and amplicon-based strategies. Hybrid capture approaches require a higher input DNA (~50–500 ng DNA) and use biotinylated probes that hybridize to the genes or exons of interest, which are subsequently purified, modified with adapters, and then sequenced [31]. In contrast, amplicon-based methods utilize PCR primers to the genes and exons of interest with multiple subsequent PCR amplification rounds, thus requiring less input DNA (~10 ng DNA) [32].

While the basic limitations of each assay are as stated above, assays can be tailored to a specific target and question. For example, new assay designs incorporating molecular barcodes allow for the removal of significant noise occurring secondary to sequencing errors and can increase the analytic sensitivity of the assay to 0.1% mutant allele fraction [33]. Particular applications for which this would be helpful include serial disease monitoring for the emergence of a recurrent or resistant clone, particularly in the form of circulating tumor DNA (ctDNA). As with any laboratory developed test, the lower limits of acceptability, with regard to sensitivity and specificity, must be determined for each assay that is developed.

DNA Yield from Archival Tissue Samples

As the majority of lung cancer specimens available for molecular diagnostic testing are formalin-fixed paraffin-embedded tissues (FFPE), the validation and use of these specimens in a variety of molecular testing platforms are central to the clinical implementation of genomic testing. Depending on the specific parameters of the specimen as detailed earlier in the chapter, the minimum tissue needed will vary based on testing platform. It has been extensively documented that multiple testing methodologies can be successfully performed on DNA isolated from archival FFPE tissues. For example, OncoPanel testing performed at the Center for Advanced Molecular Diagnostics at Brigham and Women's Hospital had success with 96% of nearly 3900 archival FFPE specimens that were run on a targeted panel massively parallel sequencing assay with a minimum input of 50 ng DNA [47]. These success rates were obtained with minimum tissue requirements of at least 20% malignant cells and at least 3 mm in greatest linear dimension of tumor [47].

Archival cytology direct smears have also been shown to be suitable for molecular testing, as adequate DNA was successfully isolated for PCR with amplicons ~200 bp in length from direct smears that were at least 1 year old [48]. Direct smears stained with both Giemsa and Diff-Quik were shown to have DNA quality equal to those from matched frozen controls and were suitable for multitargeted sequencing on a large panel of genes [49]. In contrast to direct smears, increased DNA degradation over time has been documented in liquid-based cytology (LBC) specimens, as successful PCR amplification from archival LBC specimens required primers for PCR products <200 bp [48]. It is important to note that every assay must be validated for every specimen type to be used in the clinical diagnostic test.

Tumor Enrichment Techniques

Multiple techniques exist to isolate tumor cells from FFPE tissue blocks (Table 6.2). These techniques range from simple scrolls with no attempt to enrich for tumor content to more sophisticated methodologies that can capture even a single tumor cell. While it depends on the particular specimen, in most instances, it is necessary to perform some level of enrichment for tumor cells when isolating tumor DNA from FFPE blocks. This greatly increases the number of specimens that is adequate for molecular testing and enhances the suitability of the isolated input DNA for the particular analytic sensitivity of the molecular methodology in use. However, it is also important to keep in mind that the minimum tumor cellularity requirements are based on the assumption of no tumor heterogeneity, and subclonal events may be below the analytic sensitivity.

If no tumor enrichment technique is needed, for example, in tissue blocks which consist predominantly of tumor with minimal stroma or inflammatory cells, then either using thick (e.g., 50 micron) scrolls or scraping the entirety of a series of

Table 6.2 Tumor enrichment techniques

Technique	Method	Technicality	Precision
No enrichment	Thick micron scrolls, scraping entirety of unstained slide	✓	✓
Macrodissection	H&E examined and marked by pathologist for subsequent tumor dissection without microscope	✓✓	✓✓
Manual microdissection	H&E examined and marked by pathologist for subsequent tumor dissection under light microscope	✓✓✓	✓✓✓
Laser capture microdissection	A laser-coupled microscope is used to capture the tissue of interest	✓✓✓✓	✓✓✓✓

unstained slides would be adequate. Presuming the adequacy of the slide had been previously documented, these techniques would require no additional pathology review for subsequent tumor and DNA isolation. However, it is important to remember that even tissues, which appear to be >90% tumor by light microscopy, may be significantly contaminated by inflammatory and stromal cells. Additionally, tumor content can change as deeper sections into the block are obtained.

A common tumor enrichment technique that is employed by many molecular diagnostic laboratories is that of macrodissection, as it provides an adequate amount of tumor enrichment, yet requires limited technical expertise. In this procedure, a hematoxylin and eosin (H&E)-stained slide obtained from a tissue level immediately adjacent to a series of unstained slides is examined by a pathologist, and the area of interest is circled with a marking pen by the reviewer. This technique serves to remove large areas of tissue that do not contain tumor, as well as to concentrate the isolation on viable areas of the tumor that are free of necrosis and significant inflammation. The technologist who is isolating the tumor cells will use the marked H&E as a guide to scrape the appropriate area of the unstained slide or core the appropriate area of the block. Similarly, manual microdissection can be performed, in which the technologist isolating the tumor cells uses the aid of a light microscope when performing the dissection. This latter technique may be facilitated by the use of a stereomicroscope [50].

Finally, sophisticated techniques such as laser capture microdissection (LCM) are available which are capable of precision down the single cell level. However, this is a technically complex procedure, requires expensive equipment, and is currently used by only a small number of laboratories. In clinical practice, many specimens will require some level of tumor enrichment and most are amenable to simple macro- or microdissection techniques.

Role of Cytology Specimens

In an attempt to minimize invasive procedures, a fine needle aspiration may be the procedure and specimen of choice for diagnostic testing. Additionally, there may be a concurrent fine needle aspiration performed at the time of core biopsy, and it has

Table 6.3 Cytology and surgical specimen comparison for molecular diagnostics

	Cell blocks	Direct smears	Liquid-based cytology	Surgical biopsy
Performance in molecular diagnostics	✓	✓✓	✓✓[a]	✓
On-site assessment	–	✓	–	✓
Fixative	Formalin	Alcohol	Variable	Formalin
Nuclei preservation (FISH)	–	✓	✓	–
Slide based record retained	✓	–	–	✓

[a]Archival liquid-based cytology specimens show inferior performance to archival direct smears [48]

been shown that fine needle aspiration samples frequently provide increased overall cellularity, higher tumor percentage, and improved sequencing quality than concurrently acquired core needle biopsy specimens [51]. Superior DNA retrieval has been documented in cytology specimens, with an average DNA concentration of 299 ng/μL for cytology specimens and 171 ng/μg for paired FFPE tissues in one study [52]. Therefore, if the surgical or biopsy specimens prove to be inadequate, available cytology specimens should also be reviewed for adequacy, if not prioritized for testing.

Multiple cytology specimens have been used in molecular testing, including cell blocks which are the most obvious choice due to the similarity in processing as tissue FFPE blocks, but also direct smears and liquid-based cytology specimens [48, 53, 54] (Table 6.3). Adequacy of cell blocks is determined by following the general same principles as is used in tissue samples in regard to the quantity and percent cellularity of tumor present. While adequacy assessment of smears or thin preps is similar in that there must be a specific burden of tumor present depending on the application, the use of this specimen type has generally been discouraged in the past because the use of this material necessarily destroys the physical diagnostic specimen [9]. The ready availability of digital slide scanning equipment in most pathology and/or molecular testing centers allows laboratories to circumvent this problem; laboratories are encouraged to obtain and archive a scanned slide image up to 200× magnification prior to scraping the tumor material.

Direct smears have been shown to be useful in next-generation sequencing, PCR-based assays, as well as in FISH studies, with the distinct advantage of having whole nuclei present [48, 53, 54]. Betz et al. demonstrated a 97% concordance between direct smears and cell block preparations when performing *ALK* FISH [54]. Additionally, this study also demonstrated that judicious use of a single, cellular smear can serve multiple purposes. For example, a portion of a single smear can be microdissected for use in a PCR-based molecular testing for *EGFR* testing, while the remaining slide can be destained for *ALK* FISH testing and then restained afterwards with Diff-Quik for retention of diagnostic material [54]. The availability of rapid on-site evaluation (ROSE) performed by pathologists is another advantage to using direct smears and increases the likelihood of obtaining sufficient diagnostic material.

Liquid-based cytology samples have also been used with success in molecular diagnostics and have the similar advantage to direct smears of containing whole nuclei for FISH studies. For example, *ALK* FISH studies have been successfully performed on liquid-based cytology specimens [55]. A likely source for liquid-based cytology specimens in lung cancer testing would be that of a pleural fluid specimen from a patient with stage IV disease, which may serve as an excellent specimen for molecular testing.

As increasing number of tests are required in the ongoing management of lung cancer patients, minimally invasive procedures will be relied upon heavily to obtain diagnostic material. Cytology specimens are emerging as an excellent source of material for molecular testing and incorporating them into the molecular diagnostic workflow, and validation processes will become increasingly necessary.

Importance of Communication with the Patient Care Team

One of the most crucial steps to ensuring that specimens obtained for molecular diagnostic testing are adequately triaged, processed, and allocated is the coordination and communication plan between the multidisciplinary patient care team (Fig. 6.3). All members of the patient care team, including radiology, surgery, oncology, in addition to surgical pathology, molecular pathology, and cytopathology must be in communication and aware of the need for molecular testing. As the acquisition of tissue for diagnostic studies is often an expensive and invasive medical procedure with associated risks for the patient, it is essential not to misappropriate the specimen by either mishandling or wasting precious diagnostic tissue.

The oncologist clinically managing the patient's care is most often the ordering physician for the molecular diagnostic test, and he or she must notify the interventional radiologist or surgeon that the specimen obtained will be needed for molecular diagnostics. Subsequently, communication between the interventional radiology team and cytopathology for rapid on-site specimen adequacy evaluation can ensure the presence and adequacy of tumor for molecular testing. To facilitate appropriate specimen is handled when received in either the cytology or histology laboratories, the pathology requisition form should be appropriately labeled to indicate a need for molecular testing.

The implementation of standardized up-front histology sectioning to create serial unstained slides that are cut without intervening waste and limit facing into the block on multiple occasions can greatly impact the rate of specimen adequacy. At one institution, the execution of such a policy improved the specimen adequacy results for tumor next-generation sequencing from approximately 70% to greater than 90% [56]. In addition to ensuring specimen adequacy, this also streamlines workflow and can help to improve turnaround times for molecular testing.

Another workflow implementation that can greatly improve the efficiency of molecular testing is standardized documentation of the most appropriate tissue block (smear or liquid-based cytology specimen in the case of cytology) available for molecular testing by the pathologist. This documentation should also include, at

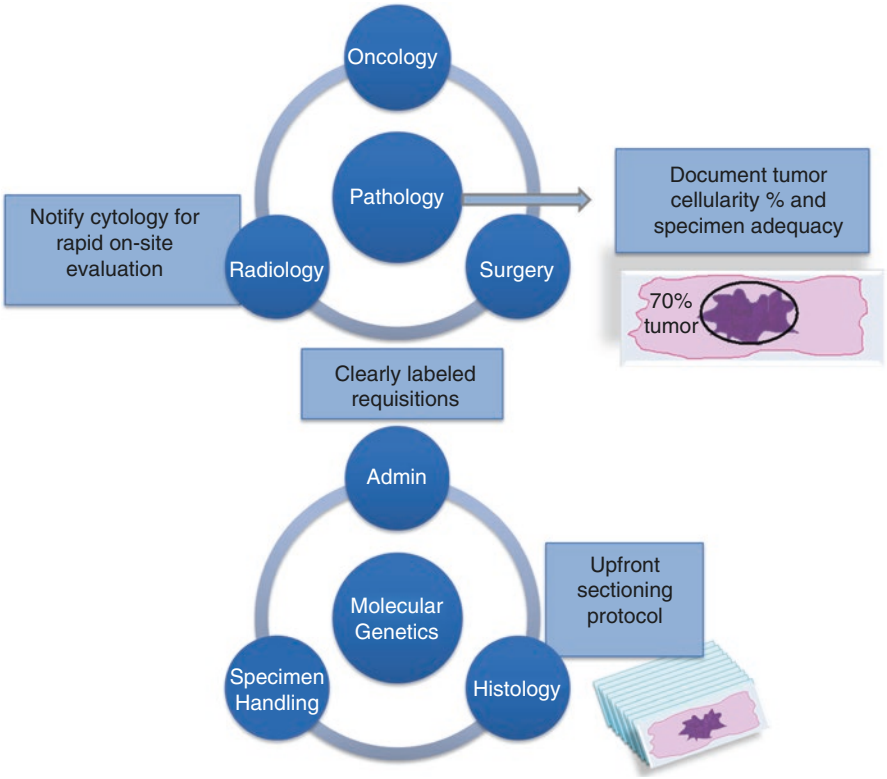

Fig. 6.3 Multidisciplinary patient care team communication. Coordination and communication is necessary for successful employment of genomic testing in lung cancer and requires joint efforts from multiple disciplines, including pathology, medical oncology, radiology, and surgery. Admin, administrative support

a minimum, the size of the tumor present and the percent tumor cellularity. This can be invaluable information when the molecular laboratory receives an order for a diagnostic test and minimizes the amount of time spent searching for an appropriate specimen. Finally, it is also important to notify the oncologist or requesting physician if there is not a specimen suitable for molecular testing and that additional material will need to be obtained if clinically indicated.

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Chapter 7
Mutation Testing of Lung Cancer Biomarkers (Excluding IHC and NGS)

Bryce Portier

Introduction

It is well established that a diagnosis of lung cancer carries significant morbidity and mortality. Worldwide there are more than 1.8 million new lung cancer cases diagnosed annually and over 1.5 million lung cancer-related deaths [1]. Biomarker testing has taken a central role in identifying genomic targets for personalized lung cancer therapy. In most cases, cell morphology examined by hematoxylin and eosin (H&E) staining facilitates classification of lung cancer as either non-small cell lung cancer (NSCLC) which accounts for 85% of new diagnosis or small cell lung cancer which accounts for approximately 15% of new diagnosis [2]. The NSCLC classification can be further divided based on morphology/immunohistochemistry into adenocarcinoma (50%), squamous cell carcinoma (35%), and large cell carcinoma (15%) [3, 4]. In the event morphologic type of NSCLC cannot be resolved by H&E, a limited panel of immunohistochemical markers (TTF1/Napsin A or p40/p63) can be utilized to resolve histologic subtype [2]. Determination of morphologic class, followed by biomarker profiling, is critical for generating accurate prognostic data and predictive selection of effective targeted therapy. The pioneering work performed on *EGFR* and *ALK* has paved the way for future advancement and improved refinement of lung cancer therapy. Current lung cancer biomarkers utilized for clinical prognostication and therapy prediction are limited and include *EGFR*, *ALK*, and recently *ROS1*. Many exploratory biomarkers are under investigation; however, there is not enough evidence to justify widespread

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_7

clinical testing. In addition to discussing current and investigational biomarkers in NSCLC, this chapter will also examine multiple methodologies currently utilized for mutation testing. Specifically, the utility of IHC and NGS will not be described in this chapter; these two topics will be explored in detail in subsequent chapters. This chapter will focus on describing mutation testing methodologies followed by an overview of lung cancer biomarkers.

Methods for Testing Lung Cancer Biomarkers

There are numerous commercial assays and methodologies available for molecular testing lung cancer biomarkers. In the last few years, there has been rapid growth in our genomic understanding of lung cancer, and as a result, the number of biomarker variants available for clinical testing has increased. Selection of a specific testing modality is largely dependent on testing volume, number of analytical targets, required sensitivity, and equipment available/expertise. The following section will highlight commonly encountered methodologies for lung biomarker testing (excluding immunohistochemistry and next-generation sequencing) and will include a discussion on minimal invasive lung biomarker testing.

Sanger Dideoxy Sequencing

Sanger sequencing is the foundational method for lung cancer biomarker testing, such as *EGFR* mutation detection, and remains the gold standard for detection of single base pair substitutions and small insertion/deletions (in/dels). Sanger sequencing is a methodology based on sequencing by synthesis [5]. Modifications to Sanger's original methodology now allow performing sequencing by synthesis utilizing detection of fluorophore-labeled dideoxy nucleotides which are incorporated through successive rounds of PCR amplification. Sanger sequencing is also referred to as "sequencing by termination" or "chain terminator sequencing" due to the incorporation of dideoxy nucleotides that halts sequence elongation and creates sequences of different lengths. After subsequent rounds of amplification, these sequences of variable length are separated by capillary electrophoresis, and the termination nucleotide is identified. The strengths of utilizing a Sanger-based assay to detect mutations in lung cancer biomarkers include relatively low cost, widespread laboratory availability, noncomplex manual interpretation, and well-established protocols/procedures. Major limitations include low multiplexing capability (not scalable), requirement of a high mutation load (approximately 20%), and inability to detect gene amplifications. Another major limitation is detecting gene translocations (also referred to as fusions such as *ALK*, *ROS1*, and *RET*) which have a myriad of translocation partners and breakpoints, making knowledge of exact breakpoints a prerequisite for detection of gene translocations by Sanger sequencing [6].

Pyro-sequencing (Non-NGS)

Pyro-sequencing offers a cost-effective lung cancer biomarker detection method which is based on sequencing by synthesis. Unlike Sanger sequencing, which requires detection of incorporated fluorophore-labeled dideoxy nucleotide, detection of base pairs in pyro-sequencing is based on chemiluminescent detection of inorganic pyrophosphate released following base pair incorporation [7]. Strengths of pyro-sequencing include simple, robust, fast, and sensitive (detects down to ~5% mutant allele burden) assay that is capable of utilizing small amounts of input DNA. Drawbacks for lung cancer biomarker detection include limited ability to multiplex, decreased sensitivity in detecting regions rich in homopolymer repeats, and short read lengths. Best applications include detection of single base pair substitutions or small in/dels (such as those found in *EGFR* or *KRAS* mutation testing) and confirmation of mutations identified by orthogonal technologies [8].

High-Resolution Melting Analysis (HRMA)

In contrast to Sanger sequencing which is time consuming and labor intensive, high-resolution melting analysis (HRMA) methodologies for lung cancer biomarker testing offer a single assay that can quickly scan and detect the presence of mutations. The HRMA assay methodology is in essence based on detection of a PCR melting (dissociation) curve [9]. This method detects sequence differences following PCR amplification and has been used successfully to screen for *EGFR* and *KRAS* mutations in formalin-fixed paraffin-embedded tissue [10]. Using HRMA, samples are also discriminated according to their sequence length, GC content, and strand complementarity. A simple HRMA method) involves the use of double-stranded DNA-binding dyes. As samples are heated, high-fluorescent double-stranded DNA denatures leaving single-stranded DNA and the dye (signal) is lost. Therefore, small temperature changes result in a detectible change in fluorescence signal, and these two parameters can be plotted (i.e., melt curve) to allow detection of the presence or absence of single nucleotide variances. Advantages include cost-efficient mutation screening, simple assay design, options for automated mutation detection calling software, and fast turnaround time. Limitations include decreased accuracy with larger amplicon fragments, and detected variants traditionally undergo confirmation testing via a secondary method (classically a Sanger sequencing-based assay).

Allele-Specific Real-Time PCR Assays (TaqMan and ARMS)

Cobas® EGFR *Mutation Test (TaqMan)*

One allele-specific real-time PCR assay utilized for clinical NSCLC testing is the cobas® *EGFR* mutation test (Roche). This assay was approved by the US FDA as a companion diagnostic test to select patients with *EGFR* exon 19 deletions or L858R

substitution in exon 21 for treatment with erlotinib, for use in first-line metastatic NSCLC [11]. This assay is based on TaqMan probe technology which utilizes a mutation-specific primer and wild-type blocking primer in conjunction with a target-specific probe. Target amplification results in Taq-based exonuclease removal of target probe from quencher and generation of a fluorescent detection signal. Strengths include high sensitivity, high specificity, and FDA approval for *EGFR* mutation detection. Utilization of this assay requires no design challenges, optimization, or melt analysis. Limitations include lack of gene translocation detection, and mutation detection is limited to 41 predefined *EGFR* variants (not able to detect novel variants).

Therascreen® EGFR *Kit (Scorpion Amplification Refractory Mutation System (ARMS))*

A second allele-specific real-time PCR assay approved by the US FDA for *EGFR* testing and as a companion diagnostic for afatinib is the Therascreen® *EGFR* assay (Qiagen) [11, 12]. This assay utilizes mutation-specific probes for amplification, and target sequence amplification results in separation of quencher from fluorophore, similar to TaqMan assay, except no secondary target probe is utilized as the Scorpion primer incorporates both fluorophore and quencher [13]. Specifically, this assay identifies 29 *EGFR* mutations in exons 18–21. Therascreen® *EGFR* assay advantages include high sensitivity due to signal generation solely by mutant target, multiplexing, no need for assay design, and no need to perform melt analysis. Limitations include not applicable for gene translocation detection or detection of novel variants.

Peptide Nucleic Acid-Locked Nucleic Acid PCR (PNA-LNA Clamp)

Peptide nucleic acid-locked nucleic acid probe (synthetic DNA analog)-mediated real-time PCR assays allow high-sensitivity mutation detection (Table 7.1) [14]. This method is well suited for applications such as *EGFR* mutation testing in small cytological specimens such as bronchial washings, pleural effusions, and sputum [15]. This methodology has successfully detected low-level (<1% mutant allele) *EGFR* and *KRAS* mutations in NSCLC [16, 17]. The PNA-LNA clamp methodology includes utilizing a probe "clamp" for wild-type sequence in order to block strand synthesis (amplification). This probe blocks wild-type allele amplification, but a single base mutation in a mutant allele will destabilize the PNA probe binding and allow selective amplification of the mutation-harboring strand. Advantages include high-sensitivity detection of mutant alleles in samples with low tumor mutation allele fractions or samples with low tumor burden. Limitations include inability to detect gene translocation or detection of novel variants.

Table 7.1 Comparison of multiple methods for detecting mutations in lung cancer (organized from low sensitivity to ultra-high sensitivity)

Method	Sensitivity (% mutant DNA)	Application
	Low sensitivity	
Sanger sequencing	20%	Tumor tissue
	Medium sensitivity	
Pyro-sequencing	5–10%	Tumor tissue
TaqMan PCR	5–10%	Tumor tissue
SNAPSHOT	5–10%	Tumor tissue
dHPLC	5%	Tumor tissue
HRMA (melt analysis)	5%	Tumor tissue
Fragment analysis	5%	Tumor tissue
MALDI-TOF MS	5%	Tumor tissue
	High sensitivity	
Scorpion ARMS	1%	Tumor tissue/CTC/CF-DNA
PNA/LNA clamp	0.1–1%	Tumor tissue/CTC/CF-DNA
	Ultra-high sensitivity	
BEAMing	0.01%	CTC/CF-DNA
Digital PCR	0.01%	CTC/CF-DNA

CTC circulating tumor DNA, *CF-DNA* cell-free DNA, PNA))

Digital Droplet PCR

Digital PCR offers an alternate methodology to that of traditional real-time PCR for lung cancer biomarker testing. Like RT-PCR, digital droplet PCR offers ultrahigh sensitivity and precision for lung cancer mutation screening (Table 7.1) [18, 19]. This method involves partitioning DNA into numerous individual or parallel PCR reactions (droplets). The result of this process is a decrease in the amount of competing DNA for amplification and the ability to quantify lung cancer mutations (such as *EGFR* or *KRAS*) at the single-molecule level. Individual droplets with more than one copy of a target molecule can be identified using a Poisson model for exclusion from analysis. Applications include detecting copy number variation and identification of low abundant mutations in *EGFR* (including T790 M mutation) in pretreatment biopsy samples [20]. Advantages include no need for standards or references, scalable assay precision (increase number of droplets), linear detection of small fold changes, and simple workflow. A major disadvantage is the requirement of dedicated instrumentation.

nCOUNTER

Methods discussed above focused primarily on mutation detection including substitution and small in/dels which are applicable for *EGFR* and *KRAS* testing in NSCLC. However, molecular methods available for detecting a single or multiple gene translocation events are limited. Florescent in situ hybridization (FISH),

immunohistochemistry (IHC), and next-generation sequencing (NGS) can be applied to detect translocations; however, each assay has its own strengths and weakness. A molecular assay for simultaneous detection of multiple gene translocations (*ALK, RET*, and *ROS1* in NSCLC) has been previously demonstrated using the nCOUNTER system (NanoString) [21]. The nCOUNTER multiplex assay utilizes detection of gene expression differences in known translocations based on non-amplified mapping of mRNA transcripts. The basic idea is in the event of a translocation, there will be an imbalance of probes in the 5′ and 3′ end of the translocated gene. The advantages include the ability to utilize formalin-fixed paraffin-embedded tissue, no amplification steps (no PCR artifacts), low hands on time, and a high level of multiplexing for simultaneous detection of all relevant NSCLC clinical translocations. Disadvantages include dependence on high-quality RNA extraction from FFPE and dedicated instrumentation.

Minimal Invasive Lung Biomarker Testing

Recently there has been a large emphasis and excitement surrounding the detection of actionable variants utilizing nucleic acid isolation following a blood draw (i.e., liquid biopsy). This has taken the form of detecting cell-free DNA (CF-DNA) and/or isolated circulating tumor cells (CTC). Both methods represent an important evolution in testing but push the limitations of assay performance, as clinical lung cancer biomarker assays need to possess both an exceptionally high sensitivity and specificity in order to limit false positive/negative interpretations. CTC methods require a CTC capture step which is most commonly performed using an antibody-mediated capture procedure (such as anti-EMT capture). This form of capture has inherent bias due to the inefficiency of capture which influences downstream molecular biomarker testing. CF-DNA methods are hindered by collection of low-quantity tumor-specific DNA released in circulation and stability of free nucleic acids in circulation. Overcoming both CF-DNA and CTC limitations requires a downstream ultrahigh sensitive molecular methodology. Mutation screening methods for *EGFR* and *KRAS* have been successfully implemented in plasma using PNA-LNA clamping and digital PCR (Table 7.1) [22–25]. While these assays offer ultrahigh sensitivity in mutation detection, their utility in comparison to standard tumor biopsy-based methodologies is still an active area of investigation.

Biomarkers Utilized for Lung Adenocarcinoma

KRAS *(Mutation; Frequency ~25%)*

Kirsten rat sarcoma (*KRAS*) viral oncogene homolog is a member of the guanosine triphosphate (GTP)-binding protein family and is responsible for pro-survival signaling. *KRAS* represents the most commonly mutated gene in NSCLC

adenocarcinomas [26]. Mutations cluster at hotspots in codons 12, 13, and 61 [27]. Mutations include substitutions, insertions, and deletions. Mutations in *KRAS* are most frequently observed in smokers; however, incidence in nonsmokers approaches 15%, and therefore smoking history provides a poor estimation of *KRAS* mutation status [28–30]. *KRAS* mutations result in stimulus-free and constitutively active signaling, which results in cell proliferation and survival. Furthermore, *KRAS* mutations are nearly mutually exclusive from *EGFR* mutations or *ALK* translocations [31]. Currently, there is no direct targeted therapy for *KRAS*-mutated NSCLC. However, MEK inhibition (selumetinib and trametinib) is under investigation as a strategy to target the downstream aberrant signaling in *KRAS*-mutated NSCLC. In addition to MEK inhibition, CDK4/CDK6 inhibition also shows antitumor activity in *KRAS*-mutated NSCLC [32]. Likely improved drug targeting will include combination therapy such as MEK or CDK4/CDK6 in combination with AKT inhibitors (MK-2206) to circumvent resistance mechanisms [33].

EGFR *(Mutation; Frequency ~15%)*

Epidermal growth factor receptor (*EGFR*), also known as HER1/ERBB1, represents one of the most highly investigated and currently utilized biomarkers in NSCLC. Presence of an *EGFR* activating mutation serves as a predictor of response to tyrosine kinase inhibitors (TKI) including erlotinib, gefitinib, and afatinib [34–36]. *EGFR* mutations most commonly occur in nonsmokers and women of Asian descent. While TKIs show clinical benefit (improved response rate and progression-free survival) [12, 15, 37], the likelihood of response is relative to location of the mutation site. *EGFR* mutations in exons 18–21 (tyrosine kinase domain of *EGFR*) are associated with sensitivity to TKIs [34, 35]. Within these exons, short in frame deletion amino acids (747–750) and a point mutation (L858R) account for approximately 90% of activating *EGFR* mutations [34, 35, 38]. One key clinical mutation for screening includes the T790 M mutation in exon 20 which is estimated to confer resistance to first-generation TKIs in approximately 50% of cases [39].

BRAF *(Mutation; Frequency ~4%)*

The v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) protein is a serine/threonine protein kinase. Mutations in *BRAF*, particularly the glutamate substitution mutation at codon 600 (V600E), are common in disease types such as papillary thyroid carcinoma and melanoma where they are observed in approximately 50% of cases [40–42]. However, in NSCLC *BRAF* mutations are relatively uncommon (approximately 3–4%) [43, 44]. In addition, only about half of the mutations in NSCLC are isolated to the single amino acid V600. While *BRAF* mutations can be targeted with inhibitors (vemurafenib and dabrafenib), the low frequency and nonuniform mutation profile in NSCLC dilutes the attractiveness of *BRAF* as a routine predictive biomarker.

MET *(Mutation; Frequency ~3%)*

The mesenchymal-to-epithelial transition (*MET*) gene was discovered in the late 1980s and encodes a RTK [45]. The MET-RTK regulates a multitude of cell processes including cell scattering, invasion, anti-apoptosis, and angiogenesis [46]. Aberrant *MET* signaling has been identified in multiple human malignancies [46–48]. Likewise, variations in *MET* signaling can occur due to increased ligand (hepatocyte growth factor, HGF) binding, mutation, amplification, or increased proteolytic degradation. *MET* overexpression is commonly seen in NSCLC (25–75% depending on antibody and cutoff criteria) and has been linked to poor clinical outcome [49, 50]. *MET* alterations in NSCLC also include mutations resulting in exon 14 skipping, which increase *MET* signaling and promote oncogenesis [51, 52]. These mutations are most common in smokers and older patients. Therapy options include multikinase TKIs [53–56]. However, alternative therapies under evaluation include targeted *MET*-TKI inhibitor (tivantinib) and monoclonal antibody (onartuzumab) [57–59].

ERBB2 *(HER2) (Mutation; Frequency ~2–4%)*

Human epidermal growth factor receptor 2 (*HER2*) belongs to a family of membrane receptors known as the erbB family. *HER2*, also known as *ERBB2*, functions by homo- and hetero-dimerization with erbB family members. Dimerization results in signaling via multiple pathways including PI3K, MAPK, and JAK/STAT [60]. *ERBB2* represents a widely utilized biomarker in breast cancer due to amplification being present in 15–25% cases. However, in NSCLC, the frequency of *ERBB2* mutation is low (2–4% exon 20 in frame insertion). *ERBB2* mutations in NSCLC occur most commonly in nonsmokers, females, and Asian ethnicity [61]. *ERBB2* amplification is observed more frequently than mutation in NSCLC, with an incidence of approximately 20% [62, 63]. Clinical response to anti-HER2-directed therapy (trastuzumab, neratinib, afatinib, and lapatinib) in NSCLC with *HER2* overexpression has shown limited therapeutic efficacy [64–69].

ALK *(Translocation; Frequency ~5%)*

Anaplastic lymphoma kinase (*ALK*) gene encodes a receptor tyrosine kinase with high sequence similarity to the insulin receptor [70]. Translocations involving *ALK* and *EML4* in NSCLC were first described in 2007 [71]. The most common gene rearrangement in NSCLC is *EML4-ALK* at a frequency of 4–5% and is most commonly observed in young women with a history of light smoking or never-smoking [26]. Subsequently, nearly 30 *ALK* translocation partners have been identified [70]. Therapy options include multikinase TKIs (crizotinib, ceritinib, and alectinib)

[72–76]. Resistance occurs in the majority of cases treated with first-line crizotinib. New second-generation TKIs including ceritinib are being investigated in patients that progress or are intolerant of crizotinib [75].

RET *(Translocation; Frequency 1–2%)*

The *RET* gene encodes a RTK that is essential for normal cell development and maturation. *RET* translocations have clinical significance in a variety of human malignancies including papillary thyroid carcinoma (20–40%) and NSCLC (1–2%) [77–79]. Like *ALK* translocations in NSCLC, *RET* translocations occur most frequently in young females with a history of light smoking or nonsmoking. In NSCLC *RET* has six identified translocation partners (*KIF5B, CCDC6, NCOA4, TRIM33, CUX1*, and *KIAA1468*) [80]. The most frequent *RET* translocation partners include *KIF5B* and *CCDC6.* In in vitro models, several TKI's (sunitinib, sorafenib, vandetanib, and cabozantinib) have shown efficacy in blocking *RET* translocation signaling [81]. However, clinical trials evaluating human therapeutic efficacy are ongoing (NCT01823068, NCT01639508, NCT01866410, and NCT01708954).

ROS1 *(Translocation; Frequency 1–2%)*

The *ROS1* gene encodes a RTK that is involved in pro-survival and anti-apoptotic signaling through multiple pathways [82]. In NSCLC, *ROS1* translocations occur with a variety of gene partners including but not limited to *CD74*, *EZR*, *SDC4*, and *TPM3* [82]. *ROS1* translocations result in neoplastic transformation both in vitro and in vivo. Translocations occur in multiple malignancies but are most prevalent in papillary thyroid carcinoma (approximately 40%) [83]. *ROS1* translocations are also observed in NSCLC adenocarcinomas at a frequency of 1–2%, but do not represent a clinically actionable finding [84, 85]. The patient population that harbors *ROS1* translocations is similar to *ALK* translocations and includes young nonsmokers or light smokers [84]. Early preclinical evaluation of *ROS1* translocation-positive NSCLC showed strong sensitivity to crizotinib [83]. Recently NCCN guidelines added *ROS1* to include testing in all NSCLCs similar to *ALK* [86].

Biomarkers Utilized for Lung Squamous Cell Carcinoma

FGFR1 *(Amplification; Frequency ~17–20%)*

The fibroblast growth factor receptor 1 (*FGFR1*) is a RTK that regulates proliferation via MAPK and PI3K signaling pathways. *FGFR1* amplification is the most common alteration seen in squamous cell carcinoma (20%) [87, 88]. *FGFR1*

amplification has oncogenic potential as seen in NSCLC cell lines, which show sensitivity to RTK targeted therapy [87]. Clinical-based RTKs targeted therapies including dovitinib and nintedanib are currently ongoing (NCT01861197 and NCT01948141) [89, 90].

PIK3CA *(Mutation; Frequency ~16%)*

There are three classes of phosphatidylinositol-3 kinases, Class 1A PI3Ks (*PIK3CA*) are the most relevant to human cancer [91]. *PIK3CA* is responsible for production of phosphatidylinositol-3,4,5-trisphosphate which activates the AKT/mTOR pathway [92]. This pathway is essential for cell growth, survival, and motility. Amplification of *PIK3CA* is more common in NSCLC squamous cell carcinoma than adenocarcinoma (33% vs. 6%, respectively) [93]. Mutations are also more prevalent in squamous cell carcinoma and occur at approximately 2–5% of cases [93–95]. Unlike most mutations in NSCLC, *PIK3CA* mutations can occur in conjunction with other genes and is therefore not always mutually exclusive [96]. Furthermore, *PIK3CA* mutations have been implicated as a resistance method to *EGFR*-based TKI therapy [97].

PTEN *(Mutation; Frequency ~10–15%)*

PTEN inhibits the PI3K/AKT/mTOR signaling cascade through dephosphorylating PI-(3,4,5)-triphosphate [98]. Inactivation of *PTEN* removes pathway inhibition and therefore leads to nonrestricted activation of AKT. Mutations are almost exclusively seen in squamous cell carcinoma (approximately 10–15%), but are rarely observed in adenocarcinoma (approximately 1–2%) [99]. Current targeted therapy under evaluation includes AKT and mTOR inhibitors (MK-2206 and ridaforolimus) [100, 101].

FGFR2/FGFR3 *(Mutation and Translocation; Frequency ~6%)*

Fibroblast growth factor receptors 2 and 3 (*FGFR2* and *FGFR3*) are RTKs that play essential roles in cell proliferation, differentiation, angiogenesis, and development. Activation of FGFR2/FGFR3 results in downstream activation of Ras/MAPK and PI3K/AKT [102]. *FGFR2* and *FGFR3* mutations are most commonly observed in endometrial carcinoma (~12%) and urothelial carcinoma (~30%) [87, 103–105]. It is worth noting that the cancer genome atlas data first reported *FGFR2/FGFR3* somatic mutations in NSCLC [26]. Targeted therapies for FGFR2/FGFR3 under investigation include multi-RTK inhibitors (sunitinib, sorafenib, pazopanib, and vandetanib). More recently, to address side effects and low efficacy of multi-TKIs,

new selective and highly potent FGFR TKIs are under evaluation (AZD4547, BGJ-398, and JNJ42756493 [106].

DDR2 *(Mutation; Frequency ~2–4%)*

Discoidin domain receptor 2 (*DDR2*) is a RTK involved with tissue repair and tumor progression [107]. *DDR2* mutation rate in squamous cell carcinoma is approximately 2–4% [108, 109]. Early clinical evidence has demonstrated potential therapeutic effectiveness with dasatinib [110, 111] and clinical trials are in progress (NCT011514864).

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Chapter 8
Translocation Testing of Lung Cancer Biomarkers

Sanja Dacic

Specimen Requirements

Specimen requirements are common to FISH and other DNA-based molecular assays. Fresh, frozen, formalin-fixed paraffin embedded (FFPE), and alcohol-fixed specimens are suitable for analysis. The most common fixatives used in clinical practice are 10% neutral buffered formalin and 70% ethanol alcohol. Both result in great morphology preservation in addition to good DNA/RNA preservation. Unlike PCR-based assays, alcohol fixatives can be a problem for FISH assays. Most problematic fixatives include acidic solutions (Bouins), heavy metal fixatives (Zenker, B5), and bone decalcifying solutions that result in technically suboptimal assays and therefore should be avoided. Time of fixation may also have impact on the assay quality. Based on the experience with *HER2* testing, formalin fixation of 6–12 h is recommended for small biopsy specimens and 8–18 h for large surgical resection specimens [1]. Many laboratories can perform successful testing on rapidly process specimens with fixation time of only 4 h. Similarly, specimens fixed between 24 and 48 even 72 h can give optimal results. Cytology specimens are suitable for gene rearrangement testing. Most of the laboratories prefer cell blocks. Although smear preparations can be used as well, FISH analysis requires non-overlapping tumor cells which could be an issue with DAPI staining. Specimen adequacy for testing in terms of processing, fixation, and tumor cellularity should be determined by each laboratory based on their internal validation.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_8

Other Technical Considerations

FFPE tissue sections used for FISH analysis are usually 4–5 microns thick and should be placed on the charged ("+") slides to prevent tissue detachment during specimen processing. Laboratories should decide what type of glass slides should be used, but several technical details should be taken into consideration. Slides with a heavy coating or slides designed for microdissection should not be used for FISH as they frequently result in a poor hybridization and tissue detachment, respectively.

FISH protocols include several steps including deparaffinization, pretreatment/target retrieval, probe and target DNA denaturation, hybridization, post-hybridization washes, detection, and interpretation. Probe and target DNA denaturation is a critical step that may require modifications depending on the tissue size and preservation, duration, and type of fixation. This is particularly true for small biopsy and cytology specimens. Time of digestions is critical and should be standardized to maintain nuclear morphology. Overdigested tissue samples frequently show chromatin artifactual "split signals" that could be interpreted as false-positive findings. Hybridization and washing steps should be standardized and protocols should be established for every probe and specimen type. Automated tissue processing and standardized commercially available tissue digestion kits can improve consistency of the FISH assay. Typically, FISH assays designed for detection of gene rearrangements take up to 2 days, although depending on the probe robustness same-day assay is possible. A variety of amplification steps are available for enhancing weak signals. Most of the probes today are of excellent quality and such steps are not necessary.

Type of Probes

Two main FISH assay strategies are used for detection of chromosomal translocations: fusion probes and break-apart probes. Fusion strategy uses two probes, one localizing centromeric to one chromosomal break point and the second probe localizing telomeric to the reciprocal break point. Translocation negative cells show separated or "split" signals (e.g., two green and two red signals) (Fig. 8.1). In contrast, translocation positive cells show a "fusion" signal (e.g., one yellow, one green, one red signal). Break-apart assay uses two probes localizing proximal and distal to

only one of the two breakpoints of interest. The two probes are in close proximity to one another, and therefore, normal cells show fusion signals only (Fig. 8.2). Translocation positive cells show at least one pair of split signal (e.g., one fusion, one green, one red). The disadvantage of break-apart probes is that the fusion partner is not identified.

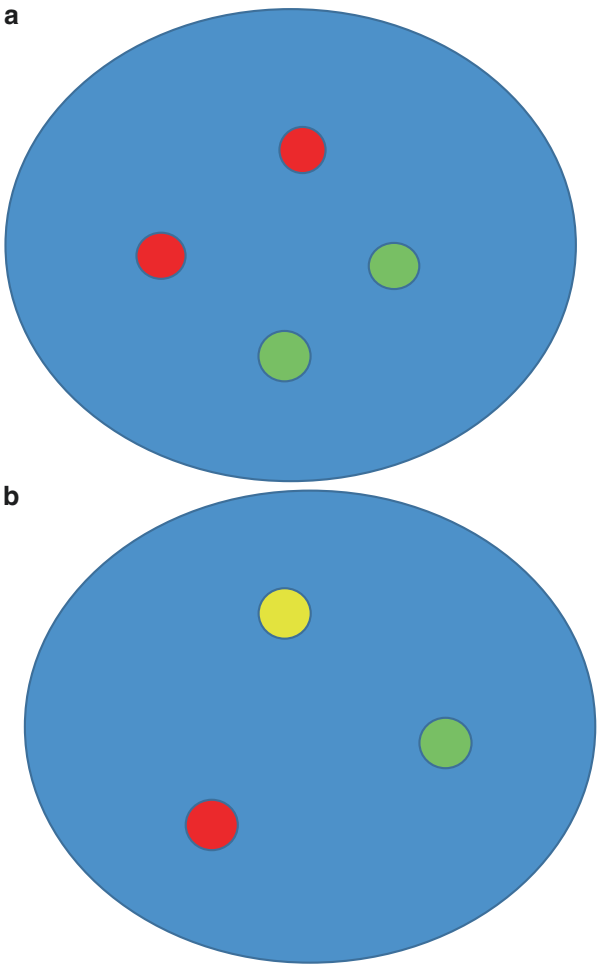

Fig. 8.1 (**a**) Fusion probe FISH assay negative for translocation ("split signal"). (**b**) Fusion probe FISH assay positive for translocation ("fusion signal")

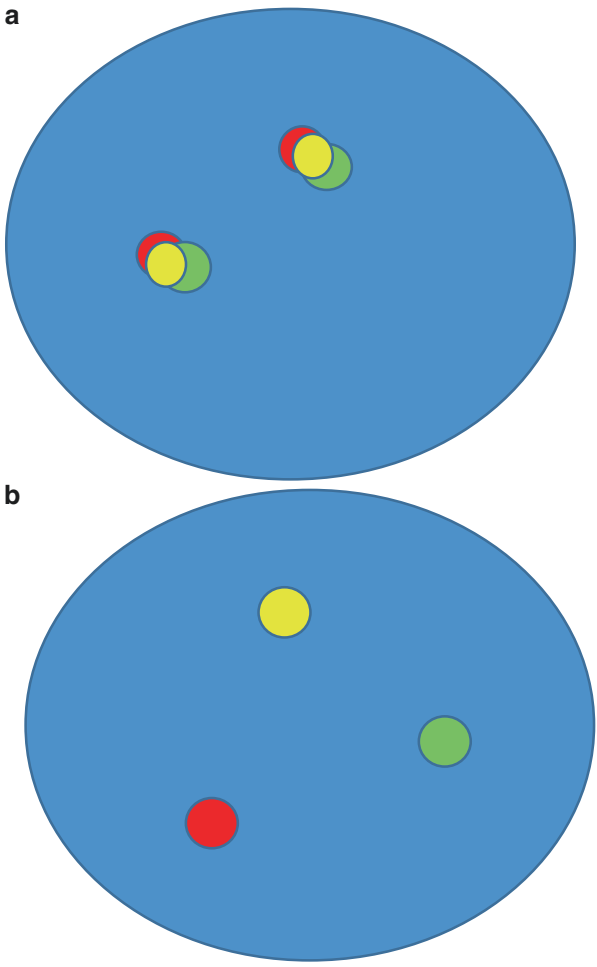

Fig. 8.2 (**a**) Break-apart probe FISH assay negative for translocation ("fusion signal"). (**b**) Break-apart probe FISH assay positive for translocation ("split signal")

Interpretation

The identification of non-overlapping cells/nuclei is the first step in the interpretation. It is essential that a pathologist identify adequate tumor areas on the routine H&E slide that is adjacent to section submitted for FISH analysis. An experienced cytotechnologist who has undergone specific FISH training in solid tumors should analyze the slides. Interpretation should be performed in areas of the slide with good signal, in which at least 50% of all nuclei are easily analyzable, with minimal background and autofluorescence. The FISH signal intensity should be consistently greater than background intensity.

Common Clinical Assays for Gene Rearrangements in Lung Cancer

ALK Gene Rearrangement

FISH Probe Design

The most common of *ALK* rearrangements involve a pericentric inversion on the short arm of chromosome 2, inv. [2] (p21p23), which creates a fusion gene encoding the amino-terminal portion of *EML4* (2p21) and the intracellular region of *ALK* (2p23), genes that are normally approximately 13 Mb apart [2]. Although the *EML4-ALK* fusion is the most common, other less common variant fusions have been reported, including translocations with other chromosomes (*KIF5B-ALK*, *TFG-ALK*) [3, 4]. The Vysis LSI *ALK* break-apart FISH probe kit (Abbott Molecular) was used to identify patients with *ALK* rearrangement positive NSCLC in the first clinical trials with ALK inhibitors, and therefore, the US Food and Drug Administration (FDA) approved this commercially available assay as a companion diagnostics for detection of *ALK* rearrangements in lung cancer [5]. The break-apart probe is designed by labeling the 3′(telomeric) 300-kb part fusion breakpoint with orange fluorochrome (Spectrum Orange, often referred as red) and the 5′ 442′-kb probe by a green signal (SpectrumGreen) (Fig. 8.3).

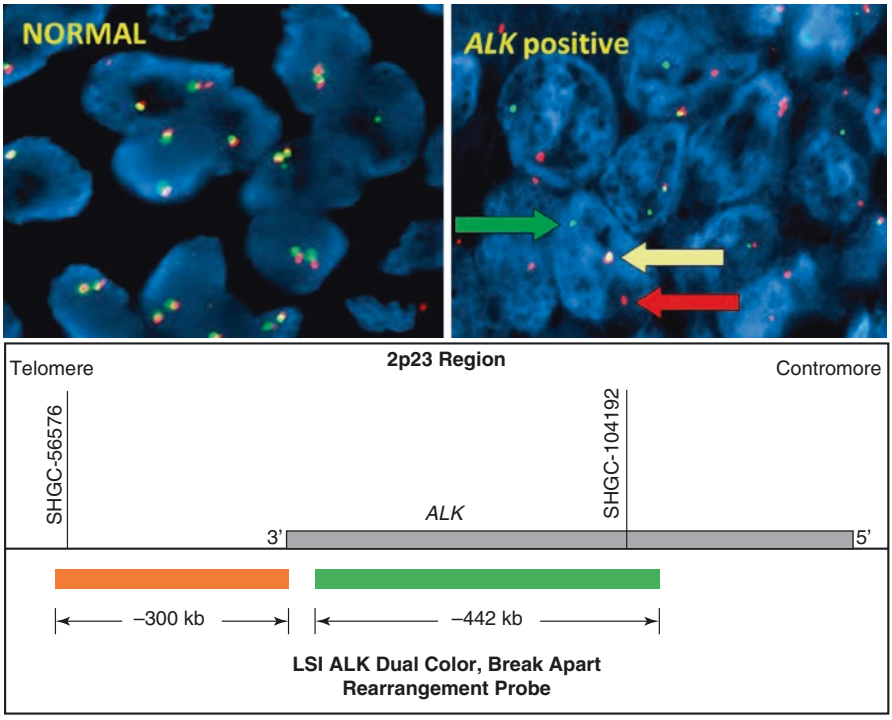

Fig. 8.3 Break-apart *ALK* FISH probe

Interpretation

The interpretation of *ALK* FISH assay may be challenging because the 5′ and 3′ probes are genetically very close [6–9]. Therefore, cells with normal pattern show fused signals. For the same reason in some cases split signal can be very narrow that the signals seems to be very close and fused in otherwise *ALK* rearranged cells. In the interpretation of *ALK* FISH, it is essential to pay attention to distance between the signals which should measure at least two signal diameters in cases positive for translocation. Any distance that is less than two signal diameter is considered to represent lack of gene rearrangement. A minimum of 50 tumor cells should be scored if there is one scorer, and a minimum of 100 tumor cells is needed if there are two scorers.

The assay is considered to be positive for *ALK* rearrangement if at least 15% of tumor cells show rearrangement. FISH patterns that are considered to represent gene rearrangement include split pattern and isolated 3′ pattern (Fig. 8.4). The number of accompanied fused 5′-3′ signals in the cell is not important for pattern classification. Isolated 5′ pattern may also be identified and is considered to represent nonfunctional reciprocal fusion product. Although this pattern has been reported to be associated with a rare *BIRC6-ALK* fusion, it should not be interpreted as rearrangement positive [10, 11]. FISH criteria particularly in respect to isolated 3′

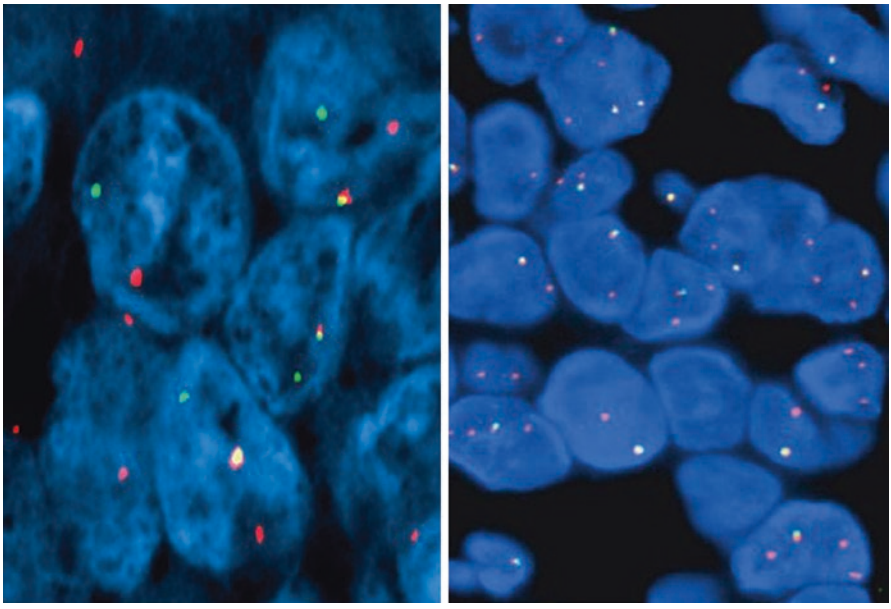

Fig. 8.4 Break-apart *ALK* FISH translocation patterns

("single orange") have been recently challenged [12, 13]. Cases classified as rearrangement positive based on isolated 3′ showed a higher rate of fusion negative cases by NGS and IHC than the group with a FISH split signal indicating that these cases may be FISH false positive [13, 14]. Isolated 3′ may be a result of technical factors such as nuclear sectioning causing loss of the 5′ (green) probe binding site, or simply observer error. Technical errors can't be reliably excluded in a case with a lower percentage of nuclei positive for rearrangement. Overall, cases with atypical signal patterns should be tested by another method such as IHC or NGS. RT-PCR may also be considered if the assay is designed to cover a large number of known fusions [15–20].

Another major source for false interpretations is the cases in which the rate of rearrangement positive cells falls within the range of 10–20%. In those cases, it is essential to recount the tumor cells and to perform a different assay as indicated above.

ALK false-negative results may also occur and are most likely caused by the complex gene rearrangements and cryptic insertions [14, 21, 22]. Recently, Wiesner et al. identified a novel *ALK* transcript, ALK^{ATI}, which arises independently of genomic aberrations at the *ALK* locus through alternative transcription initiation and which can be detected by ALK IHC, but not FISH [23]. Preliminary data showed that the patients with ALK^{ATI} may benefit from ALK inhibitors.

ALK RT-PCR

RT-PCR for detection of *ALK* rearrangements provides detailed information about *ALK* fusion partners. The risk of false-negative results and high failure rate for RNA-based assays on FFPE tissue samples make implementation of this assay in clinical practice difficult. In addition, this assay is designed to detect only known fusion partners and fusions with unknown partners would remain undetectable [1, 3, 4, 18, 24, 25].

Other Gene Rearrangements

The other most commonly identified gene fusions identified in lung carcinoma include genes *ROS1*, *RET*, *NTRK1*, and *NRG1*. Similar to *ALK*, break-apart FISH assay is currently the most commonly used method for detection of *ROS1* fusions (Fig. 8.5) [26–28]. *RET* rearrangement is also detectable by FISH or RT-PCR [26, 29–34]. Targeted NGS identified *NTRK1* rearrangements resulting in oncogenic fusion products *MPRIP-NTRK1* and *CD74-NTRK1* [35]. Whole transcriptome sequencing identified *CD74-NRG1* fusion that was also identified by RT-PCR [36].

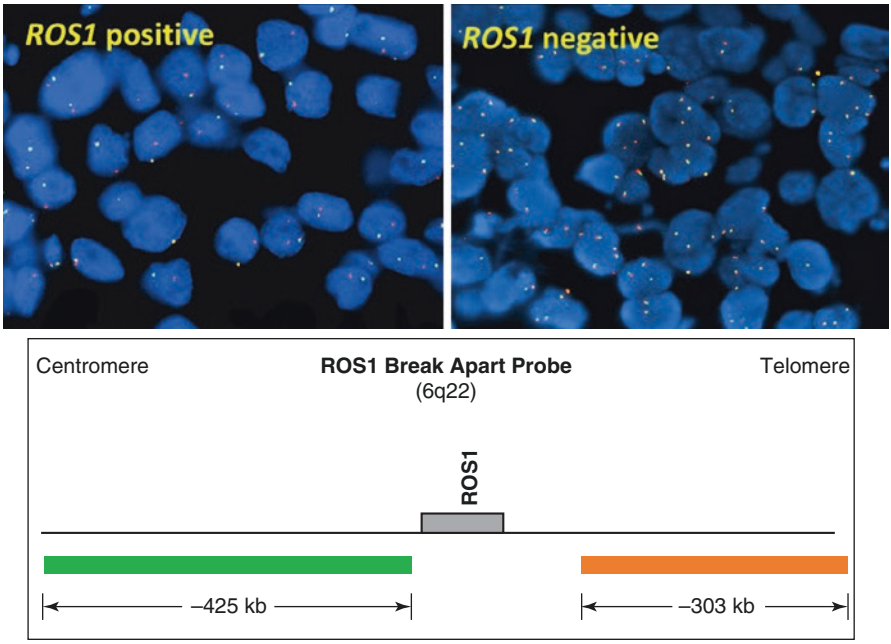

Fig. 8.5 Break-apart *ROS1* FISH probe

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Chapter 9
Immunohistochemistry of Lung Cancer Biomarkers

Mary Beth Beasley

Introduction

Predictive immunohistochemical (IHC) staining refers to stains which may be used as a screening test or surrogate test in which the results are applied to therapeutic decision-making. Immunohistochemistry is a rapid and relatively inexpensive method of detection compared to other methodologies such as fluorescence in situ hybridization (FISH) or other molecular detection methods and may potentially be interpreted with fewer malignant cells than are required for other methodologies.

IHC staining is not without its limitations. Pre-analytical variables such as fixation and cold ischemia time; analytical variables such as antibody type, dilution, and retrieval methods; and post-analytical variables such as background staining and artifacts may all influence results. Most commercially available IHC antibodies are optimized for use on formalin-fixed paraffin-embedded (FFPE) tissue samples. Alcohol-based fixatives and decalcifying solutions may alter results. Cytology samples such as fluid cytologies and fine needle aspirations can be used as long as the antibody is properly validated for the fixative used, if different from formalin, although cytology specimens are frequently not included in trial data and are currently not validated for use with programmed cell death ligand 1 (PD-L1) immunostains. Due to heterogeneity of expression, results on small biopsy specimens may not correlate with those of a larger tissue sample [1–3].

In spite of these limitations, IHC can provide a cost-effective alternative to FISH or other molecular methods in certain settings. The antibodies in clinical use primarily include those directed toward anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) in which a positive test indicates the presence of a fusion gene resulting in aberrant expression of the gene product. Immunohistochemistry for

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_9

detection of PD-L1 has expanded rapidly in recent years and has resulted in a complicated landscape of available antibodies and interpretation guidelines.

Anaplastic Lymphoma Kinase (ALK)

Genomic rearrangements of *ALK* occur in 1–5% of non-small cell lung carcinomas (NSCLC) and most commonly involve fusion with echinoderm microtubule-associated protein-like 4 (*EML4*), although fusions with other partners may occur, and over 20 fusion partners have been reported [4–10]. Crizotinib was approved by the FDA for the treatment of patients with ALK-positive metastatic NSCLC, and second-generation ALK inhibitors (ceritinib, alectinib) have subsequently been approved [9]. *ALK* rearrangements are typically found in adenocarcinomas or tumors with an adenocarcinoma component as opposed to pure squamous cell carcinomas, although testing in squamous cell carcinomas may be warranted in certain situations such as young patients or nonsmokers [9, 11]. The reader is also referred to the update of the CAP/IASLC/AMP testing guidelines which are in progress at the time of this writing [12].

Initially, ALK testing by FISH using the Vysis LSI ALK Break Apart FISH Probe Kit (Abbott Molecular) was the FDA-approved reference standard. Two commercially available antibody clones, mouse monoclonal 5A4 (Novocastra, Newcastle) and rabbit monoclonal D5F3 (Ventana, Tuscon, AZ), have both shown clinically acceptable sensitivity and specificity when compared to ALK FISH results [3, 9, 13, 14]. As such, data supports that ALK IHC is an acceptable alternative to FISH. Recently, the ALK (D5F3) IHC CDx Assay (Ventana) has received approval by the FDA to select patients for treatment with an ALK inhibitor. It is of key importance to note that due to the low level of expression or protein in lung cancer compared to anaplastic large cell lymphoma, the ALK1 antibody typically used in the diagnosis of this tumor (mouse monoclonal anti-human CD246, clone ALK1) should not be used to detect ALK rearrangements in lung cancer cases [3]. Given the relatively low prevalence of ALK-rearranged tumors, ALK IHC has become a cost-effective method to screen for the presence of *ALK* fusion and, more recently, can be used to determine eligibility for therapy. Positive ALK staining is characterized by strong granular cytoplasmic staining, with or without membrane accentuation (Fig. 9.1). ALK protein is generally not expressed in the lung tissue, so strong IHC amplification systems can be used. Light cytoplasmic stippling of alveolar macrophages, staining in nerve and ganglion cells, and staining of extracellular mucin and necrotic tumor areas may all occur. Signet ring cells, paradoxically a common histologic finding in ALK-rearranged tumors, must be interpreted carefully as a thin rim of membranous staining may be masked by an intracellular mucin vacuole. Weak staining or stains with staining artifacts or heterogenous fixation should be confirmed by an additional method such as FISH or validated PCR. Discordant results between ALK FISH and ALK IHC assays are rare. ALK IHC-negative cases with ALK-positive FISH have been shown to contain a lower percentage of tumor cells with rearrangement. Conversely, in some cases, ALK IHC

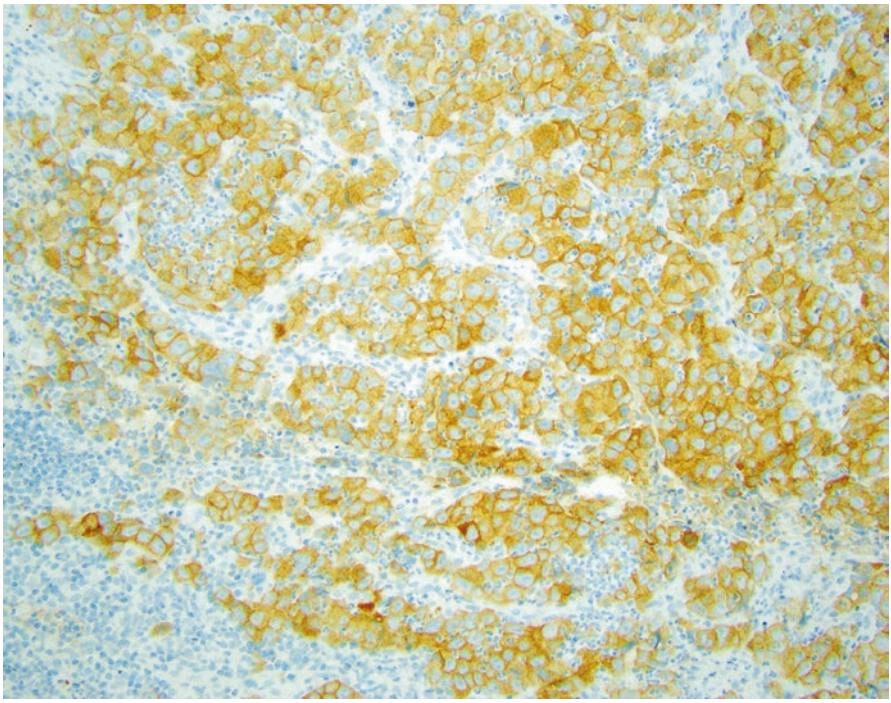

Fig. 9.1 Immunohistochemical staining for ALK is typically diffuse with granular cytoplasmic staining (ALK D5F3, 200×). Image courtesy of Dr. Natasha Rekhtman

has detected rearrangements confirmed by NGS which were not detected by FISH and were responsive to crizotinib [3, 7, 9, 13–18].

c-ros Oncogene 1 (*ROS1)*

The prevalence of patients with *ROS1* rearrangements is 2% or less. *FIG-ROS1* fusion was the first to be described although numerous other fusion partners such as *SLC34A2*, *CD74*, *and TPM3* are actually more commonly identified in lung cancer and the number of translocation partners will likely continue to grow. Like *ALK*-rearranged tumors, carcinomas with *ROS1* rearrangements are largely limited to adenocarcinomas, with similar caveats regarding testing flexibility. Patients harboring *ROS1* rearrangements have been shown to have an objective response rate of 72% to crizotinib, and as such, the drug was approved by the FDA for use in these patients in 2016 [9, 16, 19, 20].

ROS1 rearrangements are generally detected by FISH testing using a break apart probe, although RT-PCR methods, particularly those using capture-based sequencing strategies, may also be employed. Given the low prevalence of *ROS1* rearrangement in lung cancer, a cost-effective screening method is desirable. Studies evaluating ROS1 immunohistochemistry have used a single commercially available

antibody clone, D4D6 (Cell Signaling Technology). Most studies demonstrate a sensitivity of 100% relative to FISH or RT-PCR but the specificity ranges from 92 to 100%. Several cutoffs and scoring systems have been used, but a meta-analysis determined a sensitivity of 95.87% and a specificity of 93.52% when a staining intensity of at least 2+ was used. Indeed, most tumors with confirmed *ROS1* rearrangements by FISH or PCR have moderately intense staining expression; however, there is no universally accepted scoring system [9, 16, 19–24]. Further, unlike ALK IHC, ROS1 IHC can be much more difficult to interpret. While most often cytoplasmic and diffuse (Fig. 9.2), ROS1 staining can be patchy and weak and may show different staining patterns depending on the fusion present (i.e., granular/globular in CD74-ROS1 fusions and weak membranous staining in EZR-ROS1 fusions). Additionally, weak patchy staining can be seen in up to a third of tumors that do not have an underlying rearrangement. An additional complication is that the low frequency of *ROS1*-rearranged tumors limits the availability of material needed for validation [19, 20, 23–25]. Based on these limitations, ROS1 IHC may be used as a screening tool, but forthcoming guidelines recommend that ROS1 IHC-positive results undergo confirmation with FISH or a molecular method prior to considering a patient a candidate for targeted therapy. However, it is felt that there is strong enough evidence on the high sensitivity of the ROS1 IHC that tumors that lack ROS1 staining can be interpreted as negative for *ROS1* fusion [12].

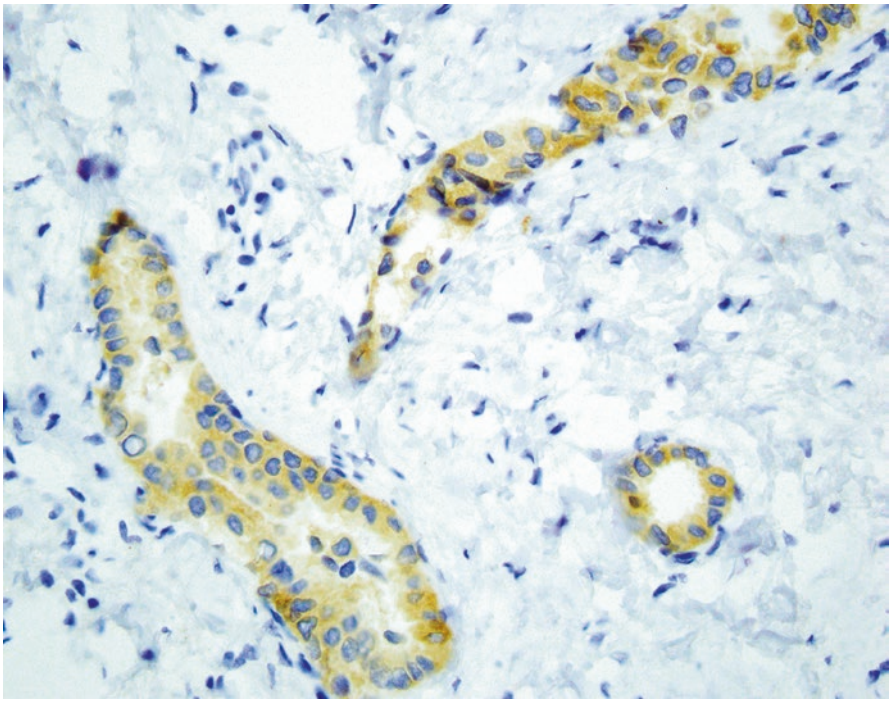

Fig. 9.2 Immunostaining for ROS-1 shows diffuse cytoplasmic staining in most positive cases (ROS-1 D4D6 400×). Image courtesy of Dr. Natasha Rekhtman

Epidermal Growth Factor Receptor (EGFR)

While the 2013 CAP/IASLC/AMP guideline allowed for the use of EGFR IHC with antibodies directed against specific mutant epitopes, the overall performance of such antibodies is generally suboptimal for consistently reliable detection of EGFR mutations with the exception of L858R which is highly specific for ELREA746_750 deletion [26–30]. While there may be a role for IHC in extremely limited samples or limited resource settings, advances in molecular technology enable analysis of very limited samples and circulating tumor DNA so routine use of EGFR antibodies is not advisable for routine use in selecting lung cancer patients for EGFR directed therapies.

BRAF

BRAF mutations in NSCLC include the c.1799 T > A9p.V600E point mutation seen in many other cancer such as melanoma; however, may other *BRAF* mutations occur in lung adenocarcinoma including both mutations in nearby amino acids as well as other substitutions at V600. Therefore, the use of immunohistochemical stains typically used in evaluation of melanoma which detect on V600E mutations will miss non-V600E mutations encountered in lung cancer and a method which evaluates the entire *BRAF* coding region should be utilized instead [31–35].

Other Genes

Potentially targetable abnormalities that occur in relatively small percentages of lung cancers are ever increasing. In general, it is recommended that these mutations should be tested as part of a multiplex testing panel and not performed routinely as a stand-alone test [12]. Immunostains applicable to most of these abnormalities (*RET*, *ERBB2*, *MET*) have generally not provided consistent results in comparison to molecular testing methodologies [25, 36]. However, similar to other low-level mutations, a low-cost screening method is attractive, particularly in low-resource settings and will likely continue to evolve. In regard to RET, mutations are rare, and while initial trials showed promise, there is currently limited evidence of therapeutic benefit although it remains under investigation [25]. RET IHC (ab134100 abcam, Cambridge, UK, with detections systems EnVision + DAKO, Denmark) produces diffusely granular cytoplasmic staining of moderate to strong intensity, occasionally with membranous or perinuclear accentuation. This antibody reported in sensitivity of 100% and a specificity of 88% in comparison to other methods but further investigation is needed [31, 36]. Conversely, due to the variety of mutations present, IHC testing for *ERBB2* (Her2) as is commonly performed in breast cancer cases is not recommended for lung cancer cases [37].

Programmed Cell Death Ligand 1 (PD-L1)

Immunotherapy for treatment of lung cancer has rapidly evolved to become part of the standard of care for many patients with advanced NSCLC. The principle of immunomodulatory therapy is based on its ability to disrupt inhibitory signals between tumor and immune cells, usually T-cells. While other signaling processes exist, current lung cancer therapies focus primarily on the interaction between PD-L1, expressed on tumor cells and programmed cell death protein 1 (PD-1) on T-cells, the interaction of which enables the tumor cell to evade the T-cell response to the tumor. By blocking either PD-1 or PD-L1, T-cells are then able to recognize and respond to foreign antigens on the cancer cells.

In recent years, the landscape of immunotherapy for lung carcinoma as well as other malignancies has evolved rapidly, but has resulted in complexities for pathologists in regard to testing tumors to determine potential eligibility for therapy. Currently, there are five different inhibitors of either PD1 or PD-L1, each of which currently has a paired proprietary anti-PD-L1 immunohistochemical antibody. Currently, in the United States, nivolumab is paired with Dako 28.8 PharmDx kit, pembrolizumab with Dako 22C3 PharmDx kit, atezolizumab with Spring Bioscience SP142 clone, durvalumab with Ventana SP263 clone, and avelumab with Dako 73-10 clone, the first three of which are currently FDA-approved for use in non-small cell lung cancer. In addition to the fact that each drug is paired with a proprietary antibody, each has different scoring systems for positivity, and the antibodies should be run on specified staining platforms (Table 9.1). Positive tumor staining for each antibody is based on a tumor proportion score defined as partial or complete membranous staining of any intensity (Figs. 9.3 and 9.4), while interpretation of the SP142 antibody additionally requires recording the percentage of positive tumor immune cells. Additionally, other non-FDA-approved antibodies also exist as laboratory developed tests (LDT), such as E1L3N rabbit monoclonal and 9A11 mouse IgG1 clones from Cell Signaling Technologies; ab58810 rabbit polyconal from Abcam; MIH1 mouse monoclonal from Thermo Fisher Scientific; 015 rabbit IgG from Sino Biological; 7G11 mouse IgG1 from Freeman Laboratory, Dana Farber Cancer Institute; and 5H1 mouse monoclonal from Lieping Chen Laboratory Yale School of Medicine [38]. Further, drug approval and availability may differ in different parts of the world. In the United States, Dako 22C3 is FDA approved as a companion diagnostic (i.e., required) for prescribing pembrolizumab, while Dako 28-8 and Ventana SP142 are FDA approved as complimentary (recommended) diagnostic tests for nivolumab and atezolizumab, respectively. The Ventana SP263 clone is currently FDA approved as a complementary diagnostic for durvalumab in the United States for bladder cancer with approval for lung cancer anticipated at the time of this writing; however, this antibody is CE marked in Europe for determining eligibility for nivolumab and, more recently, pembrolizumab in non-small cell lung cancer. While the current text is up to date at the time of writing, given the rapidly evolving approval processes and recommendations for patient testing, it is recommended that the reader additionally refer to locally applicable resources for the latest testing guidelines and recommendations.

Table 9.1 Summary of immune therapy drugs, corresponding anti-PD-L1 antibody, staining platform, and scoring parameters

Drug	Antibody clone	Developer	Platform/ detection system	Tumor cells scored	Immune cells scored	Current scoring for positive test
Nivolumab	28-8	Dako	Dako Link 48 Envision Flex	Yes	No	≥1%
Pembrolizumab	22C3	Dako	Dako Link 48 Envision Flex	Yes	No	≥50%-first line ≥1%-second line
Atezolizumab	SP142	Spring Bioscience	Ventana BenchMark	Yes	Yes	TC3/IC3 PD-L1 ≥50% TC2/IC2 PD-L1 5–49% TC1/IC1 PD-L1 1–4% TC0/IC0 PD-L1 <1%
Durvalumab	SP263	Ventana	Ventana BenchMark	Yes	No	≥25%
Avelumab	73-10	Dako	Dako Link 48 Envision Flex	Yes	No	≥1%

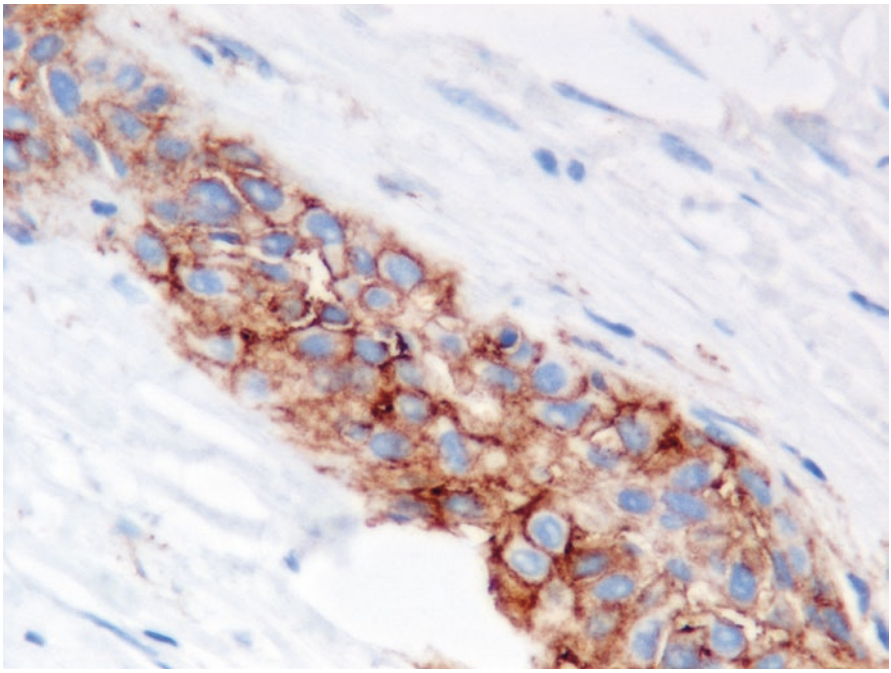

Fig. 9.3 Positive staining for PD-L1 is defined as partial or complete membranous staining of any intensity. This example shows strong membranous staining. Cytoplasmic staining should be discounted (Dako 22C3 Pharm Dx kit 400×)

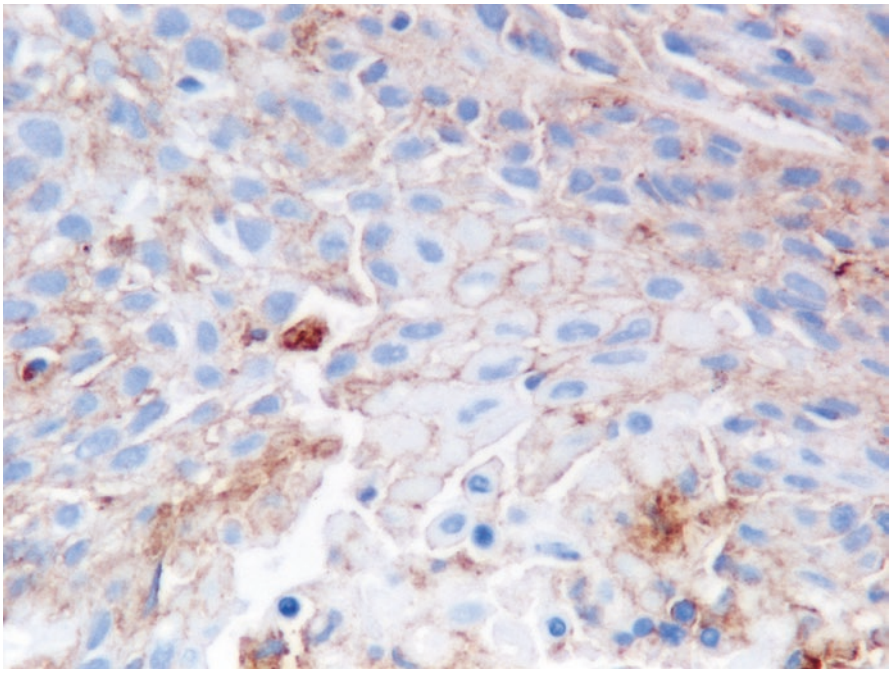

Fig. 9.4 In this example, PD-L1 staining is weaker and only partially stains the cells membrane, but would still be considered as positive staining (Dako 22C3 Pharm Dx kit 400×)

Challenges to the pathologist with the currently approved testing approach are myriad given the various antibodies, scoring systems, and testing platforms. As such, a practical solution, preferably with a single PD-L1 antibody which may be run and validated on a range of staining platforms, would be desirable. Several efforts to compare antibodies have been undertaken or are currently ongoing. To date, studies evaluating Dako 22C3 and 28-8 antibody kits have shown fairly good concordance of staining between these two antibodies. For example, in the BLUEPRINT phase 1 study, 14% of cases stained with 28-8 and 22C3 were discordant, and there was 72% concordance in the German harmonization study. Both studies demonstrate a lower percentage of staining of tumor cells with the SP142 antibody [39, 40]. SP263 has also been shown to demonstrate good concordance with 22C3 and 28-8 antibodies [39]. Similar results regarding concordance among 22C3, 28-8, and SP263 but not with SP142 have also been reported by Ratcliffe et al. and Rimm et al. [41, 42]. The Rimm study also found good concordance with the LDT E1L3N [42]. This particular LDT has been shown to have good sensitivity compared to other LDTs but further study is needed for this group of tests [38]. Another major issue is the requirements for particular staining platforms/detection system for a given antibody, a prospect that is generally not realistic for most laboratories. Initial studies evaluating cross platform concordance for 28-8, 22C3, and SP263 antibodies have shown good concordance [43].

Additional challenges include the fact that heterogeneity of staining may be present (Fig. 9.5), and while one study demonstrated 92% concordance between biopsy and resection [44], others have found a much lower concordance rate [45]. This heterogeneity may in part account for the number of patients who respond to anti-PD-1 or anti-PDL-1 treatment in spite of negative PD-L1 staining [46, 47]. Further complicating the current landscape is the fact that none of the antibodies are validated for use on cytology specimens, which is problematic as a cytology specimen may represent the only diagnostic tissue for many patients. While issues with heterogeneous sampling will not be completely eliminated, this issue and its relevance to treatment selection warrants further study. The issue of evaluating immune cells with the SP142 may prove more of a challenge with cytology specimens and lymph node samples regardless of biopsy type.

In summary, IHC has proven to be a useful methodology in certain testing situations, particularly in regard to screening for ALK and ROS1 mutations, but currently has less utility as a surrogate to molecular testing for most other molecular abnormalities. PD-L1 immunostaining has resulted in numerous challenges for pathology practice. Ongoing efforts will hopefully result in a simplified landscape in the future, but studies remain ongoing.

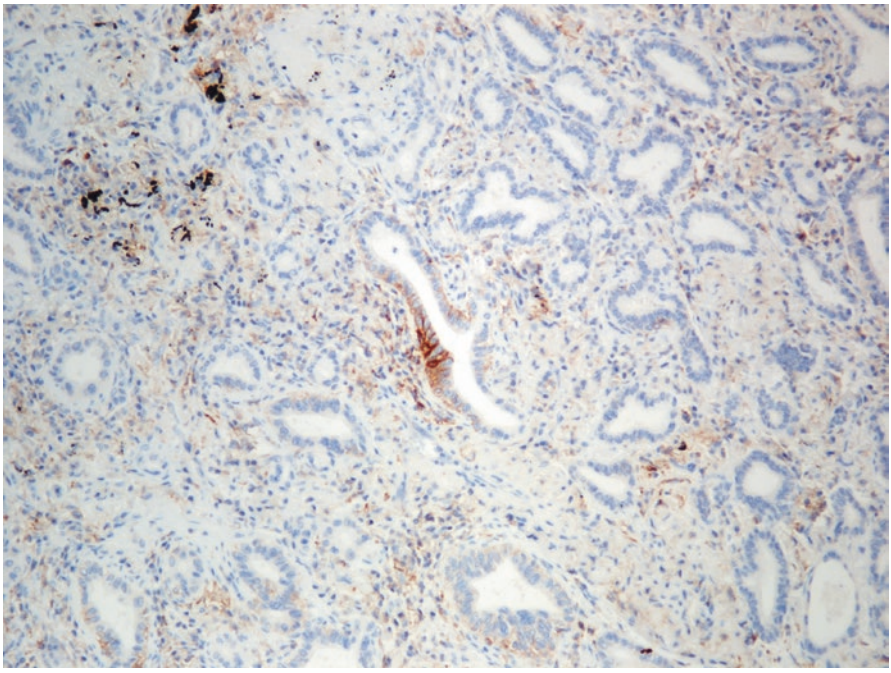

Fig. 9.5 IHC staining for PD-L1 may be heterogeneous. In this example, positive staining is only focally present in a large section of tumor, which could lead to discrepant negative results on a smaller sample (Dako 22C3 PharmDx kit 200×)

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Chapter 10
Next-Generation and Third-Generation Sequencing of Lung Cancer Biomarkers

Bryce Portier

Introduction

It is well established that lung cancer is an aggressive disease and it remains the leading cause of cancer-related deaths. Worldwide, there are more than 1.8 million new lung cancer cases diagnosed annually and over 1.5 million lung cancer-related deaths [1]. Morphologically, lung cancer is subdivided into two main types: non-small-cell lung cancer (NSCLC) which accounts for the majority 85% of new diagnosis and small-cell lung cancer which accounts for the minority 15% [2]. While NSCLC cases represent the majority of new diagnosis, this group can be further subdivided by morphologic and immunotypic methods into squamous cell carcinoma (SCC), adenocarcinoma (ADC), or large-cell lung carcinoma [3]. If all subtypes and clinical stages of lung cancer are combined, only 16% of patients achieve a benchmark of 5-year survival, which is largely due to the late stage (advanced disease progression) at the time of initial diagnosis [4]. In cases detected at an early stage (still localized), the 5-year survival rate is greatly increased to approximately 53% [5]. In an effort to improve early disease detection, new National Comprehensive Cancer Network (NCCN) guidelines recommend increasing low-dose computerized tomography (CT) screening. Furthermore, potential new diagnostic assays such as the automated three-dimensional morphologic analysis of epithelial cells in sputum (LuCED lung test) hopefully will increase the detection of early stage lung cancers [6, 7].

In addition to early detection, advances in understanding lung tumor biology and genomics are aiding in discovering new effective treatment solutions. Understanding the mechanisms and pathways that drive oncogenesis has directly led to the discovery of two predictive biomarkers in lung cancer: (1) epidermal growth factor receptor (*EGFR*) and (2) anaplastic lymphoma kinase (*ALK*) [8]. For *EGFR*, multiple

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_10

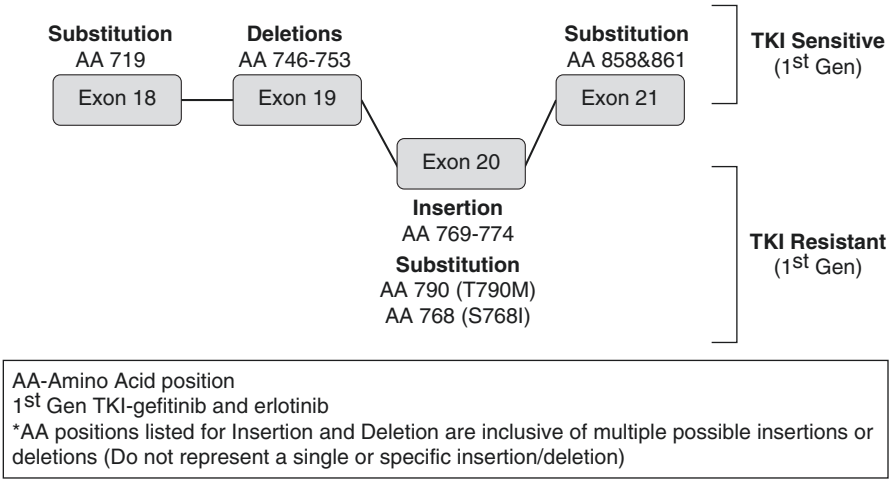

Fig. 10.1 Correlation of *EGFR* mutations and predicted TKI response

clinically significant alterations are known to occur in exons 18–21. Depending on the specific *EGFR* mutation, selection of a specific targeted therapy with sensitivity for that mutation can be determined (Fig. 10.1). One technology leading the path for new biomarker discovery and identification of driver pathways in lung cancer is next-generation sequencing (NGS). In the clinic, NGS technology is playing an essential role in interrogating large numbers of patients and screening vast portions of the genome in the search for altered genetic pathways and driver alterations in lung cancer. It is hopeful that NGS-based techniques, paired with prospective clinical trials, will expand our lung cancer biomarker knowledge and biomarker menu. Currently, only a limited set of biomarkers are routinely utilized in lung cancer clinical screening for targeted therapy selection (*EGFR*, *ALK*, and *ROS1*). In the following section, we will explore NGS with a focus on its clinical utility/benefits and variety of methodologies for addressing specific clinical questions and discuss barriers to widespread clinical adoption.

Next-Generation Sequencing (NGS) Background

The clinical use of NGS has significant benefits for diagnostic biomarker discovery and clinical screening capacity compared to traditional molecular assays such as single-gene Sanger-based sequencing (also referred to as first-generation sequencing) (Table 10.1). For clarification, the terminology "next"-generation sequencing or "NGS" refers to sequencing methodologies other than the traditional first-generation Sanger di-deoxy sequencing. NGS broadly encompasses both currently utilized methods referred to as "second-generation sequencing"

Table 10.1 Summary of NGS testing benefits and potential barriers to clinical adoption

Benefits of NGS vs. Sanger sequencing
Screen multiple genes/samples at one time (massive parallel)
Low input DNA/RNA required
Sensitivity to detect mutant allele at 2–10% (Sanger 15–25%)
Sensitive variant detection in samples with heterogeneity
Quantitative assay
Lower cost per sample/target
Ability to detect copy number alterations and gene fusions
Barriers to NGS adoption
Multiple platforms and rapidly evolving technology
Upfront cost of instrument and training
Lack of guidelines and unclear on process of LDT validation
Complex workflow with need for bioinformatic expertise
Dedicated hardware for analysis and long-term data storage

and new advancements in sequencing known as "third-generation sequencing" technologies. While debate over the exact categories for second- and third-generation sequencing Next-generation sequencing (NGS) exist, in general, second-generation sequencing represents methods that amplify DNA via emulsion PCR (e.g., Ion Torrent) or solid-phase amplification (e.g., Illumina). These methods are in contrast with third-generation sequencing which is performed utilizing non-amplified, single molecules (e.g., Pacific Biosciences and Oxford Nanopore). Regardless of classification as "second-" or "third"-generation sequencing, both methods are encompassed in the term "next-generation sequencing" in which the term "next" refers to any non-Sanger-based sequencing methodology.

The utility of NGS (second and third generation) over that of Sanger is the ability to perform massive parallel sequencing. In essence, massive parallel sequencing involves interrogation of numerous samples and numerous alterations with speed and accuracy. Ultimately, this results in higher throughput which reduces cost per sample. NGS is also highly flexible with specific applications that can be tailored to the clinical question [9]. The clinical use of NGS has fundamentally improved our understanding of lung cancer biology and has led to revolutionizing clinical molecular diagnostic testing. As diagnostic lung tissue is often limited, NGS allows interrogation of numerous targets with limited sample input secondary to its ultralow sample input requirement. It is also capable of detecting mutations below 15% mutant allele frequency (compared to Sanger which requires 15–25% mutant allele frequency) [10].

Input material for NGS can be either DNA or RNA. Multiple sequencing methods exist and include whole-genome sequencing (WGS for DNA), whole-exome sequencing (WES for DNA), whole-transcriptome sequencing (RNA-Seq for RNA), and targeted sequencing (TS either DNA or RNA). Each method WGS, WES, RNA-Seq, or TS has specific strengths and weaknesses. In general, DNA-based methods identify small base pair alterations, insertion/deletions, as well as potential copy number changes. One significant difference between WGS, WES, and TS is depth of sequencing reads generated per target, which is higher for TS assays which focus on a selection of targets that typically represent a small fraction of the exome or genome. For instance, in lung cancer, TS-based NGS assays could focus on known genomic alterations in key biomarkers. RNA-based sequencing is utilized for detection of alternative gene-spliced transcripts, posttranscriptional modifications, gene fusion, mutations/single-nucleotide polymorphisms, small and long noncoding RNAs, or changes in gene expression. These methods will be explored and described in more detail below.

NGS Methodology

Whole-Genome Sequencing (WGS)

Currently, WGS represents one of the highest cost NGS methods and is not routinely utilized in routine clinical screening or monitoring of lung cancer. However, like most technologies, the cost of WGS is declining with improvements in NGS technologies [9]. WGS can detect a wide range of genomic alterations, including known disease-associated and novel variants, a feature that makes this technique well suited for research. Barriers to routine clinical lung cancer screening include the cost, the large volume of data produced, and necessary expertise/tools for data mining. Data analysis is a significant challenge for WGS, and streamlined process needs to be generated for this method to fulfill the gaps needed in personalized medicine [11]. Clinical strengths of WGS include the ability to determine breakpoints in balanced chromosome translocations and inversions and detecting genomic alterations outside of coding regions [12]. WGS allows full interrogation of promoters, enhancers, introns, noncoding RNAs (i.e., miRNAs), and unannotated regions [13, 14]. This full view of the genomic landscape is well suited for research applications or driver pathway discovery where a comprehensive profile of point mutations, complex rearrangements, indels, and copy number alterations is required [12]. For example, The Cancer Genome Atlas (TCGA) Research Network utilized WGS for lung adenocarcinomas and identified 25 significantly mutant genes, including both known mutations, *TP53* (50%), *KRAS* (27%), *EGFR* (17%), *STK11* (15%), *KEAP1* (12%), *ATM*, *NF1* (11%), *BRAF* (8%), and *SMAD4* (3%), and unknown (never previously reported) mutations, *SMARCA4*, *ARID1A*, *RBM10*, *SETD2*, *PICK3CA*, *CBL*, *FBXW7*, *PPP2R1A*, *RB1*, *CTNNB1*, *U2AF1*, *KIAA0427*, *PTEN*, *BRD3*, *FGFR3*, and *GOPC* [15]. The trade-off for such complete genomic landscape

analysis is low sequencing coverage. This one feature greatly limits the clinical application for routine lung cancer screening. WGS coverages vary depending on methodology but on average are below 100-fold, whereas targeted sequencing assays routinely achieve greater than 1000-fold coverage. Fold coverage is directly correlated with ability to identify tumors with low mutation burden, which is especially problematic in tumors that are not clearly separated from non-tumor stroma (dilutes mutant allele burden) [10].

RNA Sequencing (RNA-Seq)

RNA-Seq is a specialized form of NGS which can be utilized to interrogate the lung cancer transcriptome (represents up to ~4% of the human genome) [16]. Following the central dogma of molecular biology, DNA is transcribed to messenger RNA (mRNA), and mRNA is translated into protein. While the human genome contains approximately 25,000 genes, not all genes will be transcribed and translated into protein. Moreover, not every coded gene will be transcribed in proper order due to alternate splicing. Therefore, sequencing RNA (specifically mRNA) allows one to address questions including what genes are being expressed and at what level of expression. RNA-Seq can generate a comprehensive profile of the complete transcriptome or be utilized for a more focused targeted sequencing application. RNA-Seq as a method allows mapping the boundaries of exons and introns for identification of splice variants, identification of gene translocations, posttranscriptional modification, mutations, and noncoding of miRNAs [9, 12, 17]. It also offers a highly sensitive assay for quantification of the abundance of a transcript, even higher than comparative microarray technology [18]. While RNA-Seq offers several options not available by DNA-based NGS, it has its own inherent challenges which include library construction (inherently more difficult due to labile RNA molecule), data mining (high number of low abundant transcripts—potential false-positive calls), and obtaining complete transcript coverage [19].

Whole-Exome Sequencing (WES)

WES is utilized to specifically sequence the coding exons (~2.5% of the human genome) or the portion of genes that form the template for mRNA and successive protein production. This methodology specifically ignores noncoding regions such as promoters, enhancers, introns, and noncoding RNAs. Elimination of sequencing in these regions decreases the number of sequencing targets and thereby allows for improved fold coverage. WES focus solely on coding exons in annotated genes and therefore only allows variant detection in known coding genes. WES can be designed to also include sequencing of selected or limited regions of noncoding DNA regions which include exon-flanking regions and potentially select miRNAs. Similar to

WGS, the amount of sequencing data can be extensive for each sample and the number of total detected variants by WES can be high (20,000–30,000 range) depending on tumor sample and NGS methods/bioinformatics utilized. This large number of variants makes detecting actionable activating mutations a challenge. While more focused than WGS, application of WES to lung carcinoma is still currently best suited for research rather than routine clinical practice. Improvements in NGS such as decreased cost, faster analysis time, increased coverage, and improved accuracy could drive increased adoption of WES into routine clinical practice [10].

Targeted Sequencing (TS)

TS represents the most clinically utilized current NGS assay for lung cancer diagnostic testing. This method focuses specifically on interrogation of known genomic regions of interest. TS limits the sequencing to a small number of targeted regions, ultimately decreasing the amount of sequencing time and data generated, while also making the assay highly cost-effective by increasing the number of samples that can be analyzed simultaneously (multiplexed). Limiting TS to known cancer-relevant alterations makes this assay highly suited for clinical use which requires detecting known alterations such as point mutations and deletions in *EGFR* or even translocations in *ALK* or *ROS1*. However, being so highly targeted, this method may miss variants that are present but not located in regions interrogated by the assay. The adoption of TS via NGS into clinical practice for lung cancer has resulted in the availability of a highly sensitive method for detecting actionable alterations in lung cancer specimens [20–22]. A recent report showed NGS-based TS was able to identify *EGFR*/*KRAS*/*ALK* alterations in up to 58% of patients that were called wild type by standard testing, which translated into improved opportunities for therapeutic intervention [23]. Since most NSCLCs are detected once locally advanced and/or inoperable tumors, often only fine needle aspirate (FNA) cytology samples of mets are available for molecular testing. FNA tumor cell content may be very limited and therefore testing by traditional Sanger sequencing would not be possible. However, TS via NGS can utilize nanogram quantities of DNA, and FNA/cytology samples have been shown to be sufficient for TS NGS analysis [24–26].

NGS Translocation Detection

Currently, the list of routinely tested and actionable translocations specific for lung cancer is small and includes *ALK*, *RET*, and *ROS1*. Other kinase gene fusions have been detected by NGS from isolated lung adenocarcinoma DNA and RNA and include *MPRIP-NTRK1*, *AXL-MBIP*, *SCAf11-PDGFRA*, and *EZR-ERBB4* [27–29]. Regardless of molecular methodology utilized for detection, accurate identification of translocations can be challenging. Utilizing in situ hybridization (ISH) is the

current gold standard, but immunohistochemistry (IHC) is often performed as it offers a faster and less burdensome screening/detection methodology. However, IHC does not actually identify the translocation; rather, it identifies overexpression of a protein that occurs secondary to the translocation. Therefore, the IHC approach is applicable for *ALK* which lacks endogenous expression in the lung, but is not a viable option for identification of *RET* translocations due to endogenous *RET* expression [30] and potentially not useful for *ROS1* due to false-positive staining and poor correlation with FISH [31]. Unlike ISH and IHC options, NGS can be applied to identify both known and de novo translocations. In addition, NGS allows the simultaneous screening of actionable gene fusions in a single assay with high specificity and low input requirements (sample preservation). The inherent difficulty in identifying translocations via NGS is the high variability of translocation partners and breakpoints along with low incidence of translocations in lung cancer. While the canonical *EML4–ALK* fusion consists of *EML4* exons 1–13 fused to *ALK* exons 20–29, over 20 different *ALK* translocation partners have been identified [32]. NGS is gaining clinical utilization for translocation detection in lung carcinoma due to its comprehensive screening of multiple low incidence translocations, paired with high sensitivity for detection, rapid assay run time, and lower cost compared to single assay/single translocation testing options such as ISH [33]. Ultimately, the goal of utilizing NGS for translocation detection is to properly and rapidly stratify patients to the proper best personalized targeted therapy (sunitinib, sorafenib, or vandetanib) [28, 34].

NGS Utilizing Liquid Biopsy

The overarching trend in molecular diagnostics is to do more with less. NGS is perfectly suited for this task, as very little material is required for testing and the methodology is flexible to allow full mutation profiling or translocation screening. However, this is only applicable when tissue or cytology samples are available, which is not the case for routine follow-up or disease management. In these cases, often minimally invasive blood draws (liquid biopsies) are performed. Recently, much interest is focused on nucleic acid isolation from liquid biopsies via capturing rare circulating tumor cells (CTCs) or cell-free DNA (CF-DNA). A detailed discussion on the advantages and disadvantages of CTCs vs. CF-DNA is outside the scope of this article; however, a good summary was recently published [35]. Both CTC and CF-DNA have been successfully applied to capture starting material for clinical NGS testing. CTCs)have already shown utility for NGS-based *EGFR* mutation testing, with one study showing an 84% match in CTC *EGFR* mutation profile compared to tissue biopsy and in addition multiple *EGFR* mutations were identified demonstrating the possibility of detecting tumor heterogeneity [36]. Likewise, CF-DNA has been successfully utilized for NGS-based lung cancer diagnostic testing for both general mutation screening and focused identification of acquired tyrosine kinase inhibitor (TKI) resistance *EGFR* mutations [37, 38]. The difficulty with

)CTC or CF-DNA applications is the very limited amount of DNA and the mixture of genomic and tumor nucleic acid. To overcome these challenges, NGS methodologies have been developed such as Tagged Amplicon Deep Sequencing (TAm-Seq), Safe Sequencing System (Safe-SeqS), and Cancer Personalized Profiling by deep sequencing (CAPP-seq) which have demonstrated up to 92% sensitivity and >99.99% specificity for *EGFR* mutation detection at the variant level [39–42]. These novel NGS methods improve the sensitivity of standard NGS by performing highly targeted hybrid capture, high-throughput deep sequencing, and utilizing bioinformatic tools to remove artifacts and discover rare mutations and potentially translocations [43].

Barriers to Adoption of Clinical NGS for Lung Cancer

While NGS has gained widespread use as a research tool, it has only been in the last few years that it has started to gain acceptance and utilization in the highly regulated clinical CAP/CLIA laboratory-based environment. Several barriers exist for widespread clinical adoption including cost, rapid technology change, lack of regulatory guidance, and complex bioinformatic data interpretation challenges (Table 10.1). These items will be discussed in detail below.

Cost of Clinical NGS Testing

Like most new technologies, NGS instrumentation and reagents can represent a high-cost burden for labs interested in undertaking the task of starting NGS testing. Instrument prices vary from sub-100,000 US dollar benchtop sequences to over 1,000,000 US dollars for high-throughput instrumentation. On top of instrument capital purchase cost, there is an annual service contract (price is highly variable). There are also costs for reagents, assay validations, personnel, and data analysis. NGS has a high upfront and operation cost relative to other molecular diagnostic equipment such as real-time PCR or Sanger-based assays. Cost can be greatly minimized per sample or test by the high degree of multiplexing that is capable, but lab volume and in-house expertise should be considered before initiating a NGS sequencing assay in the clinical setting. An additional variable that should be considered is the amount of testing reimbursement that will be generated by NGS testing. Current Procedural Terminology (CPT) codes are continually updated and in 2017 CPT codes for NGS-based testing exist [44]. However, the rate of successful reimbursement and the amount of reimbursement can be highly variable depending on geographic location and payer. This uncertainty in financial return is a direct barrier to widespread clinical adoption.

Guidelines

Although NGS is extensively used for research, its application in clinical practice has not been fully realized in part due to the lack of formalized validation and testing guidelines. NGS testing, while a promising method for lung cancer screening, is still a relatively new technology, and therefore, standards for validation in the CAP/CLIA lab are not well established. In addition, the regulation of laboratory-developed tests (LDT) in general has been a major unanswered question. The Food and Drug Administration (FDA) issued draft guidance in 2014 outlining new enforcement of testing regulation specifically targeting LDTs [45]. Based on this draft guidance, it was unclear what regulation NGS-based LDT testing would follow. However, recently, the FDA released a white paper that stated it would not issue a final guidance on the oversight of LDT [46]. This publication has largely cleared the way for NGS assay validations to move forward and fall under the regulatory guidance of CAP inspections and inclusion in proficiency testing, similar to other high complexity assays performed in the CAP/CLIA clinical laboratory setting. Moving forward, NGS still represents a unique validation challenge for molecular diagnostic labs, while well acquainted with running DNA-/RNA-based assays "wet lab," it is the post-run analytical component of NGS that is difficult to validate, due in part to the novelty of NGS analytic tools and novel skills required for NGS bioinformatic data. In addition, NGS analysis requires a multistep "pipeline" method for processing data in which small deviations in assay design or post-sequencing analytic processing (filters) can impose any number of potential downstream errors. Despite these challenges, NGS is still being adopted in academic and private hospitals and has proven to be a profitable entity for commercial companies [47].

Bioinformatics

An in-depth exploration of bioinformatic approaches utilized in clinical NGS analysis is outside the scope of this chapter. However, it is worth mentioning that the large-scale data produced by NGS is a significant obstacle to adoption of clinical-based NGS assays [48]. To identify variants from NGS data, often multiple software packages need to be stitched together into a data analysis pipeline. These programs include sequence aligners, variant callers, and variant annotation. Each software component allows modification of multiple variables that can be user altered to allow highly customized workflows but at the price of decreased standardization and ability to perform quality assessments between labs. Most "pipelines" will consist of a sequence aligner (maps sequencing reads to a reference genome), variant caller (identifies variant sites), and variant annotation (links variant calls to database with annotated lists of clinical variants such as Catalog for Somatic Mutations in Cancer (COSMIC) [49]. The performances of different aligners have been

extensively studied and each has pros/cons, making adoption of a uniform analysis pipeline unlikely [50, 51]. One common tool that offers a good introduction to NGS data analysis is the Genome Analysis Toolkit (GATK; Broad Institute, Cambridge, MA, USA) [49]. This toolkit allows multiple standardized forms of NGS analysis and has well-documented instructions for users.

In addition to software standardization, an additional hurdle with clinical NGS is the sheer volume of data produced. This can cause analytic bottlenecks, even with recent advances and lowered costs of processing power. An additional potential problem is long-term data storage in a CAP-/CLIA-approved manner. Types of files or recommended length of storage for NGS has not been standardized at this time. Lastly, how labs are reimbursed for complex NGS analysis and even how labs integrate NGS data into electronic health records is highly variable, with no set standardization or national guidance [52–54].

Conclusion

There is clear evidence that NGS can accurately identify clinically significant biomarkers for lung cancer, such as *EGFR* mutations and *ALK* rearrangements, and that this technology can help guide personalized targeted therapies [55]. NGS performed on lung biopsies or cytology specimens can identify both established and emerging biomarkers depending on selected targets for sequencing and analysis [8, 56]. Likewise, NGS performed on CTCs or CF-DNA can be utilized to identify biomarkers for guided therapy or follow patients for monitoring development of tyrosine kinase inhibitor (TKI) resistance (such as *EGFR* T790M). Ultimately, the significance of applying clinical NGS to lung cancer screening is its ability to simultaneously interrogate numerous biomarkers and rapidly/accurately direct patients to an approved efficacious targeted treatment. Ongoing exploratory research utilizing NGS will undoubtedly translate into discovery and validation of novel predictive biomarkers, which will ultimately translate into clinical NGS practice and improve lung cancer diagnosis and treatment. Furthermore, it is expected that NGS technology will continue to advance at an accelerated rate and that the tangible outcome of this will be the improvement in our understanding of causal genetic mutations/alterations in lung cancer and continued improvement in lung cancer treatment and outcome.

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Part III
Molecular Pathology of Specific Cell Types

Chapter 11
Mutations as Predictive Biomarkers for Adenocarcinoma

Navin R. Mahadevan and Lynette M. Sholl

Introduction

Lung carcinoma is one of the most common and lethal diseases afflicting the global population. Despite decades of efforts to improve outcomes through multimodal therapy, including surgery, radiotherapy, and chemotherapy, survival rates have remained dismal. Invasive lung adenocarcinoma is the most common lung carcinoma in the United States [1] and comprises a genetically, morphologically, and clinically diverse collection of tumors. In surgical pathology practice, invasive adenocarcinomas are currently subtyped according to their predominant histologic pattern (lepidic, acinar, papillary, micropapillary, or solid), some of which carry both prognostic and predictive significance [2], although historically these patterns have not significantly influenced clinical decision making. The discovery of recurrent molecular alterations in lung adenocarcinoma and subsequent development of genetically targeted therapies have revolutionized the diagnosis and treatment of these tumors. With our current understanding, approximately 60% of lung adenocarcinomas have a defined oncogenic driver mutation (Fig. 11.1) that in many cases predicts treatment response.

Molecular diagnostic techniques, including PCR and single-gene sequencing, fluorescence in situ hybridization (FISH), immunohistochemistry, and, most recently, next-generation (massively parallel) sequencing, have come to the fore in the pathologic characterization of lung adenocarcinoma, and molecular pathologists now play a key role in the multidisciplinary approach to the evaluation of this tumor type. Here we will review the most common targetable mutations in lung adenocarcinoma, the parallel development of molecularly targeted therapies, and the specific molecularly defined resistance mechanisms arising in the face of these therapies.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_11

EGFR

EGFR is a member of the ErbB transmembrane growth factor receptor family and is comprised of an extracellular ligand-binding region, a single hydrophobic transmembrane bridge connected to an intracellular juxtamembrane (JM) region, a tyrosine kinase domain, and an intracellular C-terminal tail with multiple tyrosine residues. Upon extracellular ligand binding, EGFR homodimerizes or heterodimerizes with other members of the ErbB family and autophosphorylates its C-terminal tyrosines, causing downstream intracellular activation of PI3K, JAK/STAT, and MAPK signaling pathways, ultimately leading to cellular proliferation. Approximately 20% of lung adenocarcinomas (Fig. 11.1) harbor somatic activating

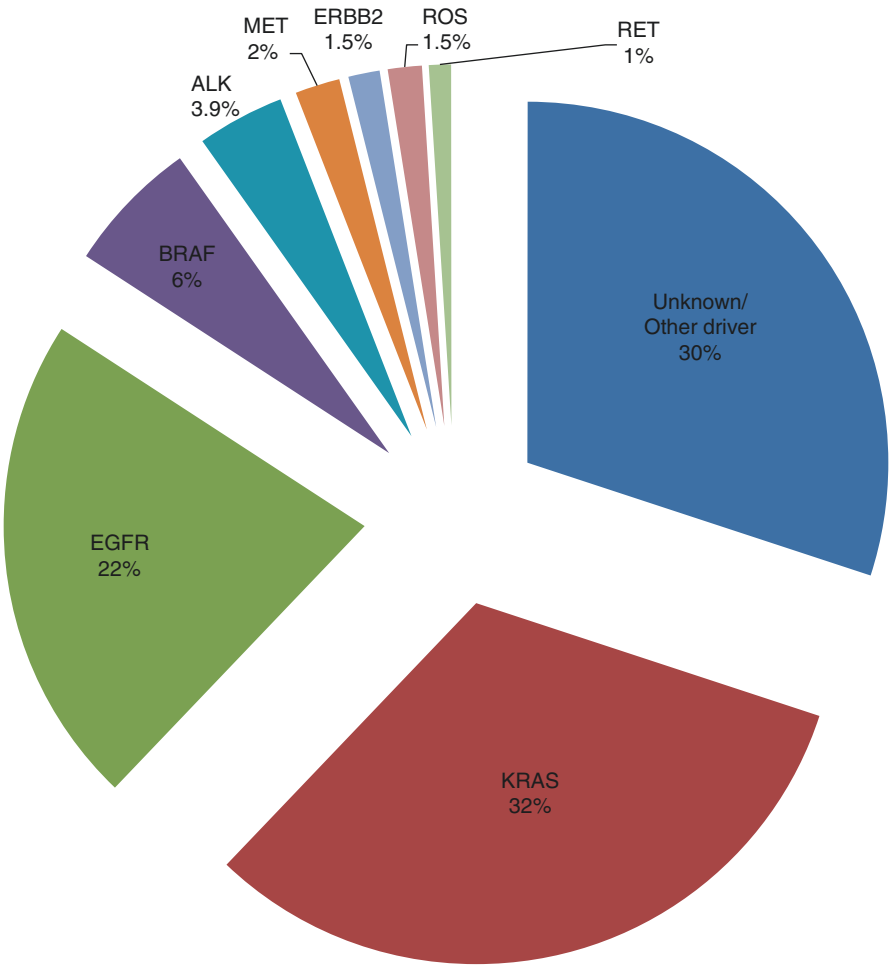

Fig. 11.1 Molecular landscape of lung adenocarcinoma. Estimation of mutation prevalence is based on targeted next generation sequencing of lung adenocarcinomas performed at Brigham and Women's Hospital and Dana–Farber Cancer Institute ("Oncopanel") from 2013–2017. Prevalence data for fusions is based on Awad MM et al. 2016 10.1200/JCO.2015.63.4600 [16]. Please see text for relevant discussion of alterations

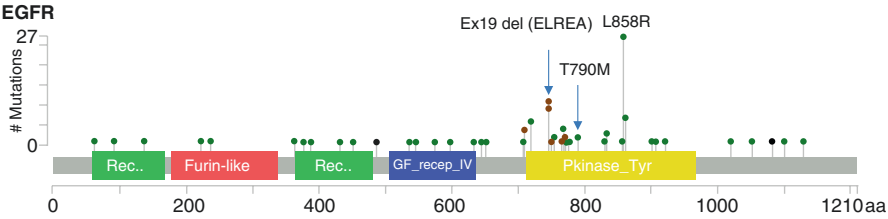

Fig. 11.2 Distribution of mutations in *EGFR* lung adenocarcinoma. Missense, in-frame deletion, and nonsense mutations are represented by *green, brown, and black lollipop symbols*, respectively. Adapted from http://www.cbioportal.org (accessed 9 Apr 2017)

mutations in *EGFR*; *EGFR* mutations more commonly occur in female patients, never-smokers, and patients of East Asian descent [3]. *EGFR* mutations cluster in the tyrosine kinase domain encoded by exons 18, 19, 20, and 21. The most common activating *EGFR* mutations (~95%) are a point substitution in exon 21 (L858R) and in-frame deletions in exon 19 involving the LREA motif (Fig. 11.2), which result in constitutive ligand-independent kinase activity [4]. The therapeutic importance of these molecular alterations was shown in the mid-2000s when it was found that lung adenocarcinomas specifically harboring these activating mutations responded to treatment with small-molecule EGFR inhibitors, such as erlotinib, gefitinib, and afatinib, whereas *EGFR* wild-type tumors did not (reviewed in [4]). This led to a paradigm shift in the diagnosis and treatment of lung adenocarcinoma wherein detection of these genetic alterations was used to select patients for targeted therapy, beginning the era of "precision medicine" in solid tumor oncology.

Acquired resistance to EGFR-targeted therapy invariably arises in patients at a median of 12 months following initiation of therapy [5]. The most common mechanism (~50%) is the T790M exon 20 mutation (see Fig. 11.2) in the ATP-binding site of the kinase domain, which results in the normalization of increased ATP affinity enabled by the activating *EGFR* mutation (e.g., L858R) to near wild-type levels [6]. Recently, third-generation EGFR inhibitors (e.g., osimertinib) designed to overcome the T790M-mediated resistance have been approved by the FDA based on reported 95% clinical efficacy of these drugs in phase I clinical trials of patients with EGFR T790M mutations [7]. Phase 3 randomized trials have confirmed the superiority of osimertinib over platinum-based chemotherapy in the relapse setting in terms of disease response, progression-free survival, and adverse events [8]. Thus, detection of the T790M mutation at the time of relapse has become critical to guide further targeted therapy. Non-T790M mechanisms of EGFR resistance also exist, including *MET* and *ERBB2* (HER2/neu) amplification, *PIK3CA* mutation, and evolution to small cell carcinoma [9], some of which may be amenable to targeted therapy where such agents exist (e.g., MET, ERBB2 inhibitors [10, 11]).

Testing for *EGFR* mutations is recommended for all patients with lung adenocarcinoma at time of diagnosis ([3]-guidelines update, in preparation) and is routinely

performed using a variety of methods, including Sanger sequencing with and without mutated allele enrichment, the amplification refractory mutation system, fragment length analysis, restriction fragment length polymorphism, real-time PCR, and digital droplet PCR. More recently, *EGFR* mutation testing has been incorporated into next-generation panel-based sequencing platforms allowing for co-detection of potentially targetable molecular alterations and resistance mechanisms. Furthermore, plasma-based PCR testing for circulating cell-free DNA (cfDNA) can detect T790M in circulating tumor DNA from EFGR tyrosine kinase inhibitor (TKI)-resistant patients and is associated with clinical response to osimertinib [12]. Assays using next-generation sequencing of cfDNA are currently widely available; however, the clinical performance of this approach to tumor genotyping in different settings is still under investigation.

MET Exon 14 Skipping

c-Met encodes MET, which belongs to a family of receptor tyrosine kinases that play a crucial role in cell growth and proliferation, survival and apoptosis, epithelial-mesenchymal transition (EMT) and invasion, tissue remodeling, and morphogenesis. MET is physiologically activated by the binding of its specific ligand, hepatocyte growth factor (HGF), leading to the downstream activation of PI3K, STAT, and MAPK pathways [13]. The intracellular MET juxtamembrane domain is partially encoded by exon 14 and contains critical regulatory elements, including a tyrosine at position 1003 (Y1003), which is the direct binding site for Cbl, an E3 ubiquitin ligase that promotes MET protein degradation [14]. Somatic substitution and small deletion mutations at the exon 14 splice acceptor and donor sites leading to exon 14 skipping, as well as rare missense substitutions involving Y1003 (Fig. 11.3), lead to increased stability and oncogenic potential of the MET receptor ([15]). *MET* exon 14 skipping mutations are seen in 3–4% of non-squamous lung carcinomas (Fig. 11.1), the majority of which are adenocarcinomas. Stage IV tumors with *MET* exon 14 skipping mutations tend also to show concurrent MET copy number amplification and strong protein expression by immunohistochemistry. *MET* exon 14 skipping mutations are associated with older age and female gender and are enriched in tumors with a poorly differentiated (see Fig. 11.4) or pleomorphic histomorphology [16, 17]. Isolated case series have shown partial responses of MET exon 14 skipping mutant lung adenocarcinomas to the multitargeted tyrosine kinase inhibitors, crizotinib and cabozantinib [16, 18].

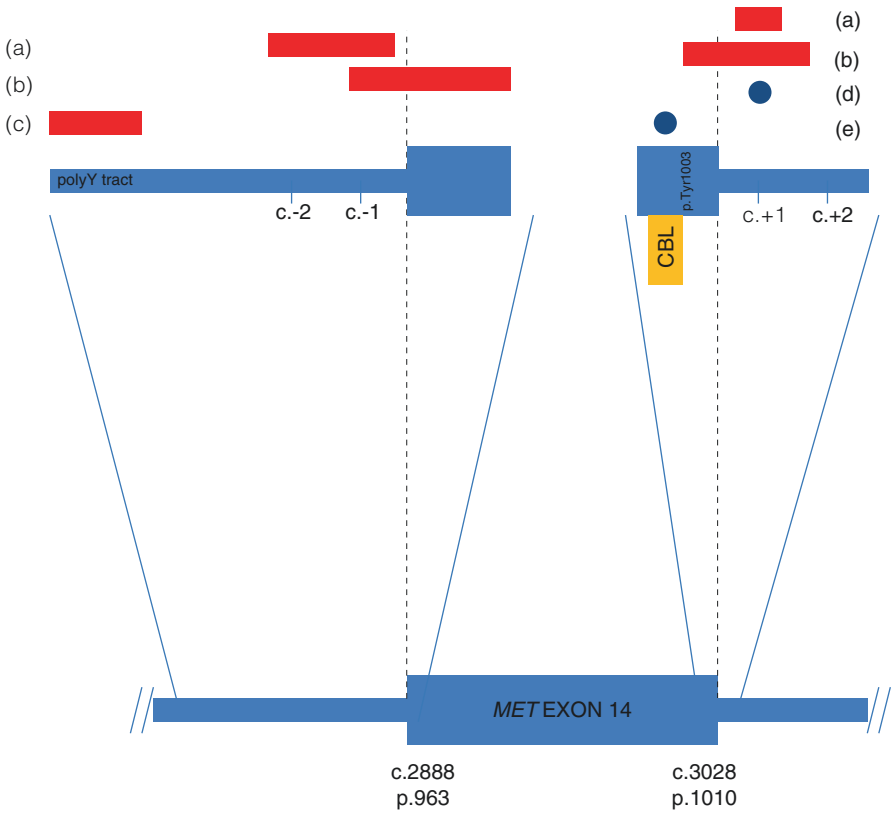

Fig. 11.3 Schematic of mutations in *MET* (NM_001127500.2) that result in exon 14 skipping in lung adenocarcinoma. *Black dotted lines* represent intron/exon boundaries. Deletions are shown as red *rectangles* and point mutations are shown as *blue circles*. Deletions can occur at the (**a**) canonical splice donor and acceptor sites, (**b**) span the intron/exon boundaries, or involve the (**c**) polypyrimidine (polyY) tract in intron 13. Point mutations can occur at the (**d**) splice donor site or at a (**e**) key tyrosine residue (p.Tyr1003) that mediates CBL binding

Resistance mechanisms related to MET inhibitors specifically in the context of exon 14 skipping have not been well described; however, kinase domain mutations such as *MET* p.D1228V that interfere with inhibitor binding have been demonstrated to confer resistance to MET inhibitors when applied in the context of *MET* gene amplification [19].

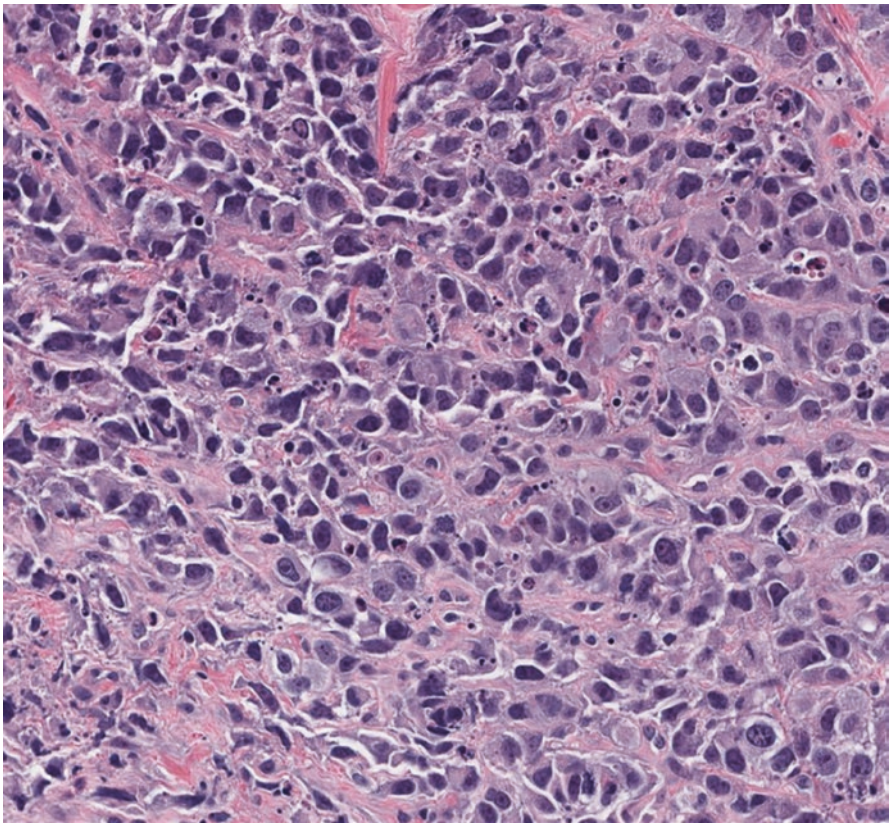

Fig. 11.4 Morphology of lung adenocarcinoma with *MET* exon 14 skipping mutation. This lung adenocarcinoma shows poorly differentiated features, which is often associated with MET exon 14 skipping (and gene amplification). Next-generation sequencing revealed a canonical exon 14 skipping mutation, *MET* p.D1010N (c.3028G>A), and gene amplification (hematoxylin and eosin staining, 200× magnification)

BRAF

BRAF encodes a serine/threonine kinase that is a key member of the RAS/RAF/MAPK growth and proliferation signaling pathway. While activating mutations of BRAF are very common in certain tumor types (e.g., cutaneous melanoma), *BRAF* mutations overall are relatively rare in lung adenocarcinoma (~4%, Fig. 11.1). The activating V600E mutation in exon 15 predominates in other tumor types, whereas only about half of lung adenocarcinomas harbor this mutation. About half of the *BRAF* mutations seen in lung adenocarcinoma occur at other codons in exon 15 or at several hotspots in exon 11 (Fig. 11.5) [20]. *BRAF* V600E mutations are generally present in a mutually exclusive fashion with other oncogenic drivers, but recurrent non-V600 are more likely to be observed as co-mutations, including with

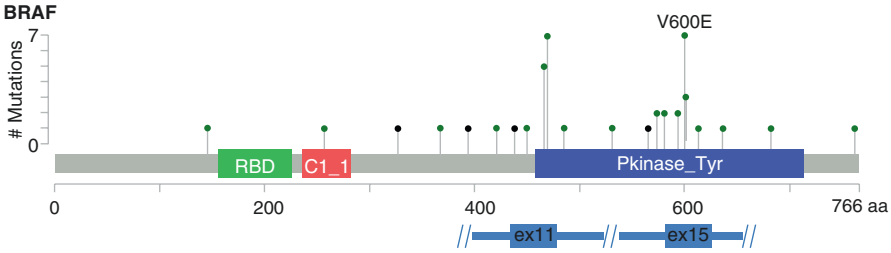

Fig. 11.5 Distribution of mutations in *BRAF* in lung adenocarcinoma. Missense and nonsense mutations are represented by*green* and *black lollipop symbols*, respectively. Adapted from http://www.cbioportal.org (accessed 9 Apr 2017)

EGFR and *KRAS* [21]. In contrast to the success of other molecularly targeted therapies, stand-alone BRAF-specific inhibitors have had generally disappointing results in lung adenocarcinoma, with resistance developing within 6 months despite significant initial responses (reviewed in [22]). Resistance mechanisms include constitutive autocrine signaling through EGFR or loss of the full-length BRAF V600E-mutated protein. However, phase 2 trials of the BRAF inhibitor dabrafenib together with the MEK inhibitor trametinib have demonstrated a 63% response rate in patients with *BRAF* V600E-mutated lung adenocarcinoma with a median duration of response of 9 months [23]; therefore, targeted therapies do hold promise in this setting. Current clinical guidelines do not recommend up-front, stand-alone testing for *BRAF* mutations, but molecular labs should incorporate testing of the *BRAF* gene, ideally to include both exons 11 and 15, into multiplex genotyping or next-generation sequencing panel testing [guidelines in preparation].

ERBB2

Like EGFR, ERBB2, also known as HER2/neu, is a member of the ErbB family of growth factor receptors and a well-recognized oncogene in a variety of tumor types. In breast and gastrointestinal carcinomas, *ERBB2* amplification is relatively common, leading to activation of downstream MEK and AKT pathways and predicting response to targeted therapies such as trastuzumab and lapatinib. The predominant mechanism of ERBB2 activation in lung takes the form of mutations, rather than amplification. The most common activating mutations of *ERBB2* in lung adenocarcinoma occur as small exon 20 in-frame insertion mutations occurring at or around codon 775 in the kinase domain and leading to duplications of some or all of the YVMA amino acid sequence [24]. These mutations lead to constitutive phosphorylation and activation of HER2 and downstream signaling pathways (AKT, MAPK; [25]). *ERBB2* exon 20 mutations have been reported in approximately 2% of lung adenocarcinomas (Fig. 11.1) and tend to occur in female nonsmokers and patients of East Asian ethnicity, similar to the characteristics of patients with *EGFR* mutations [26–28]. Several case studies have shown responses in patients harboring

ERBB2-mutant lung adenocarcinomas to ERBB2-inhibitors in combination therapies [29–31]. The largest study to date examining the efficacy of ERBB2-targeted agents in this tumor type is a retrospective study that included a total of 101 patients with *ERBB2*-mutated lung adenocarcinoma, of which 65 patients received ERBB2-directed therapies with conventional platinum-based chemotherapy. ERBB2-targeted therapies (trastuzumab and trastuzumab-emtansine) in combination with chemotherapy were associated with a 51% overall response rate and 4.8-month median progression-free survival, which was similar to patients receiving chemotherapy alone in the first line [32]. Together, these data suggest that *ERBB2*-mutant lung adenocarcinoma may be sensitive to targeted therapy, though larger prospective studies are needed to determine true clinical efficacy. Studies are conflicting on the association between *ERBB2* activating mutations and copy number changes in lung adenocarcinoma. Limited studies examining *ERBB2*-mutated tumors suggest a high frequency of concomitant *ERBB2* gene polysomy or amplification [33]. However, other studies show that, overall, ERBB2 amplification is rare in lung adenocarcinoma and may co-occur with other oncogenic driver alterations. Further, these studies show that ERBB2 amplification and mutation are largely nonoverlapping, suggesting that these represent distinct mechanisms of oncogene activation and tumorigenesis in the lung [34]. Overall, the number of cases analyzed to date limits the conclusions that can be drawn on this topic. There is little evidence to support routine testing for *ERBB2* amplification; however, *ERBB2* exon 20 mutation detection should be incorporated into genotyping or sequencing panels for lung adenocarcinoma [CAP/IASLC/AMP Lung Cancer Testing Guidelines, in preparation].

PD-L1

PD-L1 (also known as CD274) is a transmembrane protein expressed on epithelial and antigen-presenting cells that serves as the ligand for PD-1 on T cells. Engagement of PD-L1 and PD-1 during T-cell activation by antigen-MHC complexes leads to inhibitory signal transduction within T cells resulting in decreased T-cell proliferation and, ultimately, anergy/exhaustion or apoptosis. The PD-L1/PD-1 axis serves as one of the several immunosuppressive effectors in the tumor microenvironment, decreasing cell-extrinsic immune control of tumor growth. Recently, monoclonal antibodies that block the interaction of PD-L1 and PD-1 (nivolumab and pembrolizumab, which bind PD-1, and atezolizumab, which binds PD-L1—known collectively as immune checkpoint blockade) have been shown to have durable clinical efficacy in approximately 20–30% of non-small cell lung carcinoma, including adenocarcinoma. PD-L1 expression on tumor cells (and in some cases infiltrating immune cells) as assessed by immunohistochemistry has been used as a biomarker to predict response to these agents, though these efforts have been complicated by the use of different antibodies on different staining platforms, which has led to a variety of cutoffs for PD-L1 expression based on each immune checkpoint inhibitor trial (for review, see [35]). When integrating

the data over the different clinical trials run for each agent, there is a consistent trend that higher PD-L1 expression predicts higher objective response rate; however, for each agent, there still remains a ~10% response rate in PD-L1 negative lung carcinoma [35].

As discussed above, while individual mutations predict responses to genetically targeted therapies, one would hypothesize that an overall higher mutational burden (perhaps regardless of the identity of individual mutations) would lead to a greater number of neoantigens and an increased probability of response to immune checkpoint blockade. Testing this hypothesis, Rizvi et al. [36] examined the relationship between non-synonymous mutation burden as determined by whole-exome sequencing and response to pembrolizumab in advanced NSCLC. They found that increased mutation burden was associated with increased progression-free survival and durable clinical benefit (>6 months of stable disease or partial response) to pembrolizumab. In addition, a transversion-high mutation signature hallmark of exposure to tobacco smoke, which positively correlated with mutation burden, was associated with increased progression-free survival. Congruently, patients who experienced a durable clinical benefit had tumors that were enriched for activating KRAS mutations, which are more common in smoking-associated tumors [36, 37].

Sequencing algorithms that can assess the presence of tumor neoantigens and the intratumoral or systemic T-cell repertoire may also be leveraged to identify likely responders to immunotherapy [38, 39]. KRAS is the most commonly mutated oncogene in lung adenocarcinoma (see Fig. 11.1), yet has proven to be the most difficult to target. Immune checkpoint blockade may prove to be an effective approach to treating at least a subset of patients with KRAS-mutated lung adenocarcinomas, and KRAS hotspots (codons 12, 13, 61) should be incorporated into routine molecular panel tests [guidelines in preparation].

Conclusions

Lung adenocarcinoma stands as the paradigm for genetically targeted “precision” therapy in solid tumors, as it has been found to harbor multiple oncogenic driver mutations for which targeted therapies exist. Multiple molecular methods (single-gene sequencing, FISH, immunohistochemistry) have been used clinically to interrogate which of these alterations exist in any given tumor at diagnosis (EGFR, ALK, ROS1, PD-L1). However, as next-generation sequencing becomes more commonly adopted, targeted gene panels or exome interrogation will allow more unbiased discovery of molecular alterations in lung adenocarcinoma, including oncogenic drivers, important tumor suppressors—and, perhaps, mutation signatures that predispose to an efficacious antitumor immune response—as well as possible mechanisms of resistance that may arise during therapy. The evaluation of lung adenocarcinoma will become ever more multidisciplinary, requiring the coordination of surgeons, oncologists, surgical pathologists, and molecular pathologists to ensure accurate diagnosis and appropriate treatment.

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Chapter 12
Translocations as Predictive Biomarkers in Lung Cancer

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Overview

Oncogenic fusion events involving *ALK*, *ROS1*, *RET*, and other genes can be identified in up to 10% of non-small cell lung cancers, the vast majority of which are adenocarcinomas. Clinical trials have established a clear role for targeted therapies in ALK- and ROS1-rearranged lung tumors, and the FDA has approved the use of certain tyrosine kinase inhibitors in the first line for patients with demonstrated rearrangements by immunohistochemistry and/or cytogenetic and/or molecular methods. Both *ALK*- and *ROS1*-rearranged lung tumors are significantly more likely to occur in non-smokers. *RET* rearrangements, in contrast, have a less striking association with never-smoking status. An optimal targeted therapy has not yet been identified for *RET*-rearranged lung carcinomas. Tumors with *ALK*, *ROS1*, and *RET* fusions have some common histologic features, including extracellular mucin and prominent signet ring cells, as well as aggressive morphologic patterns including solid, cribriform, and micropapillary growth. *NTRK* and *NRG1* fusions are exceptionally rare in primary lung tumors, but may predict response to targeted therapies.

ALK

Oncogenic fusions involving anaplastic lymphoma kinase (ALK) were originally described in anaplastic large cell lymphoma [1], subsequently in inflammatory myofibroblastic tumor, [2] and then in 2007 in a subset of lung carcinomas [3].

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_12

The dominant 5′ fusion partner is EML4, although other rare fusion partners have been described, including TFG and KIF5B. Most fusions arise from a pericentric inversion on the short arm of chromosome 2. The resulting in-frame fusion links the N-terminal coiled-coil basic domain from EML4 (2p21) with the intracellular (C-terminal) region of ALK (2p23), which may facilitate activation of the ALK tyrosine kinase by promoting dimerization [3]. The most common rearrangement in lung tumors involves a small intrachromosomal inversion event on chromosome 2 fusing *ALK* to *EML4* [3]. ALK-rearranged lung carcinoma cells are sensitive to treatment with a multitargeted tyrosine kinase inhibitor, crizotinib, originally designed to target Met activity [4]. The availability of this agent at the time of discovery of ALK rearrangement in lung cancers permitted rapid translation of ALK-targeted therapy into clinical practice. Clinical trials demonstrating the efficacy of crizotinib in ALK-rearranged lung carcinomas were published in 2010, with FDA approval of crizotinib therapy based on detection of *ALK* rearrangement by FISH following shortly thereafter [5]. Using FISH, Kwak et al. identified *ALK* rearrangements in ~5% of lung cancers and demonstrated a 57% response rate to crizotinib in *ALK*-positive patients, with many experiencing dramatic radiographic and clinical response [5]. Crizotinib was shown to be superior to chemotherapy in regard to response rates, progression-free survival, and quality of life in both the first- and second-line settings (Table 12.1) [6, 7]. Clinical practice guidelines therefore recommend first-line testing for *ALK* rearrangements in patients with advanced lung adenocarcinoma [8].

Table 12.1 Outcomes from ALK-targeted inhibitor studies

Inhibitor	Study design	Response rate	Median PFS (months)	Median OS (months)	Reference
Crizotinib	Phase 1	57%	Not reached	n/a	Kwak et al. [5]
Crizotinib	Phase 1	60.8%	9.7	Not reached	Camidge et al. [58]
Crizotinib	Pooled retrospective in patients with brain metastases	Untreated: 53% SYS vs. 18% IC Previously treated: 46% SYS vs. 33% IC	Untreated: 12.5 SYS vs. 7 IC Previously treated: 14 SYS vs. 13.2 IC	Not reached	Costa et al. [25]
Crizotinib	Phase 3 vs. chemotherapy in the second line	65%	7.7	20.3	Shaw et al. [6]
Crizotinib	Phase 3 vs. platinum chemotherapy	74%	10.9	Not reached	Soloman et al. [7]
Alectinib	Phase 1, following crizotinib therapy	55%	n/a	n/a	Gadgeel et al. [59]

Table 12.1 (continued)

Inhibitor	Study design	Response rate	Median PFS (months)	Median OS (months)	Reference
Alectinib	Phase 2	93.5%	n/a	n/a	Seto et al. [60]
Alectinib	Phase 2, following crizotinib therapy	48%	8.1 (estimated)	Not reached	Shaw et al. [61]
Alectinib	Phase 2, following crizotinib therapy	50%	8.9	Not reached	Ou et al. [62]
Ceritinib	Phase 1	58%	7.0	Not reached	Shaw et al. [63]
Crizotinib then Ceritinib	Retrospective	n/a	17.4	49.4	Gainor et al. [64]

n/a not applicable, *SYS* systemic response, *IC* intracranial response

Selection of Patients for ALK-Targeted Therapies

Patients with ALK-rearranged lung carcinomas tend to be young non-smokers presenting with advanced disease [9–11]; however, use of clinical or demographic criteria to select patients for ALK-targeted therapy or testing is not recommended [8]. FISH is generally considered the gold standard for detection of *ALK* rearrangements in lung ACA, as a result of its use in the selection of patients for crizotinib therapy in the original clinical trials. The FDA has approved the *ALK* Break Apart FISH Probe Kit (Abbott Molecular, Des Plaines, IL) as a companion diagnostic for use of this drug in lung cancers. *ALK* fusion events across human tumors lead to increased ALK transcription and protein expression by immunohistochemistry (IHC); however, the level of protein expression varies according to tumor type. Commercially available IHC antibodies optimized for use in lung cancer include clones 5A4 and D5F3. The FDA has approved an antibody kit (Ventana ALK (D5F3) CDx Assay, Roche Diagnostics, Indianapolis, IN) for use on an automated staining platform for selection of patients with crizotinib therapy. However, neither FISH nor IHC has demonstrated perfect sensitivity or specificity and may be used in a complementary fashion in practice, especially in the context of unexpected or discordant results. Molecular methods for rearrangement detection including anchored multiplex PCR and hybrid capture/next-generation sequencing are employed in some settings [12, 13]; DNA-based sequencing approaches in particular enable detection of ALK rearrangements from liquid-based specimens such as plasma [14].

With few exceptions, most published studies demonstrate that *ALK* rearrangements and other oncogenic driver mutations occur in a mutually exclusive fashion [15]. On occasions where both *ALK* rearrangement and another driver oncogenic mutation are detected, multimodality evaluation may help to clarify false-positive

results [16, 17]. Large series suggest that *ALK* and *EGFR* alterations may occur concomitantly in ~1% of selected populations, but these dually altered tumors are differentially responsive to EGFR and/or ALK inhibition; EGFR and ALK phosphorylation status may help guide selection of an appropriate inhibitor [18].

Mechanisms of Resistance and Therapeutic Options

Resistance to the ALK inhibitor crizotinib emerges almost inevitably within 1–2 years of initiating therapy. The mechanisms of resistance include secondary mutations in the *ALK* tyrosine kinase domain, fusion gene amplification, and upregulation of bypass signaling pathways (Table 12.2) [19]. Alternative, ALK-independent survival pathways that can hamper the effectiveness of crizotinib include the epidermal growth factor pathway, insulin-like growth factor pathway, RAS/SRC signaling, and AKT/mTOR signaling, among others [20]. Importantly, the type of resistance mechanism

Table 12.2 Mechanisms of ALK inhibitor resistance

Alteration	Biological impact	Confers resistance to	Reference
On target resistance mechanisms			
ALK 1151Tins	Altered ATP affinity	Crizotinib	Katayama et al. [65]
ALK C1156Y		Ceritinib	Gainor et al. [19]
ALK I1171X	Decreased TKI binding affinity	Crizotinib, alectinib	Katayama et al. [21] Gainor et al. [19]
ALK F1174C		Crizotinib	Ou et al. [24]
ALK L1196M	Gatekeeper: TKI binding interference	Crizotinib, alectinib	Choi NEJM 2010 [66]
ALK L1198F		Ceritinib	Gainor et al. [19]
ALK G1202R	Solvent-front: diminish TKI binding affinity	Crizotinib, ceritinib, Alectinib, brigatinib	Katayama et al. [65] Gainor et al. [19]
ALK G1202del	Disrupted TKI binding	Crizotinib, ceritinib, alectinib, brigatinib	Gainor et al. [19]
ALK D1203N		Ceritinib, alectinib, brigatinib	Gainor et al. [19]
ALK G1269A		Crizotinib	Doebele et al. [67]
ALK fusion gene amplification			Katayama et al. [65], Doebele et al. [67]
Off target resistance mechanisms			
EGFR mutation	EGFR pathway dependence	Crizotinib	Doebele et al. [67]
MAP2K1 mutation	MAPK pathway dependence	MEK inhibitors	Gainor et al. [19]
KIT amplification	KIT pathway dependence	Crizotinib	Katayama et al. [65]
Small cell transformation	Histologic transformation	Chemotherapy	Levacq et al. [68]

often dictates the efficacy of subsequent lines of therapy. Dual ALK and EGFR inhibition may be active against crizotinib-resistant tumor cells driven by EGFR pathway activation, whereas combined ALK and KIT inhibition may overcome KIT-amplification driven resistance [21]. Despite the diversity of resistance mechanisms, most crizotinib-resistant tumors continue to depend on ALK signaling and are sensitive to more potent, structurally distinct, second-generation ALK inhibitors, such as ceritinib, alectinib, brigatinib, and lorlatinib, which may be effective in the context of acquired resistance mutations arising in the ALK tyrosine kinase domain [19]. Certain mutations appear to arise preferentially following use of specific first- and second-generation inhibitors, and multiple resistance mechanisms may be detected in an individual tumor (Table 12.2). The location of an *ALK* kinase domain resistance mutation may have significant implications for ALK-targeted therapy. Gatekeeper mutations that influence the kinase-ATP interaction occur at codon 1196 and are analogous to the T790M mutation in *EGFR*-mutated lung adenocarcinomas and *ABL* T315I mutations in *BCR-ABL* in chronic myelogenous leukemia [22]. For ALK, the effects of this gatekeeper mutation may be overcome by using potent inhibitors. In contrast, mutations arising at the solvent front (codon 1202) appear to lead to steric hindrance of and resistance to most of the available ALK inhibitors, with the exception of the highly selective third-generation inhibitor, lorlatinib [19]. Given the differential patterns of resistance and unique sensitivities of individual inhibitors, some authors have argued for routine biopsies at the time of relapse on ALK-targeted therapies [19, 23, 24]. This approach has not been adopted in routine practice, however, and has not yet been endorsed in testing guidelines [in preparation]. In the context of crizotinib resistance, outcomes following treatment with second-generation ALK inhibitors are promising. Brain metastases are common in ALK-rearranged lung carcinomas [25], and some second-generation inhibitors, including alectinib, effectively penetrate the blood-brain barrier and demonstrate excellent activity in the central nervous system. In phases I and II trials of alectinib in the crizotinib-resistance setting, about half of patients responded, but interestingly tumor shrinkage did not correlate with survival outcomes, with 78% of patients still alive after 3 years on therapy [26].

ROS1

ROS1 is a transmembrane receptor tyrosine kinase similar in structure to ALK, consisting of an extracellular ligand-binding domain, a short transmembrane domain, and an intracellular tyrosine kinase domain. While its extracellular ligand remains unknown, it is thought to function like other receptor tyrosine kinases by intracellular tyrosine phosphorylation, resulting in activation of downstream PI3K, STAT3, and RAS/MAPK signaling with effects on cell proliferation, survival, and cell cycling [27]. ROS1 fusion events have oncogenic activity in a variety of tumor types including glioblastoma, cholangiocarcinoma, inflammatory myofibroblastic tumor, and lung adenocarcinoma. Using FISH- and IHC-based screening programs, *ROS1* rearrangements involving a variety of fusion partners including *CD74*,

Table 12.3 Clinical outcomes with ROS1 inhibitors

Inhibitor	Study design	Response rate (%)	Median PFS (months)	Median OS	Reference
Crizotinib	Phase 1	72	19.2	n/a	Shaw et al. [30]
Crizotinib	Retrospective	80	9.1	n/a	Mazieres et al. [31]

SLC34A2, *SDC4*, *EZR*, and *FIG1* have been reported in approximately 1–2% of lung adenocarcinoma [28, 29]. Similar to ALK rearrangement, these fusions result in the placement of the ROS1 kinase domain downstream of a coiled-coil domain of the 5′ fusion partner, although activation of ROS1 fusion signaling does not appear to involve dimerization [27]. Crizotinib, the same multitargeted inhibitor effective in ALK-rearranged tumors, has been shown to be effective in treating ROS1-rearranged lung adenocarcinomas. In a phase 1 trial, 72% of patients with ROS1-rearranged lung carcinomas responded to crizotinib therapy [30]. A retrospective analysis in a European cohort confirmed this robust response pattern (Table 12.3) [31]. ROS1-rearrangement also appears to be a positive predictor of response to pemetrexed-based chemotherapy regimens [32].

Selection of Patients for ROS1-Targeted Therapies

As with ALK, ROS1 rearrangements are significantly more common in young never-smokers whose tumors lack other oncogenic driver mutations [33]. Although focused testing of cohorts containing patients fitting these characteristics will enrich for ROS1-rearranged tumors, tumors from older patients and smokers may also harbor these alterations; therefore, selection of patients for testing or treatment based on clinical or demographic features is discouraged.

In contrast to ALK, there is no companion diagnostic required for use of crizotinib therapy in patients with ROS1-rearranged tumors. FISH is often considered the gold standard for detection of *ROS1* fusions given a heavy reliance on this technology in the original clinical trial of crizotinib for ROS1-rearranged lung adenocarcinomas [30]. However, in light of the rarity of ROS1 rearrangements in lung cancers, more economical and less technically demanding screening approaches may be preferable to FISH for many laboratories. ROS1 immunohistochemistry using the commercially available D4D6 clone has a sensitivity for detection of ROS1-rearranged lung adenocarcinomas approaching 100% according to most studies, with variable specificity (pooled estimate of 93%) (guidelines, in preparation). The lower specificity results from occasional low-level expression of ROS1 protein in lung tumors with other known oncogenic drivers as well as in benign reactive pneumocyte proliferations [34, 35]. As a result, use of FISH or molecular methods is recommended to confirm a positive immunohistochemical result. Targeted real-time PCR, anchored multiplex PCR, and hybrid capture/next-generation sequencing have all been reported as parallel or stand-alone testing approaches for *ROS1* fusion detection, with good concordance with FISH and IHC [36].

ROS1 rearrangements generally occur in a mutually exclusive fashion with other driving molecular alterations; however, rare instances of combined *ROS1* fusion and EGFR or KRAS mutations have been reported [37]. The clinical significance of these combined alterations, including outcomes from ROS1-targeted therapy, is currently unknown. In most cases, multimodality testing including specific sequencing-based methods can identify falsely positive ROS1 findings by FISH or IHC.

RET *Rearrangements*

The *RET* proto-oncogene encodes a receptor tyrosine kinase with an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Binding of GDNF ligands to its GPI-linked co-receptor (GFRα) causes homodimerization of RET and autophosphorylation of its intracellular tyrosine residues and downstream RAS/MAPK, PI3K, and STAT pathway activation [38]. Several 5′ partners are involved in *RET* fusion events, including *KIF5B*, *CCDC6*, and *NCOA4*. Similar to ALK, the breakpoints in RET (at exons 11 or 12) unite its tyrosine kinase domain with the coiled-coil domain of its upstream partner, allowing for ligand-independent homodimerization and downstream signaling [29]. Vandetanib, a multi-specific tyrosine kinase inhibitor, including VEGFR-2, VEGFR-3, EGFR, and RET, was able to inhibit RET fusion-bearing tumor cell growth in vitro [29]. In phase 2 trials, patients with RET-rearranged lung adenocarcinomas show partial responses or disease stabilization with the MET and VEGFR2 inhibitor, cabozantinib [39, 40], as well as favorable responses to pemetrexed-based chemotherapies [41].

The relative rarity of RET-rearranged lung adenocarcinoma and obstacles to widespread screening have led to relatively limited literature on the clinicopathologic features of this tumor type; however, RET rearrangements appear to occur more commonly in lung cancer patients with a smoking history as compared to ALK and ROS1 cohorts [42]. As in thyroid carcinomas, RET rearrangements may occur more commonly in patients with a prior history of locoregional radiation therapy [43]. *RET* rearrangement is detectable by FISH; however, the most commonly described alteration, a small intrachromosomal inversion on chromosome 10, leading to the *KIF5B-RET* fusion, leads to a subtle split in the FISH probe signals that can be difficult to consistently detect in practice. Immunohistochemistry-based RET protein detection appears robust in other tumor types with RET alterations [44], but reports on the use of RET IHC in lung adenocarcinoma are limited, with variable sensitivity and specificity for *RET* rearrangements using different commercially available antibodies [45–47]. Targeted RT-PCR and next-generation sequencing methods can be used to detect *RET* fusions. While the current clinical outcome data is too limited to support routine stand-alone testing for RET rearrangement detection, laboratories that are implementing multiplexed RNA- or DNA-based sequencing assays should incorporate RET rearrangement testing into their testing platform [AMP/IASLC/CAP guidelines in preparation].

Other Fusions

Oncogenic translocations in *NTRK1* (*TRKA)*, a member of the NTRK/TRK family of growth factor receptor kinases, have been described in a range of both adult and pediatric tumors from multiple organ sites. In small cohorts selected for the lack of other common oncogenic drivers (EGFR, KRAS, ALK, ROS1, RET), NTRK fusions can be detected in 3% of lung adenocarcinomas [48]. However, in routine clinical cohorts screened using anchored multiplex PCR, the frequency of NTRK fusions is close to 0.1% [49]. One report describes a lung adenocarcinoma patient with an *STSQM1-NTRK1* fusion who experienced dramatic response to the TRK/ALK/ROS1 inhibitor entrectinib. In contrast to most patients with ALK- and ROS1-rearranged tumors, this individual had a significant smoking history [49]. As a result of the exceptional rarity of TRK fusions in lung cancer, the clinicopathologic correlates are undefined.

NRG1 fusions have been reported in a substantial (>15%) percentage of invasive mucinous adenocarcinomas of the lung in a mutually exclusive fashion with *KRAS* mutations [50]—the most common oncogenic alteration described in this unique tumor type [51]. In vitro studies indicate that NRG1 fusions activate a HER2-HER3 signaling program, raising the possibility that HER family blockade may serve as a therapeutic avenue in these tumors. BRAF fusions have also been described in invasive mucinous adenocarcinoma and may prove amenable to RAF/MEK-targeted therapies [50].

Histopathology of Translocation-Positive Lung Tumors

ALK- and ROS1-rearranged lung adenocarcinomas are among the most well pathologically described fusion tumors. These characteristically show solid and papillary growth with cribriform pattern and prominent mucin production (Fig. 12.1a) [9, 34, 52]. Morphologic features that appear to be significantly enriched in both ALK and ROS1 fusion-positive tumors include signet ring cells (Fig. 12.1b) and hepatoid features [52, 53]. Although ALK and ROS1 fusions are reported primarily in adenocarcinomas, these events may be seen in large cell carcinomas or in carcinomas with predominantly undifferentiated features (Fig. 12.1c) [54]. Several reports have described a significant association between psammomatous calcifications (Fig. 12.1d) and the presence of ROS1 fusions [34, 55]. However, none of these patterns are sufficiently sensitive or specific to allow for a histopathology-based approach to patient selection for ALK or ROS1 inhibitors. Histopathologic features of RET-rearranged tumors are less well described; however, one series reported solid predominant growth with frequent psammomatous calcifications and mucin production [56]. Thus, the morphologic features of ALK, ROS1, and RET fusion-positive lung cancers show remarkable overlap. NRG1 fusions appear to correlate with invasive mucinous adenocarcinoma histology; however, to date, many of the studies reporting these fusions are largely biased toward this tumor type.

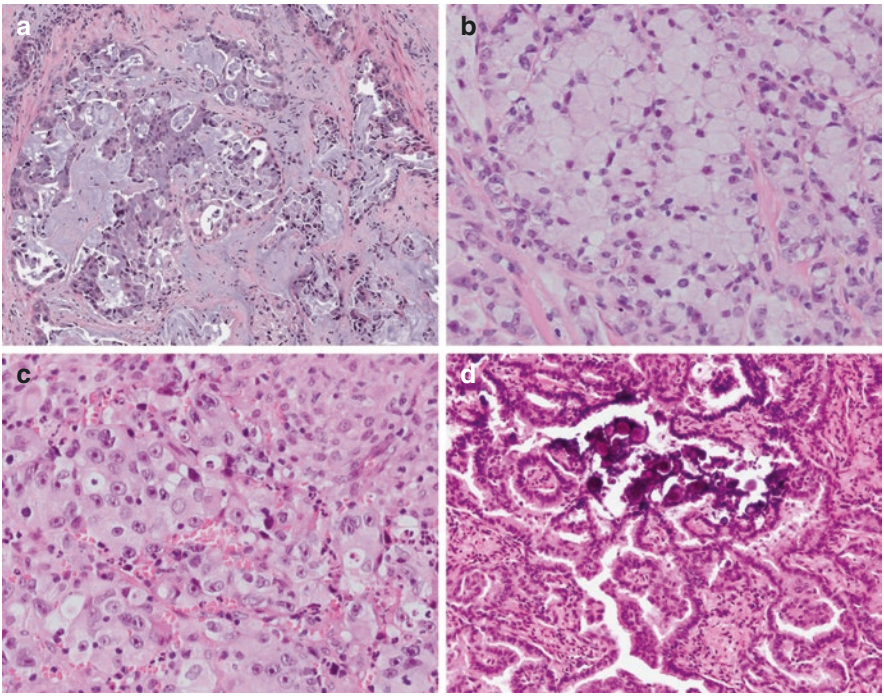

Fig. 12.1 (**a**) ALK-, ROS1-, and RET-rearranged lung tumors often show marked intra- and extracellular mucin with signet ring cells and cribriform pattern of growth. This image is taken from a lymph node metastasis of an ALK-rearranged lung adenocarcinoma (200× magnification). (**b**) Prominent signet ring cell features in a ROS1-rearranged lung adenocarcinoma (400× magnification). (**c**) ALK- and ROS1-rearranged adenocarcinomas may show high-grade growth patterns and in some cases appear undifferentiated. This is an ALK-rearranged lung adenocarcinoma with areas of solid pattern histology and high-grade nuclear features including large, irregular nuclei and prominent nucleoli (400× magnification). (**d**) Psammomatous calcifications have been associated with ROS1 rearrangements in lung adenocarcinoma, although they may be seen in lung adenocarcinomas with other oncogenic alterations, including ALK and RET rearrangement. This is a ROS1-rearranged lung adenocarcinoma with prominent papillary histology and frequent clusters of psammomatous calcifications (200× magnification)

Conclusions

Fusion-positive adenocarcinomas of the lung appear to represent a relatively unique subset of lung tumors with a predilection for younger never-smokers, often with prominent mucinous histology. The identification of this molecular subgroup has led to significant advances in targeted therapies and personalized medicine for this subset of lung cancer patients both in the first-line setting and at relapse when unique resistance mechanisms drive the biology of tumor progression. This population is also largely distinct from the more common tobacco-associated carcinomas of the lung and may be relatively refractory to the immune checkpoint inhibitor

therapies that are showing promise in some non-small cell lung cancer patients [57]. Based on the efficacy of tyrosine kinase inhibitor therapy in ALK and ROS1 fusion-positive tumors, routine testing of all patients with advanced lung adenocarcinoma, or other subtypes of lung cancer arising in light- or never-smokers, should be carried out for both of these alterations [AMP/IASLC/CAP guidelines, in preparation]. As comprehensive genome profiling techniques become widespread in clinical practice, it is likely that we will see more routine detection of defined fusion events, improved clinicopathologic characterization of these tumor subtypes, and an expanding catalog of oncogenic fusions in lung cancers.

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Chapter 13
Predictive Biomarkers for Squamous Cell Carcinoma

Ross A. Miller and Philip T. Cagle

Therapeutic options for non-small cell lung cancer (NSCLC) have significantly grown in the last years; however, these novel therapeutic options have almost exclusively targeted non-squamous NSCLC. Therapeutic advancements made over the last decade have focused on the concept of "driver mutations," particularly alterations in kinase genes or other pathways thought to be essential for tumor cell growth and development [1]. Kinase inhibitors have improved survival in patients with the corresponding actionable mutations or translocations after laboratory testing for these predictive biomarkers in tumor samples; however, these actionable mutations and translocations are almost exclusively seen in patients with non-squamous NSCLC histology.

Squamous cell carcinoma of the lung has a very high relative mutational burden when compared to other malignancies [2]. Despite this high mutational burden, a targetable mutation has not been identified to date [3]. The high mutational burden, high immunogenicity (relevance discussed later), and lack of a currently identifiable actionable driver mutation correlate with cigarette smoking exposure [4, 5] typical of lung squamous cell carcinomas [1]. Although the number of lung squamous cell carcinomas has fallen over the last decades, it remains a common cancer annually accounting for over 400,000 new cancer cases globally and around 85,000 new cancer cases in the United States [1]. As such, more promising therapeutic options are sought compared to the current standard chemotherapy regiments.

A variety of common genomic alterations have been identified in squamous cell carcinoma of the lung, the most common being loss of TP53 and CDKN2A [6, 7].

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_13

Numerous other alterations are frequently identified, and some have been identified as potential therapeutic targets. For example, FGFR1 amplification (the most studied to date), PIK3CA mutation and PTEN mutation/deletion, EGFR amplification/protein overexpression, PDGFRA amplification/mutation, and DDR2 mutation [8] have all been identified as potential targets with potential corresponding predictive biomarkers. Unfortunately, clinical trials with novel therapies have been plagued by low response rates to date. Despite the lackluster success seen thus far, there is ongoing research in the field of squamous NSCLC. One such example being SOX2 overexpression in mice models seemingly promotes the development of squamous cell carcinoma over adenocarcinoma [9]. Perhaps as we begin to further understand the intricacies of known and currently unknown molecular and cellular pathways, more promising targeted treatments with corresponding predictive biomarkers will emerge for squamous NSCLC.

As stated earlier, tumors related to cigarette smoking exposure (like squamous NSCLC) tend to be highly immunogenic. A relatively recent advancement in the treatment of NSCLC in general (both squamous and adenocarcinoma histology) is the development of immune checkpoint therapy, particularly therapy blocking the interaction between PD-1 and PD-L1. PD-1 is expressed on T-cells, and the T-cell immune response is dampened when PD-1 is bound to its ligand (PD-L1) [10] essentially resulting in a T-cell anergic response. As such, upregulation of PD-L1 expression in tumor cells enables them to escape the immune response in a process sometimes referred to as "adaptive resistance" [11]. Monoclonal antibodies targeting PD-1 or PD-L1 block the interaction and allow the immune system to recognize and attack tumor cells; these checkpoint inhibitor immunotherapies have shown remarkable responses in some patients [12, 13]. Immunohistochemistry for PD-L1 has been used as a predictive biomarker to identify patients who may respond favorably to therapy. However, immunohistochemical evaluation has its challenges. Firstly, there are multiple anti-PD-L1 antibodies available or in development, each approved for a different drug. It is not practical or feasible for pathology laboratories to perform multiple different immunohistochemical assays for a single protein. Performing multiple assays would require numerous tissue sections, potentially exhausting tissue blocks for other testing (i.e., other molecular studies for therapeutic decision making). Additionally, none of the available immunohistochemistry assays have been validated for cross-utilization at this time. Secondly, there is significant heterogeneity of PD-L1 expression in tumors [14], and often only a small biopsy specimen is tested in practice; as such the PD-L1 protein expression may be misrepresented on small biopsy specimens. Thirdly, positive or negative staining does not always correspond to therapeutic response [15–17]. Despite these limitations, immune checkpoint therapy associated with PD-L1 biomarker testing is another treatment option for squamous cell carcinoma and other NSCLC with some patients having dramatic responses. As our knowledge of immunotherapy advances, additional predictive markers may emerge, and evaluation of single or multiple proteins expressed by squamous cell carcinomas and other NSCLC may help better identify patients who will respond to such treatment regimens.

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Chapter 14
Molecular Pathology of Small Cell Carcinoma

Mary Beth Beasley

Introduction

Small cell carcinoma (SCLC) comprises approximately 15% of all lung carcinomas, although the incidence has reportedly declined in recent years [1]. SCLC has traditionally been regarded as distinct from "non-small cell carcinomas (NSCLC)" (i.e., adenocarcinoma, squamous cell carcinoma, and undifferentiated large cell carcinoma), in large part due to the traditional treatment of SCLC with chemotherapy in contrast to surgery. However, more recent literature supports that surgery may produce a survival benefit in low-stage disease [2, 3]. SCLC, along with large-cell neuroendocrine carcinoma (LCNEC), is high-grade neuroendocrine carcinoma with a poor prognosis. While SCLC and LCNEC are often considered as one end of a spectrum of pulmonary neuroendocrine carcinomas including the low-grade typical carcinoid (TC) and the intermediate-grade atypical carcinoid (AC), recent studies have shown distinct differences in clinical, immunohistochemical, and molecular features of the high-grade tumors and TC/AC, challenging the concept that these represent a spectrum of tumors arising from a common precursor cell [4, 5].

Molecular Analysis of Small Cell Carcinoma

SCLC harbors many of the common genetic alterations found in other carcinomas; however, as would be expected, the frequency of their occurrence differs, and SCLC additionally harbors mutations not typically found in other lung carcinomas.

In general, SCLC typically shows a very high frequency of p53 mutations and Rb inactivation. Rb inactivation is found in 80–100% of SCLC, and, due to their

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_14

inverse relationship, loss of $p16^{INK4}$ is infrequently encountered. As would be expected, Rb loss leads to overexpression of E2F and loss of cell cycle arrest. The finding of a high level of Rb inactivation in SCLC is different from non-small cell carcinomas, which tend to have lower levels of Rb loss and higher levels of p16 loss or cyclin D1 overexpression [6–8]. While a precise precursor lesion for SCLC has not been identified, it has been suggested that Rb inactivation occurs as an early event, followed by LOH mutation of 5q and/or 22q followed by C-MYC amplification [9].

In contrast to the carcinoid tumors, SCLC has a higher frequency of deletions on chromosomes 3p and 17p and has an inverted bcl-2/Bax ratio [7, 10]. SCLC also has a higher level of telomerase activity [11]. *TERT* copy gain has more recently been shown to be an independent predictor of poor prognosis [12]. Loss of heterozygosity (LOH) for 3p, 5q21, and 9p has also been found [7, 8]. Mutations of the *MEN1* gene, as seen in TC and AC, are not seen in SCLC [13]. In regard to gene copy number specifically, SCLC has been found to have fairly consistent increased copy number on chromosomes 1, 3q, 5p, 6p, 12, 14, 17q, 18, 19, and 20 while showing copy number loss on 3p, 4, 5q, 10, 13, 16q, and 17p [14–16]. In an array-based study, Voortman et al. demonstrated copy number gains in at least 1 MYC family member in 27/33 SCLCs but in only 1/19 carcinoids. This study also demonstrated copy number increases in the fibroblast growth factor receptor 1 (*FGFR1*) gene and for the Janus kinase 2 (*JAK2*) gene in one SCLC case each but in no carcinoid tumors [17].

More recently, studies have examined the genomic profile of SCLC with more extensive profiling techniques [18–21]. Comprehensive genomic profiling of 110 SCLCs using whole-genome sequencing was reported by George et al. [18]. This analysis confirmed previously known genomic losses in 3p, particularly 3p14.3-3p14.2 and 3p12.3-3p12.2 harboring *FHIT* and *ROBO1*, respectively. This study also confirmed that nearly all SCLC harbored bi-allelic inactivation of *TP53* and *RB1*. Interestingly, the two cases which harbored a wild-type *RB1* were found to have abnormalities leading to overexpression of cyclin D1, thus providing an alternate pathway for Rb inactivation. The authors concluded that complete genomic loss of both *TP53* and *RB1* was obligatory in the pathogenesis of SCLC [18].

Another interesting finding in this study was that mutations affecting the *NOTCH* family genes were found in 25% of SCLC. Additionally, the majority of cases demonstrated high levels of *DLK1*, an inhibitor of Notch signaling, while a second subset of tumors instead expressed *ASCL1*. *ASCL1* is a lineage oncogene of neuroendocrine cells, the expression of which is inhibited by active Notch signaling. As such, both patterns suggest low Notch pathway activity in SCLC. Expanded studies in mouse models suggest activated Notch signaling may serve as a tumor suppressor in SCLC [18]. Interestingly, mutations in *NOTCH* family genes appeared to be mutually exclusive from mutations in *CREBBP*, *EP300*, *TP73*, *RBL1*, and *RBL2* which have also been reported in other studies in addition to mutations in *PTEN* and *PIK3CA* [18, 22].

As would be expected, the two high-grade neuroendocrine carcinomas, SCLC and LCNEC, have many similarities from a molecular standpoint. Indeed, Jones et al. demonstrated that SCLC and LCNEC were indistinguishable by gene profiling analysis [23]. However, differences between the two tumors have been demonstrated. An extensive array-based study by Peng et al. [24] demonstrated a large number of common mutations but also noted some statistically significant differences, specifically that losses at 3p26-22, 4q21, 4q24, and 4q31 were seen more frequently in SCLC, while gains at 2q31, 2q32, and 2q33 along with loss at 6p21.3 were associated with LCNEC. A study by Hiroshima et al. [25] reported a statistically significant difference in allelic loss of 5q33 between SCLC and LCNEC. Both LCNEC and SCLC show high expression of hASH1 which is involved in neuroendocrine differentiation; however, a study by Nasgashio et al. [26] demonstrated that while both tumors showed high expression levels of hASH1 mRNA, the staining score of hASH1 was higher in SCLC. Additionally, this study also found that expression of hairy/enhancer of split 1 (*HES1*), a negative regulator of neuroendocrine differentiation, was more highly expressed in LCNEC, suggesting that SCLC more strongly expressed a neuroendocrine phenotype, while LCNEC retained characteristics more similar to bronchial epithelium [26].

It is known that LCNEC and SCLC may be combined with one another, and an elegant study by D'Adda et al. [27] was the first to study genetic alterations in combined SCLC/LCNEC using microdissection to evaluate the components separately and compared the results with pure SCLC or LCNEC. In this study, six combined SCLC/LCNEC were compared with eight pure SCLC and eight pure LCNEC. In the combined tumors, both components demonstrated a common pattern of genetic alterations involving 17p13.1, 3p14.2-3p21.2, 5q21, and 9p21. The authors note that these alterations are usually involved in early carcinogenesis and therefore suggest a close relationship between the two components and further hypothesize they may be evidence of a monoclonal carcinogenesis mechanism. The authors did find differences between the two components. The LCNEC component had more frequent alterations in 6q, 10q, and 16q, while alterations in the 6p and X-Y PAR regions were seen more frequently in the small cell component, although none reached statistical significance. Interestingly, alterations were reported in both components of the combined tumors which have not been highly reported in either tumor in pure form, implying that the two components of the combined tumors potentially have more commonality with each other than they have due to their respective pure forms. The authors conclude that combined SCLC/LCNEC represent "transition" carcinomas in the spectrum of high-grade neuroendocrine pulmonary tumors, with pure forms representing the two extremes of this differentiation [27]. Buys et al., in contrast, reported a combined SCLC, LCNEC, and adenocarcinoma and evaluated the genomic profiles of each tumor component. In this study they failed to find shared genetic alterations between the SCLC and LCNEC components and suggested the two components evolved independently, as did the adenocarcinoma component [28].

Molecular Abnormalities in Small Cell Carcinoma in Relation to Targeted Therapy

In regard to SCLC and potential targeted therapies, SCLC has not been shown to harbor EGFR mutations, and there is currently no evidence to support that EGFR tyrosine kinase inhibitors have a role in the treatment of SCLC outside of isolated case reports [29, 22, 30]. However, Voortman et al. did demonstrate that SCLC harbored copy number alterations in genes encoding proteins in the PI3K-AKT pathway and apoptosis pathway genes such as *BCL- 2*, *MCL1*, and *PMAIP1*, suggesting these may be potential drug targets [17]. Abnormalities in the PI3K/AKT/mTOR pathway have been reported by multiple authors, and it has been observed that deletion of *PTEN*, which acts as a suppressor of this pathway, may be an important driver of SCLC [21, 22, 31]. Studies evaluating everolimus and temsirolimus, inhibitors of mTORC1, in SCLC have thus far demonstrated only limited antitumor activity, and evaluation of drugs directed toward other pathway targets is needed [22, 31].

Rossi et al. as well as others have demonstrated that SCLC has high levels of c-kit overexpression in addition to platelet-derived growth factor receptors [32–34]. However, imatinib, while showing initial promising results in preclinical trials, has shown a lack of efficacy in subsequent studies [22, 35].

Insulin-like growth factor has been a focus of interest in SCLC. Xu et al. reported that K-homology domain-containing protein, a member of the insulin-like growth factor RNA-binding protein family, was highly expressed in both SCLC and LCNEC [36]. Yazawa et al. [37] demonstrated that SCLCs overexpress insulin-like growth factor binding protein-2 via Neuro-D, a neuroendocrine cell-specific transcription factor. Badzio et al. (JTO 2010) reported high insulin-like growth factor 1 receptor (IGFR1) expression by immunohistochemistry as well as increased gene copy number by in situ hybridization, but only rare true occurrence of gene amplification. Unfortunately, clinical trials have thus far failed to demonstrate an improved progression-free survival with anti-IGF1 therapy [22].

Other potential targets under investigation include inhibition of *FGFR1*, which is amplified in up to 9% of SCLC, inhibition of *PARP1*, use of Aurora kinase inhibitors in SCLC with *MYC* amplification, and use of RET tyrosine kinase inhibitors in tumors with *RET* mutations. Given the essentially ubiquitous presence of *TP53* and *RB1* mutations, drugs targeting these pathways are under development and investigation [19, 22, 31, 38]. Rudin et al. have additionally documented the amplification of *SOX2* and noted that inhibiting *SOX2* using short hairpin RNA decreases proliferation in SCLC cell lines with *SOX2* amplification [20].

In summary, SCLC is a high-grade neuroendocrine carcinoma which harbors similar, but not identical, molecular abnormalities to LCNEC, but differs from NSCLC. Effective targeted molecular therapy has thus far remained elusive although many targets hold potential promise and are under investigation.

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Chapter 15
Molecular Pathology of Uncommon Carcinomas

Alain C. Borczuk

Introduction

Morphologic classification of lung carcinoma has broad categories of common carcinomas that include small cell carcinoma, adenocarcinoma, and squamous cell carcinoma. Uncommon carcinomas include variants of the above categories, many of which have been covered in the respective chapters. For this section, the focus will be on sarcomatoid carcinoma which includes pleomorphic carcinoma, a distinct group of tumors in which spindle or giant cells represent a reproducibly recognizable component (over 10%), pulmonary blastoma, and carcinosarcoma. Tumors of bronchial gland/salivary gland type will be covered, with a focus on the distinction of mucoepidermoid carcinoma from adenosquamous carcinoma. Other tumors of interest as uncommon carcinomas of the lung include NUT carcinoma and lymphoepithelioma-like carcinoma, two entities more frequently described as part of their extrapulmonary counterparts.

Sarcomatoid Carcinoma

Tumors in this category are defined as having a sarcomatoid or giant cell component [1] and are considered to be poorly differentiated. While uncommon (less than 1% of lung malignancy) [2, 3] they are thought to have a higher rate of adverse outcome than other lung carcinomas. Not only known for aggressive invasive behavior, these tumors have also been notoriously treatment refractory [4]. They are associated with tobacco smoking [1]. Some cases may be related to asbestos exposure [5].

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_15

The subcategories of this group reflect differences in tumor composition. A combination of adenocarcinoma, squamous carcinoma, or large cell carcinoma with spindle or giant cell component is designated as pleomorphic carcinoma (PC), while tumors with pure spindle or giant cell component are designated as spindle cell carcinoma or giant cell carcinoma accordingly (Fig. 15.1a–c). The morphologic

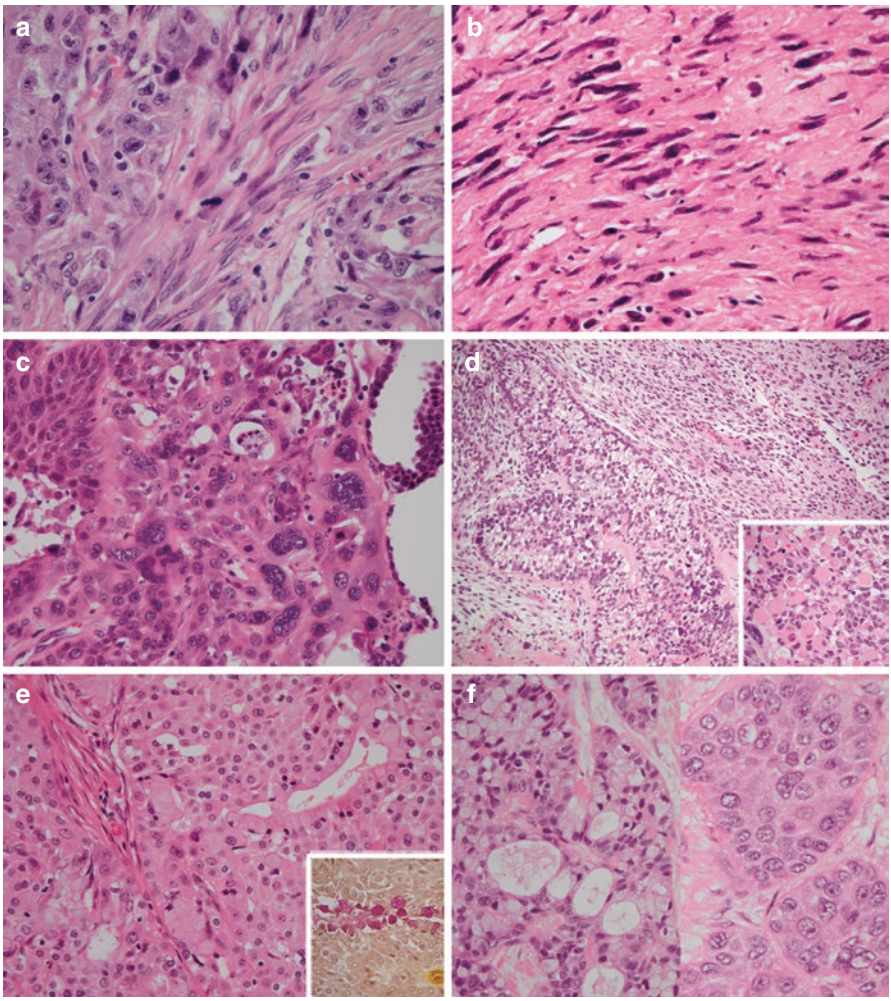

Fig. 15.1 Uncommon carcinomas. (**a**) Sarcomatoid carcinoma, pleomorphic carcinoma type with large cell undifferentiated carcinoma (*left*) and spindle cell pattern on the *right*. (**b**) Sarcomatoid carcinoma, spindle cell type. (**c**) Sarcomatoid carcinoma, pleomorphic carcinoma type, with squamous cell carcinoma (*left*) and giant cell carcinoma (*center*). (**d**) A pulmonary blastoma with fetal-type epithelial component (*left*), spindle cell component (*right*), and rhabdomyosarcomatous differentiation (*inset*). (**e**) Mucoepidermoid carcinoma with solid areas of bland intermediate cells and mucous cells with low-grade nuclei. Inset highlights mucicarmine-positive cells. (**f**) An adenosquamous carcinoma with adenocarcinoma with signet ring cells (*left*) and squamous cell carcinoma (*right*)

definition of a tumor giant cell includes not only enlargement of the nucleus and cytoplasm but frequently incorporates multi-nucleation and significant emperipolesis. It has been proposed that these three subgroups of tumors are similar to each other and published series have described them together as sarcomatoid carcinoma. Carcinosarcoma is reserved for tumors with a mixture of carcinoma and sarcoma in which the sarcomatous component is a recognizable differentiated element, such as malignant cartilage, skeletal muscle, or the bone also described as a heterologous element. Pulmonary blastomas (PB) are defined as a combination of carcinoma and sarcoma in which the epithelial component is a specific pattern—well-differentiated fetal adenocarcinoma (WDFA, Fig. 15.1d)—but also have a sarcomatous heterologous element (Fig. 15.1d, inset).

It has been proposed that this category of tumors represents a manifestation of epithelial-mesenchymal transition—that is, the potential of an epithelial cell to lose characteristic features of epithelium, becoming less cohesive and more like a mesenchymal cell. Evidence for common clonal origin of different components of these tumors and data favoring carcinomatous origins for these tumors will be discussed in the sections to follow.

Sarcomatoid Carcinoma (Except Pulmonary Blastoma)

As the name implies, these tumors have varied histology and include mixtures of carcinomatous components along with spindle or giant cell elements. While in the past this observation was of pathological classification and communication relevance alone, more recently, the recognition of the importance of adenocarcinoma (AdCa) and squamous cell carcinoma (SqCa) categories has revived interest in characterizing those components in PC. Mochizuki et al. [6] reviewed 68 cases of PC with AdCa component seen in 50%, SqCa in 16% and LCC in 34%. This distribution of AdCa component in PC was similarly reported by Nakajima et al. [7], with 49% containing AdCa, 22% SqCa and 16% LCC. Rossi et al. reviewed 75 sarcomatoid carcinomas with 51 PC showing 39% AdCa, 27% SqCa, and 41% LCC; their series had many cases with more than one pattern [8]. Overall, these data are similar to the original series of Fishback et al. [1]; among 61 cases of PC, 57% had AdCa, 10% had SqCa, and 32% had LCC.

IHC Characterization

The immunohistochemical characterization of PC suggests an epithelial origin with transition/progression to sarcomatous areas. In their analysis of 31 cases of PC, Pelosi et al. [9] showed cytokeratin, carcinoembryonic antigen (CEA), and epithelial membrane antigen (EMA) were more reactive in epithelial components and vimentin, fascin, and microvessel density greater in the sarcomatous component.

However, immunoreactivity for cytokeratin and EMA were identified in pleomorphic components, and vimentin was seen in epithelial component. CEA was not positive in pleomorphic components. In a study extending IHC markers to lung-specific markers, Rossi et al. [8] showed spindle cell reactivity for thyroid transcription factor 1 (43%), cytokeratin 7 (62%), and surfactant protein A (6%) in PC, with similar rate of TTF1 (55%) and CK7 (70%) in spindle or giant cell carcinoma. In the same series, the epithelial component of PC was positive for CK7 (76%), TTF1 (59%), and surfactant protein A (39%); interestingly 39% of cases had AdCa histologically.

In a series of 19 cases of lung sarcomatoid carcinoma/PC [10], spindle or giant cell component was positive for keratin (80%), EMA (50%), p63 (50%), TTF1 (26%), and MOC31 (42%). Specifically in the ten cases of PC, the epithelial component was positive for keratin (100%), EMA (100%), TTF1 (70%), p63 (50%), and MOC31 (100%).

Histogenesis

Collectively, the IHC studies support the impression of epithelial/carcinomatous origin of PC. Existing data support a single clonal origin for sarcomatoid carcinoma, and therefore these tumors are carcinomas with sarcomatoid differentiation rather than sarcomas. While data on specific molecular events will be discussed in later sections, those relevant to histogenesis will be mentioned here.

In pleomorphic carcinoma, there is evidence that in the majority of cases, both epithelial and spindle cell component harbor the same KRAS mutation [11, 12] when a KRAS mutation is present. Individual cases have been reported in which a mutation was identified only in one component [13]. In carcinosarcomas, p53 mutational status was identical in both components [14].

Studies examining loss of heterozygosity at various loci support the contention that both elements in these biphasic tumors arise from the same clonal origin. The presence of more complex DNA changes in the mesenchymal component of PC and carcinosarcoma [15, 16] supports the view that the carcinoma component temporally precedes the mesenchymal component in the progression of the tumor.

Cytogenetics

Few comparative genomic hybridization studies have been reported in sarcomatoid carcinoma. Yakut et al. [17] performed CGH on AdCa, SqCa, LCC, and sarcomatoid carcinoma and showed that 5p gains were common to all groups, 3q gains seen in SqCa and in 2 of 4 sarcomatoid carcinomas, 14q gains in 3 of 4 sarcomatoid carcinomas which had overlap with AdCa, and 12p gains in LCC but not in other groups.

Specific Mutations in Sarcomatoid Carcinoma

TP53

Mutations in P53 were reported [14] in 4 of 9 spindle cell ca. (exon 5, 7, and 8) and 1 of 6 carcinosarcomas (exon 7). In a series of 22 PC, p53 mutation was seen in 14% of cases, most commonly exon 7 mutation [18]. Additional recent studies have shown a higher rate of TP53 mutations, from 58% [19] to 74% [20].

EGFR/KRAS Mutation

The identification of activating EGFR and KRAS mutations in lung AdCa and the association with response to EGFR-targeting tyrosine kinase inhibitors (TKI) have led to interest in the identification of these mutations in other histologic patterns of lung cancer. Given the admixture of patterns in pleomorphic carcinomas including cases with AdCa component and the proposed sequence of carcinoma to sarcomatoid pattern in these tumors, it would be expected that a proportion of PC would harbor these activating oncogenic mutations. Overall, in Asian populations in which EGFR mutations are seen at a higher rate than in Western populations, the rate of EGFR mutation is lower in PC when compared to AdCa. In 23 SC/PC, one European study [11, 21] identified two cases with EGFR mutation (9%); in 17 PC from Japan [13], three cases were identified (18%). Interestingly, all five of these cases had AdCa as the epithelial component. In a series of 22 cases of SC, Italiano et al. [21] reported no EGFR mutations; in that series, the histology of the epithelial component was not specified and the subtype of sarcomatoid carcinoma not reported. In one case report, an AdCa with exon 19 EGFR mutation was reported at autopsy as a pleomorphic CA with both the same activating exon 19 mutation and an acquired resistance T790 M mutation [22]. In two additional North American series of 33 and 36 cases, no EGFR mutations were identified [19, 23]. An EGFR mutation was described in one patient with carcinosarcoma, found in both adenocarcinoma and chondrosarcomatous elements [24].

In contrast, the rate of KRAS mutation in SC/PC has been similar to that of AdCa. In the aforementioned Japanese series of PC [13], no KRAS mutations were identified, similar to the relatively low rate reported in non-mucinous AdCa from Japan. KRAS mutation rates in Western series of SC have varied from 9 to 38%, with a combined overall rate of about 25% [11, 12, 19, 21–23, 25]. In the series of Pelosi et al. [12], the KRAS mutations were typical smoking-associated transversion mutations; of the mutation cases reported, four had an AdCa component, and two had LCC component. In a recent large study of 125 pulmonary sarcomatoid carcinoma, 34% harbored KRAS mutations, while EGFR mutations were seen in 5.6% (two were resistance-associated exon 20 insertions) [20]. One series suggested an adverse outcome among tumors with KRAS mutation [26].

Therefore, a subset of PC harbor mutations similar to AdCa, albeit with a relatively lower rate of EGFR mutation than seen in adenocarcinoma. This may be in part due to the smoking association of this tumor type. While EGFR mutation may suggest that PC/SC patients may benefit from EGFR-targeting TKI therapy, one caveat to this was reported by Shukuya et al. [27]. Response rate to TKI therapy among non-AdCa lung cancers harboring EGFR mutations was only 27% when compared to a 66% response rate in AdCa. While most of these cases were squamous, adenosquamous, and large cell histology, the three reported EGFR-mutated sarcomatoid carcinomas showed no response to EGFR TKI therapy. More recent studies support that response and durability of response in sarcomatoid carcinoma with EGFR mutations may be lower than in adenocarcinomas with these alterations [13, 22].

MET Exon 14 Skipping Mutations

Alterations in MET have been proposed as a precision medicine target for therapy with crizotinib. In 2009 [28], splice site and intronic mutations were identified in roughly 3% of lung AdCa that resulted in exclusion of exon 14 from the ultimate mRNA and resultant protein. These were exclusive of other driver alterations and are thought to activate MET by the loss of a critical Y1003 that is required for degradation of wild-type MET by CBL ubiquitin ligase. This loss results in resistance to degradation and persistence of MET activation. More recent studies confirmed this alteration in about 4% of lung adenocarcinomas [29–31].

A promising finding of activating MET exon 14 skipping mutations was reported in 8 of 36 sarcomatoid carcinomas (22%), with response to crizotinib in one patient [23]. This high rate in pulmonary sarcomatoid carcinoma was confirmed by two additional studies (27 and 32%, respectively) [32, 33], with a similar mutual exclusivity with other driver mutations. In a recent study of 125 pulmonary sarcomatoid carcinomas, 12% harbored MET exon 14 mutations, which was significantly enriched when compared to other carcinomas (3%). Of note these tumors more frequently contained an adenocarcinoma component as their epithelial component [20].

Some series have not identified MET exon 14 skipping mutations, but the unique characteristics of these often intronic mutations make it essential that the methodologies used had sensitivity for detection of these mutations [19, 26, 34].

ALK Translocation and Amplification

While most series have not found ALK translocations in pulmonary sarcomatoid carcinoma [20, 23, 35], one study found three pleomorphic carcinomas and two carcinosarcomas with IHC reactivity for ALK (overall 3.5%) and FISH translocation, with one patient showing response to crizotinib. These patients were younger

and had a lower level of cigarette smoke exposure [36]. In another series, one of 33 tumors (3%) harbored ALK translocation [19].

PDL1 Status

Sarcomatoid carcinoma has a higher rate of PDL1 immunoreactivity than other non-small cell lung carcinomas, with a rate over 50%. This, in addition to the presence of tumor-infiltrating lymphocytes [37], raises the possibility that these tumors will respond to immune checkpoint inhibitor therapies.

Sarcomatoid Carcinoma and Pulmonary Blastoma

The category of pulmonary blastoma (not pleuropulmonary blastoma, which is a pediatric tumor) will be separately discussed because of its relationship to well-differentiated fetal-type adenocarcinoma and, as a result, its unique molecular profile.

β-Catenin Mutations

The transcriptional effects of β-catenin promote decrease in cellular adhesion and increase in cellular migration. In nonneoplastic epithelial cells, β-catenin is part of a complex of proteins at the cell membrane; this complex includes E-cadherin, a critical component of intercellular adhesion. If β-catenin becomes disassociated from this complex, it is rapidly phosphorylated and targeted for degradation. With signaling via the Wnt pathway, this phosphorylation is inhibited, allowing accumulation of β-catenin and subsequent movement into the cell nucleus where it can exact its transcriptional effect. Mutations in β-catenin prevent its phosphorylation and therefore allow β-catenin accumulation and transcription effect in the absence of Wnt signaling.

For the diagnostic pathologist, non-mutated β-catenin localization by immunohistochemistry should be membranous; as a result of mutation, the nuclear immunoreactivity results from abnormal nuclear localization of β-catenin.

Nakatani et al. [38] described nuclear localization of β-catenin and β-catenin mutation in well-differentiated fetal adenocarcinoma and pulmonary blastoma. In a related study, Sekine et al. [39] demonstrated β-catenin mutations in 3 of 3 WDFA and 2 of 6 pulmonary blastomas. In a similar logic paralleling the identification of adenocarcinoma in PC and the association with KRAS/EGFR mutation, the presence of β-catenin mutations in WDFA and pulmonary blastoma suggests common histogenesis, arising from the carcinoma component.

Other Mutations

TP53 mutations were not identified in WDFA and were seen in one of seven PB studied (exon 6) [14]. In a series of five PB, three cases had β-catenin mutations, and no mutations were identified in KIT and KRAS. One case had both β-catenin mutation and an exon 19 EGFR mutation [40].

In a next-generation sequencing study of pulmonary blastoma, five patients showed mutations in BRCA2, BRAF, PTEN, EGFR, and PIK3CA. However β-catenin mutation or immunohistochemistry was not examined in this series [41].

In a study of three pulmonary blastomas (two of which harbored a β-catenin mutation), somatic DICER1 mutations were identified [42]. This is of interest as pleuropulmonary blastoma, the fetal/embryonic sarcomatous tumor of infancy, is associated with somatic and germline mutations in DICER1.

Carcinomas with Salivary Gland-Like Morphology

Less than 1% of lung carcinomas represent tumors of bronchial gland origin that resemble their counterparts in the salivary gland. While they can occur in patients of any age, a large proportion occur in patients under the age of 30. As a result, they enter the differential diagnosis of carcinoid tumor, clinically and sometimes histologically. They are not thought to be smoking associated.

Mucoepidermoid Ca

The histology of mucoepidermoid carcinoma of the bronchus is similar to that of the salivary gland (Fig. 15.1e). Lower-grade tumors have cystic areas lined with mucinous cells with admixed intermediate cells; higher-grade tumors have less mucin, more intermediate cells, and atypical squamous cells resembling squamous cell carcinoma. In cases without clear-cut transition from low-grade mucoepidermoid carcinoma, the diagnosis of high-grade mucoepidermoid carcinoma shows considerable overlap with adenosquamous carcinoma.

Cytogenetics

The recognition of a recurrent translocation in mucoepidermoid carcinoma of the salivary gland (t(11:19)(q21;p13) involving chromosomes 11 and 19 [43] and the subsequent identification of a mucoepidermoid carcinoma translocated-1-mammalian mastermind like 2 fusion (MECT1-MAML2 fusion) [44] have

introduced a relatively specific molecular marker for mucoepidermoid carcinoma. This has been subsequently examined in low- and high-grade tumors as well as tumors of different histologic subtypes. While lower-grade tumors harbor the translocation at a higher rate than the higher-grade tumors (75 vs 46%), other salivary gland and head and neck tumors do not demonstrate this translocation [36, 45]. As a result, there is speculation that some high-grade tumors that receive the diagnosis of MEC are in fact misclassified, and these misclassifications include adenosquamous carcinoma, squamous carcinoma, and salivary duct carcinoma.

Stenman [46] reported the same translocation in a child with pulmonary MEC, and this observation was confirmed in the lung [47] by the study of 17 pulmonary MEC by FISH and RT-PCR for MECT1-MAML2. An MAML2 rearrangement was confirmed in all low grade and 3 of 7 high-grade MEC by FISH (13 of 17 cases in all), but RT-PCR detected the fusion in only 6 of 14 cases. This difference in testing result may reflect variability in the fusion partner with MAML2. All cell types (mucous, intermediate, squamous) harbored the rearrangement. No cases of pulmonary AdSq (16), SqCa (24), or AdCa (41) had evidence of the translocation.

In a series of 18 pulmonary mucoepidermoid carcinomas, 12 of 18 were positive for MAML2 translocation, and this occurred in both low- and high-grade tumors [48]. Differences in survival were not seen based on MAML2 translocation status. In a detailed study of morphology, IHC, and cytogenetics, 24 mucoepidermoid carcinomas of the lung were associated with MAML2 translocation, and none of these had immunoreactivity for TTF1 or Napsin A [49]. This latter point is important in the distinction with adenosquamous carcinoma. In another study, MAML2 translocation was seen in 50% of the pulmonary MEC and tended to occur in younger patients, with tumors of low to intermediate grade [50].

While it appears that MEC is distinct from other lung tumors molecularly, the progression from low grade to high grade is not as clearly determined. Similar to the salivary gland, it raises the possibility that some of the high-grade MEC may be misclassified AdSq or SqCa.

EGFR/KRAS Mutation

Again, the attention regarding EGFR mutations in the treatment of adenocarcinomas of the lung has led to investigation of other tumors types. This has led to some interesting observations in MEC. In vitro data suggests a sensitivity to gefitinib in cells derived from MEC containing MAML2 translocation without EGFR mutation [51], and two reports of MEC tumors with gefitinib response in the absence of EGFR mutation [52, 53] have led to speculation that this tumor type may be responsive to EGFR TKI therapy. One confusing aspect of the latter study in the two reports is the discovery of L858R mutations in MEC in one series but no EGFR or KRAS mutation in another [53]. An additional study of 12 MEC showed chromosome 7 polysomy, EGFR immunoreactivity, but no amplifications or mutations in EGFR [54]. One question in these studies is again one of the misclassifications in

high-grade tumors: without the identification of MECT1-MAML2 fusion, is the high-grade MEC actually AdCa or SqCa?

EML4-ALK Translocation

There is one report [55] of a low-grade MEC positive for EML4-ALK translocation. This report did not investigate MAML2 translocation.

Adenoid Cystic

The histology of adenoid cystic carcinoma of the lung is the same as its salivary gland counterpart, with cribriform structures, tubules, and nests. Some structures show "basement membrane" like material at the center of a nest of cells. These are also central tumors, without smoking association but with a slightly older average age at presentation than MEC.

There are few molecular studies of adenoid cystic carcinoma (ACC) of the lung. Because of frequent CD117 immunoreactivity in adenoid cystic carcinoma of salivary gland, Aubry et al. [56] studied pulmonary ACC with the finding that CD117 IHC is frequently seen. Mutations in KIT exons 9, 11, 13, and 17 were not identified in the12 cases studied. In a separate series of 12 adenoid cystic carcinomas, no EGFR amplification or mutation was identified [54]. In 24 cases, no mutations were seen in EGFR, KRAS, BRAF, PIK3CA, ALK, DDR2, or PDGFRA [57].

Alterations in chromosome 6 and chromosome 9 have been reported in adenoid cystic carcinoma and specifically in a bronchial ACC [58]. The finding of a MYB-NFIB fusion t(6:9)(q22–23;p23–24) in salivary gland type adenoid cystic carcinoma [59] has been reported and shown in pulmonary adenoid cystic carcinoma in 41% of cases; however, there does not appear to be a difference in morphology or outcome between translocation-positive or translocation-negative cases [60].

Adenosquamous Carcinoma

The definition of adenosquamous (AdSq) carcinoma requires presence of both adenocarcinoma and squamous cell carcinoma histology, with at least 10% of either component (Fig. 15.1f). Unlike MEC, these tumors show an association with cigarette smoking. This tumor type may become more frequent than previously reported (up to 4% of lung carcinoma) with the emphasis on histologic distinction of adenocarcinoma and squamous carcinoma with ancillary IHC. Also its distinction from high-grade MEC becomes relevant given the differences in molecular profiles and their impact on therapeutic decisions.

EGFR/KRAS Mutation

Kang et al. [61] reported a series of 25 Korean patients with AdSq carcinoma with an EGFR mutation rate of 44%, seen in both the AdCa and SqCa components. In two different Japanese series of 26 patients and 11 patients, 15 and 27% of patients, respectively, had EGFR mutation, also with confirmation of mutation in both components [62, 63]. For KRAS mutation, the two mentioned Japanese series had a low rate of mutation in AdSq (5%) typical of Asian series of adenocarcinoma. In a European series, 2 of 20 AdSq had KRAS mutations (10%) [64]. In a series from North America, KRAS mutations were seen in 33% of cases and EGFR in 11% [65], while in another KRAS, rate was lower [66] (13%), with confirmation of mutations in both components demonstrated by both studies.

EML4-ALK

All eight cases of AdSq were negative for EML4-ALK translocation [55].

NUT Carcinoma

Poorly differentiated, high-grade carcinomas have been described that contain a translocation t(15:19)(q13,p13.1) resulting in a BRD4-NUT fusion that is oncogenic [67]. These tumors are often in midline and, while poorly differentiated, can show squamous differentiation. They have been described at all ages and are associated with poor survival. They are not smoking associated. In addition to translocation detection, these tumors can be identified by NUT immunohistochemistry [68]. While NUT tumors in the lung are rare, they have been reported [69–72]. They can be p16 positive but are HPV negative.

Lymphoepithelioma-Like Carcinoma

Lymphoepithelioma-like carcinoma (LELC) is an uncommon tumor. It is an undifferentiated tumor, with large cells with vesicular chromatin, prominent nucleoli, and indistinct cellular borders imparting a syncytial growth pattern. The most distinctive feature of this tumor is infiltrating lymphocytes that can be seen surrounding tumor nests and intermingled between tumor cells. This inflammatory population is reactive but is seen in primary and metastatic sites and is therefore integral to the tumor histology. LELC is not smoking associated but associated with Epstein-Barr virus (EBV) infection in Asian but not Western populations.

Carcinoma cells in these tumors are cytokeratin positive, while the accompanying lymphoid infiltrate is CD45- and CD8-positive T cells. Studies from Western countries do not demonstrate evidence of Epstein-Barr virus by in situ hybridization, except for individual case reports. Latent membrane protein 1 (LMP-1) can be detected in some cases. In studies from Asia, the association with EBV is strong and the LMP-1 detection higher.

For EGFR mutation, the COSMIC dataset shows 1 of 18 cases with these specific mutations. There is variability in this result as LELC are not associated with EGFR mutations [73] in some series and reported in up to 18% in other series [74]; the reason for these differences is not clear, as both series were from areas in which EBV association was seen in all cases. Of 11 cases of LELC studied, no KRAS mutations were reported. In one series, 11 tumors studied showed no evidence of EML4-ALK translocation [55].

PDL1 overexpression is frequent in this tumor subtype (74.3%) [75].

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Chapter 16
Biology of Lung Cancer Metastases

Lucian R. Chirieac

Introduction

Non-small cell lung carcinoma (NSCLC) remains the leading cause of death from cancer in both men and women [1]. Distant reoccurrence remains the major cause of morbidity and mortality in the patients with lung cancer [2]. The term metastasis was coined in 1829 by Jean Claude Récamier [3]. Today it is defined as the transfer of disease from one organ to another not directly connected to it. Metastasis is the primary clinical challenge as it is unpredictable in onset and it exponentially increases the clinical impact to the host [4]. Tumor metastasis is a multistage process in which malignant cells spread from primary tumor to discontiguous organs [2, 5]. It involves a rest and growth in different micro-environments, which are treated clinically with different strategies depending on the tumor histotype and anatomic location of the metastases. Because of the cellular heterogeneity therapies have varying efficacy challenging not only the oncologist but also our understanding of the metastatic process. Each step is rate limiting and is influenced by the interaction between tumor cells and the local micro-environment [3]. If a cell fails each one of the steps, the process stops. Therefore, development of each metastasis represents the survival of selected population of cells that preexist in the primary tumors. Tumor formation starts with cellular selection and transformation, resulting in the growth of the tumor. When the tumor reaches a critical mass new vascularization occurs through an intricate interaction of angiogenesis phenomena. Tumors acquire the propensity to invade through the basement membrane into the stroma, lymphatics, and capillaries through a process of motility and intravasation. Tumor cells migrate in the capillaries, venules, lymphatic

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_16

vessels to form microemboli and cellular aggregates that have the property to spread and disseminate to distant organ sites. At the distant organ sites the tumor emboli arrest in the capillary beds, they adhere to the vascular walls and when they reach a critical mass they start to extravasate into the neighboring organ parenchyma through a process similar to the intravasation into the capillaries. Through complex interactions between the tumor cells and the local micro-environment the tumor cells start to proliferate, form new vessels through angiogenesis, and acquire properties that are significant for the formation of metastases in distant organs [3].

In many patients the process of metastasis has occurred by the time of diagnosis, even if this is not apparent clinically. In some instances the tumor metastases can occur early in the tumor progression stages, when the primary tumor is small or undetectable. However, in the majority of tumors the process of metastasis occurs later in the tumor progression stages when the primary tumor is much larger. The process of tumor metastasis has important features that have recently been uncovered and explored [5, 6].

Lung Cancer Metastases

Lung cancer can spread to any part of the human body. Metastatic spread may result in the presenting symptoms or may occur later in the course of disease. The most frequent sites of distant metastases are the brain, liver, adrenal glands, and bones [7] (Fig. 16.1). Surprisingly the distal recurrences and metastasis to distant organs for

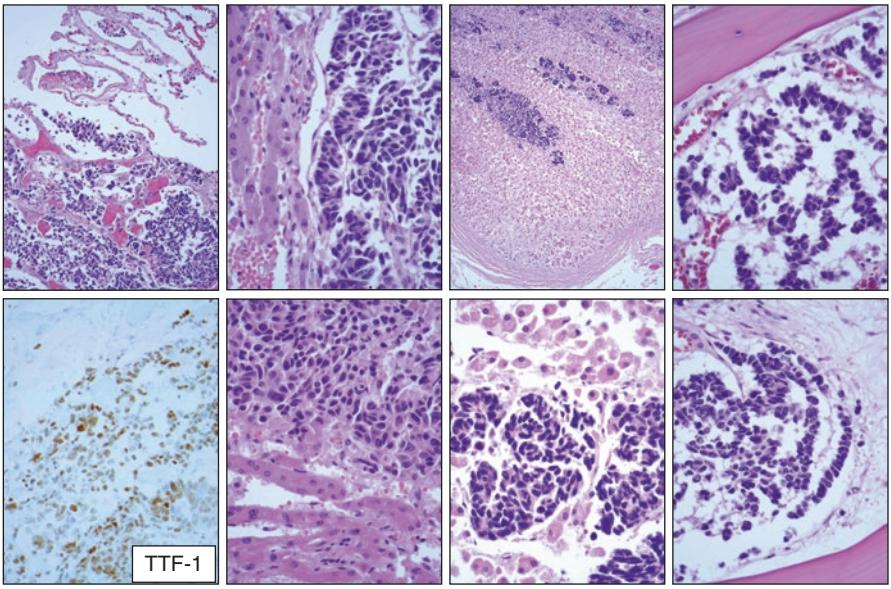

Fig. 16.1 A primary lung cancer (*left panel*) involving the lung of a non-smoker patient treated for recurrent NSCLC with multiple cycles of chemotherapy and radiation. The patient had involvement of multiple organs (liver, adrenal, and bone) that led to multiple organ failure and disseminated disease in multiple organs

Table 16.1 In the International Adjuvant Lung Cancer Trial IALT study, the incidence of either local or distant recurrence was significantly lower in the chemotherapy arm compared with the control arm. The brain was the most frequent site of metastasis (30%) and the incidence of brain metastasis (BM) was not significantly different between the two arms, whereas the incidence of metastases at other sites was significantly lower in the chemotherapy arm compared with the control arm

	Total no. events	Cisplatin-based chemotherapy (n = 932) (%)	Control (n = 935) (%)	Hazard rate	P
Overall survival rate 5 years	973	44.5	40.4	0.86	0.03
Disease-free survival rate	1095	39.4	34.3	0.83	0.003
Local recurrence incidence	379	24.3	28.9	0.72	0.003
Distant recurrence incidence	655	40.8	44.3	0.84	0.03
Brain as first metastasis incidence	227	18.1	16.3	1.07	0.61
Non-brain as first metastasis incidence	456	29.4	34.9	0.75	0.003
Second primary incidence	78	6.0	6.9	0.90	0.64

lung cancer are extremely high (41–45%) and although studies have shown that chemotherapy, targeted therapy, or systemic immune checkpoint inhibitors (preferred) could lower the incidence of metastasis (Table 16.1), the recurrence rate still remains high [8, 9]. The IALT study [9] shows that the incidence of brain metastases is the most common among the patients with lung cancer (irrespective of the type of treatment) and represents almost a third of all the tumors that spread to distant organs.

Liver Metastases

Symptomatic hepatic metastases are uncommon early in the course of the disease; asymptomatic liver metastases may be detected at presentation by liver enzyme abnormalities, CT or PET imaging. Among the patients with otherwise resectable NSCLC in the chest, CT evidence of liver metastases has been identified in approximately 3% of the cases. Newer imaging techniques (PET or integrated PET/CT) identify unsuspected metastases in the liver or the adrenal glands respectively in about 4% of the patients. The incidence of liver metastasis is much higher later in the course of disease once the tumor progresses and spreads to distant organs. Autopsy studies have shown that hepatic metastases are present in more than 50% of the patients with either NSCLC or small cell cancer.

Adrenal Metastases

The adrenal glands are a frequent site of metastasis but are rarely symptomatic. Only a fraction of adrenal masses detected on staging scans represent metastases. In a series of 330 patients with operable NSCLC, 10% had isolated adrenal masses [10]. Only 8 out of 32 (25%) were malignant, while the remainder had benign lesions like adrenal adenoma, adrenal nodule hyperplasia, or hemorrhagic cysts. Conversely, negative imaging studies do not exclude adrenal masses and a study of patients that had SCC found that at least 17% of adrenal biopsies showed metastatic involvement despite normal CT scans [11]. The lack of specificity of initial CT identifying an adrenal mass creates a special problem in patients with an otherwise resectable lung cancer. Involvement of the adrenal glands is more frequent in patients with widely disseminated disease. In an autopsy series that have been previously published, adrenal gland metastases have been identified in 40% of patients with lung cancer.

Bone Metastases

Metastases from lung cancer to bone are frequently symptomatic. Patients present with pain and elevated levels of alkaline phosphatases. Twenty percent of patients with NSCLC have bone metastases at presentation and osteolytic appearances are more common than osteoblastic ones. The most common sites of involvement are the vertebral bodies. Bone metastases are even more common in patients with SCLC, and represent 30–40%. Modern imaging studies (PET and PET/CT) have improved the ability to identify metastases to many organs including bone, with greater sensitivity than CT or bone scan.

Brain Metastases

Lung cancer is the malignancy that most commonly gives rise to brain metastasis which is a devastating complication [12]. Brain metastases are a major cause of morbidity and mortality in human malignancies in patients with NSCLC. The frequency of brain metastasis is greatest with adenocarcinoma and least with squamous cell carcinoma. Approximately 10% of the patients have brain metastases at the time of diagnosis, and approximately 40% of all patients with lung cancer will develop brain metastases during the course of the disease [13]. Patients with locally advanced NSCLC who are treated with chemotherapy and chest radiotherapy with or without surgery have a very high rate of developing brain metastases [14–17].

These patients also have a risk that ranges from 15 to 30% of failing first in the brain. Brain metastases from NSCLC have received increasing attention, because combined-modality therapy has led to improvements in intrathoracic local control and prolonged overall survival [18–20]. The risk for brain metastasis increases with larger primary tumor size and regional node involvement (which is a well-known phenomenon at the basis TNM staging system). For carefully selected patients, surgical resection may be feasible. Surgical resection of brain metastases may be feasible in cases that have operable NSCLC in the chest and solitary brain metastases. In patients with SCLC, metastases to brain are present in 20–30% at initial diagnosis. Without prophylactic irradiation, relapse in the brain occurs in 50% within the next 2 years after the diagnosis. Randomized trials have shown that the frequency of brain metastases can be significantly reduced with prophylactic cranial irradiation. It is important to identify the patients with NSCLC who are at greater risk of developing metastases because such metastases may exist in the absence of neurologic symptoms [21]. Furthermore, prophylactic cranial irradiation may be an effective modality preventing brain metastases in patients with NSCLC who receive adjuvant chemoradiation [16]. Despite advances in diagnosis, therapeutic modalities, and clinical practice guidelines, it remains unclear whether patients with NSCLC should be screened for brain metastases or not [22, 23].

Molecular Characteristics of Metastases in Comparison with Primary Tumor

Recent studies have advanced the hypothesis that there may be important differences in the primary tumor, lung tumor, and metastases of lung adenocarcinoma, regarding morphology, biomarker expression, and genotype [24]. The mutation status of metastases can differ from the primary tumors and also among metastases [3, 25]. The frequency of differences and the significance of the differences in pathologic variables between primary lung tumors and metastases and also previously systemically treated tumors have yet to be fully investigated [25, 26]. Both the cells within the primary tumor and the metastatic lesions can continue to diversify if the lesions grow and result in molecular differences between the primary and the metastatic tumor. To determine whether the genetic profiles are similar between the primary lung cancer and their paired metastases to the brain, we examined pairs of primary metastatic lung carcinomas by high-throughput genetic mutation profiling. We evaluated four-micron formalin-fixed paraffin embedded specimens from patients with lung cancer (women 52% and men 48%) with a median age of 65 years. The tumors investigated were 12 adenocarcinomas and nine squamous cell carcinomas and the corresponding brain metastases they developed after a median of

Table 16.2 Molecular characteristics of matched primary NSCLC and brain metastases

Gene	Present *only* in primary NSCLC	Present *only* in metastasis to brain	Present in *both* primary NSCLC and brain
ABL1	Y253F	G250E	
BRAF	D594G	D594G	D594G (one case)
EGFR	Exon19 del, D770_N771>AGG		
FGFR3		K650T	
HRAS	G13D		
KIT		V559I	
KRAS	G12C	G12S; G12D; G12C	G12C (two cases)
PDGFRA	T674I		
PI3K	G1049R		
RET	E632_L633del		

12.5 months (range 2–90 months) over a 35-month median follow-up time. We employed the sequenom mass spectrometry-based system (IPLEX protocol-oncomap analyses) for 252 genetic mutations in the following genes: ABL1, BRAF, EGFR, FGFR3, HRAS, KRAS, MET, N-ras, PBGFRA, PI3K, and RET. Some of the lower confidence mutations identified by IPLEX protocol were validated by homogeneous mass-extended (HME) technology. We found that nine patients (39.1%) had mutations only in the primary tumors Table 16.2. In five patients (21.7%) mutations were identified only in the brain metastases and in only three patients (13%) mutations were identified in both lung and brain metastases (Table 16.2). Except K-ras G12C mutation that was identified in two patients, all mutations were unique in each patient.

In summary, there is a great variation in the molecular abnormalities between individual primary NSCLC and their metastases to the brain. Understanding these differences will allow us to clarify the mechanism of metastatic progression of NSCLC to brain and potentially identify novel targets of therapy.

Protein Expression Characteristics of Metastatic Lung Carcinoma to Brain and Primary Metastases

We compared the expression of certain proteins between brain metastases and the primary tumors (Fig. 16.2). The results of the study showed that metastatic NSCLC to the brain have a higher expression of MIB1 ($p = 0.02$), a lower VEGF-A ($p = 0.03$), and a higher EGFR ($p = 0.03$) expression in brain metastases than the matched primary NSCL cancers (Fig. 16.2).

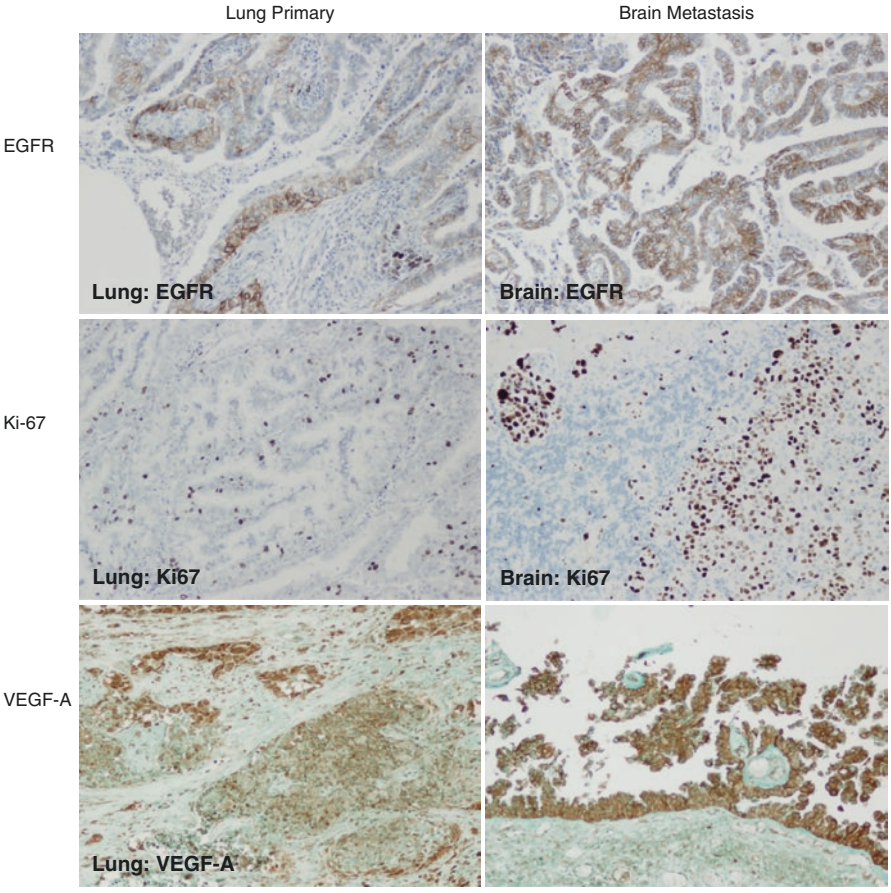

Fig. 16.2 Immunohistochemical characteristics of metastatic NSCLC to the brain

MET in Primary Lung Cancers and Corresponding Distant Brain Metastases

MET amplification has been detected in 20% of NSCL cancers with EGFR mutations progressing after an initial response to tyrosine-kinase inhibitors (TKI) therapy. MET is amplified, mutated, and overexpressed or uniquely activated in many tumors. MET expression was associated with worse prognosis in many cancers including NSCLC [27]. We investigated MET expression, phosphorylation, and gene copy gain in both primary NSCLC and brain metastases. MET FISH reveals a lower copy gain in the primary lung tumors versus a higher copy gain in the corresponding metastatic lesions

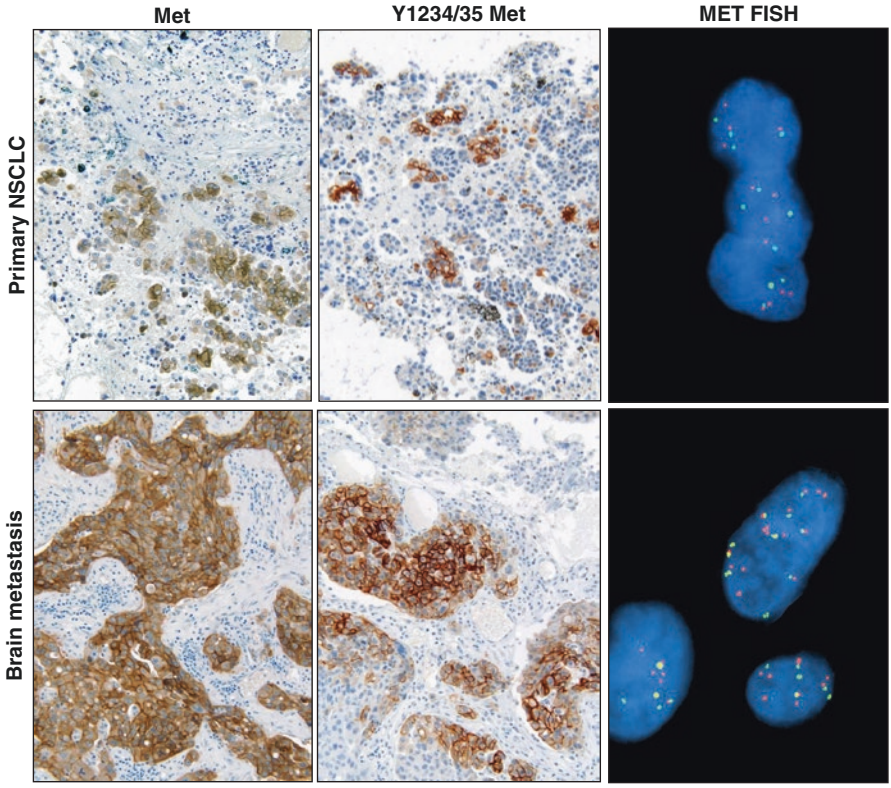

Fig. 16.3 MET expression by immunohistochemistry (first column), MET activation (Phospho MET, second column), MET amplification (third column). MET and phospho-MET staining were heterogeneous and focal in the primary cancer, but more widespread and diffuse in the paired brain metastases

(Fig. 16.3). Surprisingly we found that the expression of both the receptors is focal and heterogeneous. Furthermore, immunohistochemistry images on consecutive sections revealed colocalization of deletion of EGFR mutated cells and activated MET cells. Our studies confirm the hypothesis of clonal selection and the genotype differences between primary tumors and brain metastases (Fig. 16.4). We found that the heterogeneous Met expression, activation, and gene copy gain in primary NSCLC is significantly enriched in paired brain metastases. These results suggest that the enrichment of Met-activated lung tumor cells in brain metastases may result from an increased capacity for Met activated primary tumor cells to migrate and establish metastases. The initial response to EGFR tyrosine-kinase therapy and the initial disease control (partial response or stable disease as defined by RECIST criteria) is

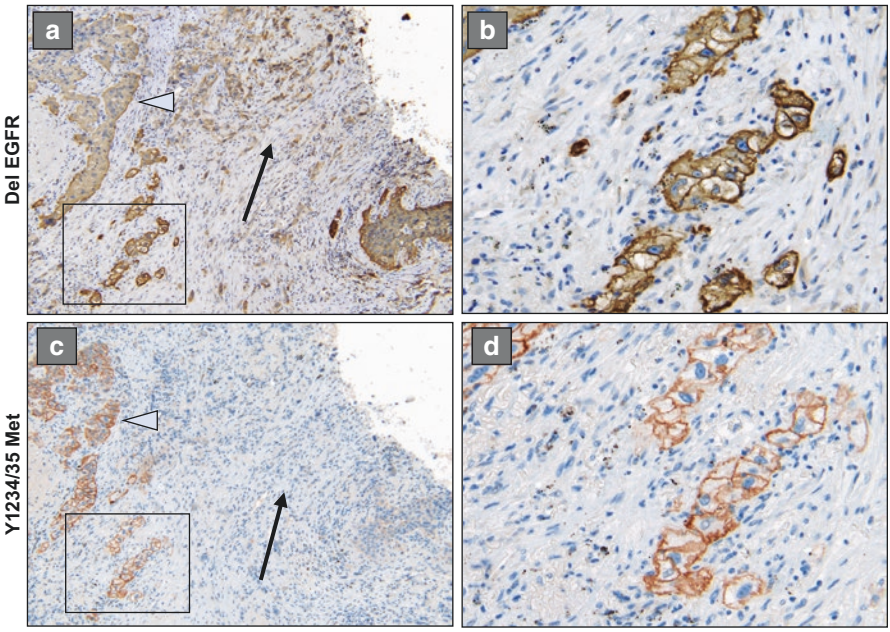

Fig. 16.4 Colocalization studies to identify the EGFR mutations have shown that the EGFR mutated cells and the MET activated tumor cells (with deletion 19 specific EGFR antibodies and phospho MET antibodies)

anticipated in tumors harboring no MET activation (scenario A, Fig. 16.5) or a low percentage of MET activation (scenario B, Fig. 16.5). By contrast, primary resistance (progressive diseases defined by RECIST criteria) is consistent with tumors harboring a high percentage of MET activated cells with concomitant EGFR tyrosine-kinase resistant cells (scenario C, Fig. 16.5) [27].

Genetic Abnormalities in Primary Lung Cancers and Locoregional Lymph Node Metastases

Recent studies looked into the EGFR, KRAS, and BRAF mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases [28]. The study revealed that 72% (31 out of 43 patients with mutated tumors in total 32% of all investigated cases) with mutations showed discordant results. The discordant mutational status in the primary tumor and the corresponding lymph node metastases were 6 out of 7 cases with EGFR mutation and 25 out of 36 cases

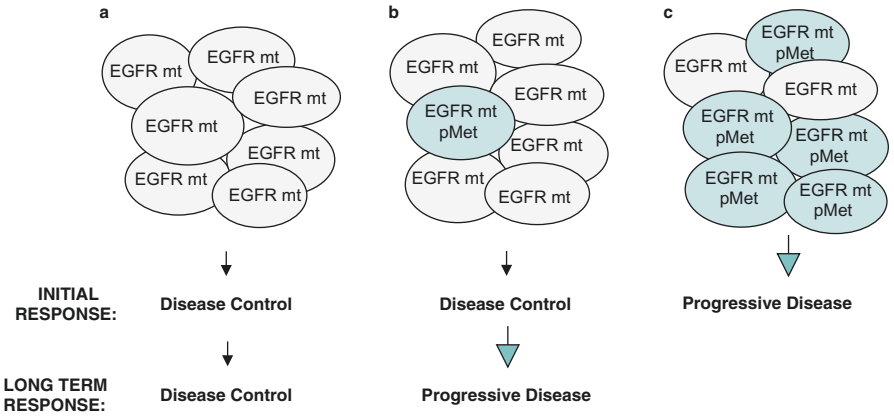

Fig. 16.5 Preexisting Met activation may predict poor response to subsequent EGFR TKI therapy in EGFR mutant NSCLC. Initial response to EGFR TKI therapy: Initial disease control (partial response or stable disease, as defined by RECIST criteria) is anticipated in tumors harboring no Met activation (A) or a low percentage of Met activation (B). By contrast, primary resistance (progressive disease as defined by RECIST) is consistent with tumors harboring a high percentage of Met activated, EGFR TKI resistant cells. (C) Disease control in tumors without Met activation may remain relatively durable but, initial disease control in tumors with low level Met activation is not durable, as focal regions of Met activated, EGFR TKI resistant cells can proliferate despite EGFR TKI therapy

with KRAS mutations. The lack of the correlation in the mutation status between primary tumors and metastases is most likely real and not due to technical problems for several reasons: (a) all tumor specimens analyzed were required to contain at least 70% tumor cells, (b) the results were confirmed by a second run, (c) the mutant rate in the primary tumors was not different to previous published data, and (d) the results were in accordance with those from other reports.

Predictive Markers Associated with an Increased Risk of Brain Metastases

We performed a controlled study of patients who were newly diagnosed with NSCLC, who developed brain metastases. These patients were initially diagnosed with early stage operable lung cancer. After surgical removal of the primary tumor the patients were followed up for a median period of 35.5 months [12]. These patients developed brain metastases as a site of distant release after a median period of 12.5 months. These patients were compared with a control group of patients who had NSCLC and no evidence of brain metastases in the same follow-up period.

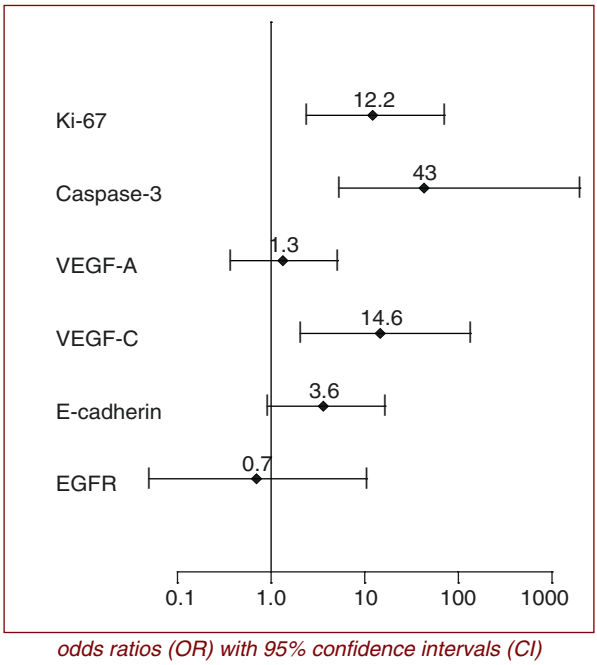

Fig. 16.6 Risk of developing brain metastasis according to expression of Ki-67, Caspase-3, VEGF-A, VEGF-C, E-Cadherin, and EGFR in the Primary NSCLC

NSCLC and their corresponding metastases were examined for expression levels of Ki-67, caspase-3, VEGF-A, VEGF-C, E-cadherin, and EGFR respectively. The study showed an increased risk of developing brain metastases in patients who had a high expression of Ki-67, caspase-3, VEGF-C, and e-cadherin but not with VEGF-A and EGFR (Fig. 16.6). Patients with an increased Ki-67 labeling index developed metastases after a median time of 1.2 years as opposed to 5 years for the patients with the low labeling index. Furthermore, patients with a lower caspase labeling index had an increased rate of developing brain metastases as opposed to patients with a high labeling index. The results of the study indicated that patients with NSCLC and high Ki-67, low caspase-3, high VEGF-C, and low e-cadherin in their tumors may benefit from close surveillance because they may have an increased risk of developing brain metastases. Higher Ki-67 and lower caspase labeling indices characterize patients who are at greater risk of developing metastatic NSCLC to the brain. The identification of this subgroup of patients is very important as these patients may benefit from early and close physical and imaging follow-up. In addition, this subset of patients may benefit from prophylactic brain irradiation. Another study looked into 100 consecutive patients with EGFR mutations that were treated

with gefitinib or erlotinib that followed the patients for a period of time and looked into the incidence of brain metastases [29]. The authors have surprisingly shown that there are differences between different types of EGFR mutations (exon 19 deletion versus L858R point mutation) in the characteristics of the primary tumors and propensity to spread to brain. The time to progression was 16.2 months with exon 19 deletions versus 11.8 months in patients with l858R ($p = 0.026$) also, the overall survival was much longer in patients with exon-19 deletions than L858R (40.6 versus 23.9 months, $p = 0.014$).

Conclusion

There may be important differences between the primary tumor and metastases of lung adenocarcinoma regarding morphology, biomarker expression and genotype. The mutation status of metastases can differ from that of the primary tumor and also among metastases. The frequency of differences and the significance of the differences in pathologic variables between the primary tumors and metastases and also previously systemically treated tumors have also yet to be investigated.

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Chapter 17
Precursor and Preinvasive Lesions

Alain C. Borczuk

Introduction

Malignant neoplasms of the lung, once mass lesions, have already undergone a set of molecular oncogenic events that sustain continued cellular survival and growth. These events include a combination of oncogene activations that are considered driver events in tumor formation, as well as accumulation of losses of tumor suppressor genes that normally regulate the proliferation of cells in response to injury. The increase in pathway activation can be the result of gain of function point mutations and in frame insertions and deletions, as well as gains in gene copy number. Activation of tumor-related genes can also be the result of gene expression changes that lead to constitutive function in cellular mechanisms of growth or prevention of cell death. Alternatively, loss of function mutations, indels, and larger regions of deletion can result in the loss of key regulators of growth and cell death. Loss of these tumor suppressor genes can also be achieved through epigenetic mechanisms that affect their promoters through methylation or regulate their expression through miRNA.

Greater understanding of the stepwise progression of molecular events that must take place for normal cells to transform into malignant cells is critical in developing better tests for the early detection of lung cancer. It may also provide clues as to the pathogenesis of cancer. In studying the histopathology of lung tissues associated with resected lung cancer, potential precursor lesions of these neoplasms have been identified and further studied.

The recent classification of lung tumors identifies three precursor lesions of lung neoplasia, that is, diffuse idiopathic pulmonary neuroendocrine cell hyperplasia leading to carcinoid tumors, atypical adenomatous hyperplasia leading to adenocarcinoma, and squamous dysplasia and squamous carcinoma in situ leading to invasive

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_17

squamous cell carcinoma. Other entities that harbor oncogenic alterations have been identified, although whether they progress to malignant neoplasms remains unknown.

Neuroendocrine Cell Hyperplasia

Neuroendocrine cells are normally present in the airway. Hyperplasia of neuroendocrine cells can be seen in association with pulmonary diseases such as chronic infection, bronchiectasis, and chronic obstructive pulmonary disease. Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) is a bilateral condition of neuroendocrine cellular proliferation which can be symptomatic, with airway obstruction and with distinctive imaging correlates. It was first described in 1992 in six patients with airway obstruction [1], and more recently, a definition that includes neuroendocrine cell hyperplasia associated with more than two carcinoid tumorlets has been proposed [2]. With such a definition, the progression of neuroendocrine cell hyperplasia into tumorlets which are aggregates of neuroendocrine cells under 5.0 mm in DIPNECH is indicative of a growing cellular population as a precursor to carcinoid tumors.

The histology of neuroendocrine cell hyperplasia is that of an increased number of these cells within the airway epithelium (Fig. 17.1). This can occur in

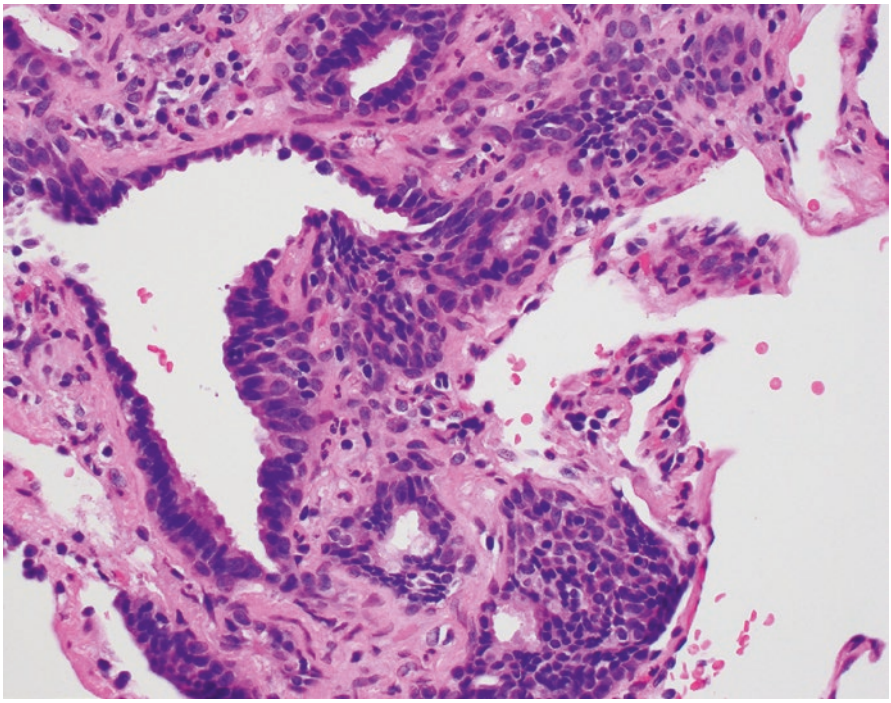

Fig. 17.1 Neuroendocrine cell hyperplasia: Bronchiolar epithelium is undermined by a uniform proliferation of round to oval neuroendocrine cells, without stromal invasion

small aggregates or can manifest as rows of neuroendocrine cells. In some instances, these proliferations can be somewhat exophytic, growing into the airway lumen (Fig. 17.2). Once they invade outside of the epithelium, they can be seen as cellular aggregates associated with peribronchiolar fibrosis known as carcinoid tumorlets. If these tumorlets exceed 5.0 mm, they are renamed carcinoid tumors.

Carcinoid tumors are characterized by an absence of 3p loss, normal FHIT, low telomerase, and MEN1 mutations. Molecular alterations in these tumors are different than those of adenocarcinomas and squamous carcinomas. While 11q 13 allelic imbalance is seen in carcinoids, it is not present in tumorlets [3]. Developing neural transcription factors are seen in neuroendocrine proliferations but not in normal neuroendocrine cells. These include TTF1, ASCL1, and POU3F2. VGF peptides increase in hyperplasia and neoplasia [4].

There are mouse models of small cell carcinoma that attempt to shed some light on potential cells of origin for neuroendocrine tumors. An animal model inducing p53 loss and RB loss in epithelial cells induced small cell carcinoma expressing ASCL1 [5]. By using adenovirus cre recombinase delivery into the airway of mice that are engineered to induce loss of p53 and RB in different types of epithelial cells, the loss of P53 and RB directed toward neuroendocrine cells led to small cell carcinoma. Only rare neuroendocrine tumors were derived from targeting type 2 pneumocytes than produce surfactant protein C [6].

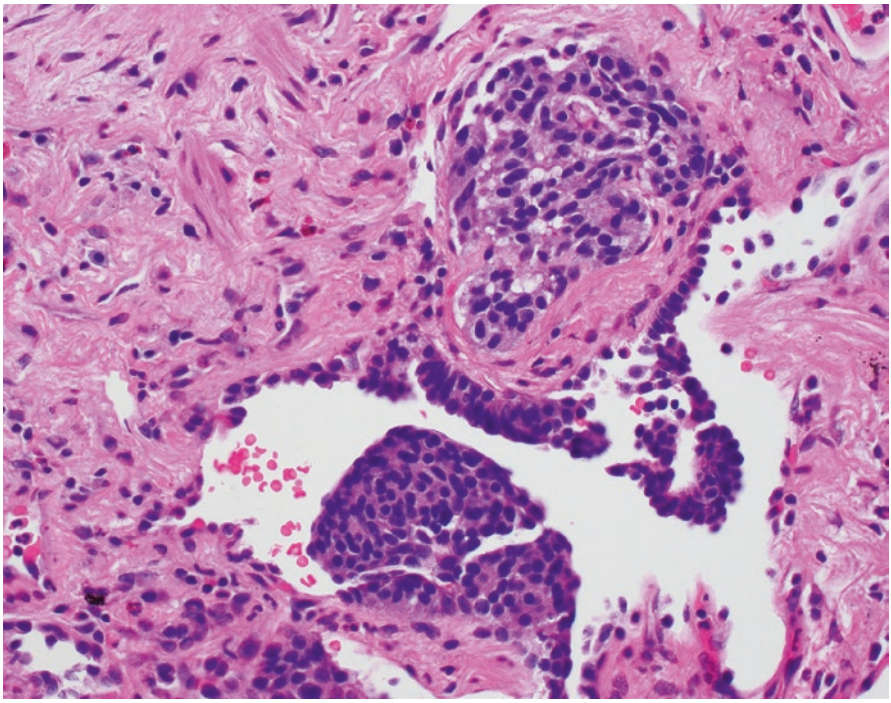

Fig. 17.2 Neuroendocrine cell hyperplasia. Proliferations of cells can be exuberant, causing polypoid projections into the airway lumen

Atypical Adenomatous Hyperplasia

Atypical adenomatous hyperplasia (AAH) is a proliferation of type 2 pneumocytes that is generally small and incidentally discovered. While a size criterion has not been absolutely set, these are usually under 5.0 mm. They are characterized by a proliferation of pneumocytes with mild to moderate atypia, but importantly these proliferations show variability from cell to cell and leave residual type 1 pneumocytes along the alveolar walls, seen as gaps in the proliferation (Fig. 17.3). Alveolar walls may be mildly thickened, but they are not invaded. Such lesions were described by Roberta Miller in 1988 as possible precursor lesions, akin to adenomas of the colon [7].

These AAH lesions are frequently found in adjacent lung tissue of patients with pulmonary adenocarcinoma but also can be found in lung tissues removed for nonneoplastic indications [8]. AAH is found more frequently in lungs from patients with adenocarcinoma (23.2%) and in women with adenocarcinoma (30.2%). AAH is seen more frequently in patients with multiple carcinomas, usually multiple adenocarcinomas [9].

An important question in the study of precursor lesions of neoplasia is their clonality. AAH lesions in women were studied, and their pattern of X-inactivation supports the clonality of these lesions [10]. In addition, the evidence supports that multiple AAH are independent foci.

These observations point to the conclusion that atypical adenomatous hyperplasia is neoplastic and has a similar pathogenesis as adenocarcinoma. Its morphologic

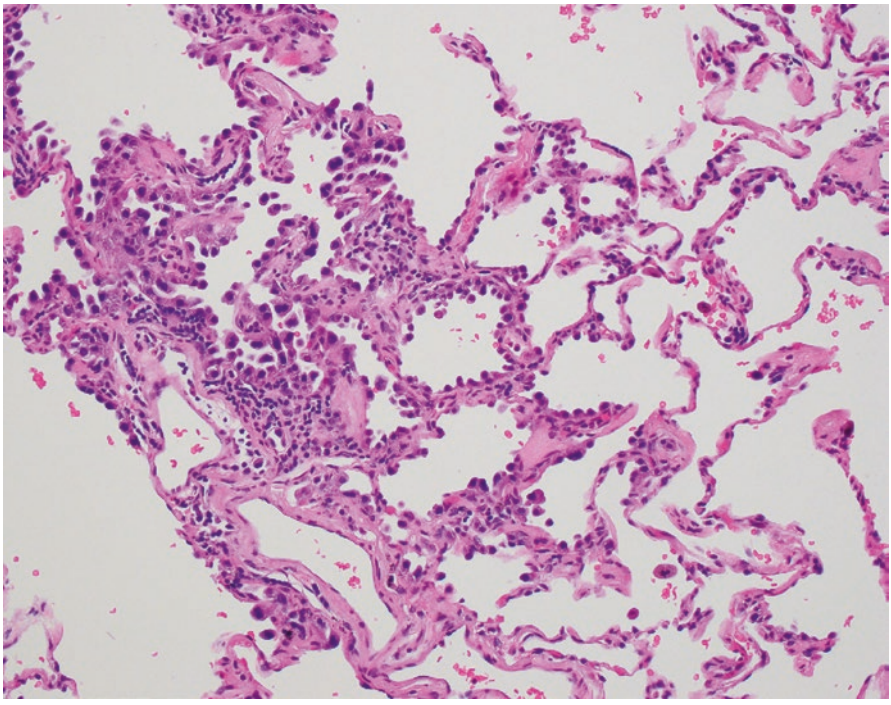

Fig. 17.3 Atypical adenomatous hyperplasia. Alveolar lining proliferations of hobnail and atypical pneumocytes, with cell to cell variability and gaps of residual type 1 cells

resemblance to early adenocarcinoma such as adenocarcinoma in situ and minimally invasive adenocarcinoma, albeit smaller and with a less uniform proliferation, is also indirect evidence of AAH as a precursor lesion to subtypes of adenocarcinoma. However, the percentage of AAH lesions that progress to adenocarcinoma is unknown; in other words, it is not known whether this is an obligate precursor to adenocarcinoma or a non-obligate one. The latter seems more likely given the number of such lesions that can be present in an individual lung resection. The latency of progression is also not known. Although authors have described a connection with TTF1-positive peripheral adenocarcinoma and have coined the term terminal respiratory unit type adenocarcinoma, what proportion of adenocarcinomas arises from a progression of AAH to adenocarcinoma in situ is also speculative. The non-terminal respiratory unit adenocarcinoma has a less well-defined precursor. It is likely that a non-lepidic precursor exists, but its histologic appearance remains poorly described [11, 12].

Loss of Heterozygosity (LOH) Data and Loss of Tumor Suppressor Activity

Loss of heterozygosity has been reported in atypical adenomatous hyperplasia at several loci, and these are seen at increased frequency in adenocarcinoma. For example, in a study of 18 AAH and 17 AdCa, loss of heterozygosity at a 16p locus was seen in 22% of atypical adenomatous hyperplasia while in 35% of adenocarcinomas and at a 9q locus in 28% of AAH and in 41% of AdCa. The locus in 9q included the TSC1 gene [13].

Loss in 3p is a commonly reported finding in 10–15% of AAH [14–16]. However, loss of FHIT does not appear to occur in AAH (see "Squamous Dysplasia" section), suggesting that the 3p loss involves a different locus. Losses in 9p in roughly 13% of cases and in 17p in 5% of cases have been described. Allele-specific LOH at 9p locus was seen in preinvasive lesions and identified at an increasing rate in invasive carcinoma [17]. The likely target of this LOH is CDKN2a (p16), but other pathways of silencing may also be in play (see section on "Promoter Hypermethylation"). In addition to 3p, 9p, and 17p, LOH at 5q, 7p, and 10q in AAH is described [18]. Overall, these studies suggest that there are common regions of loss in AAH and adenocarcinoma and that the regions of loss increase during progression.

P53 Mutations

P53 IHC was studied in AAH, with p53 overexpression in 17–53% of cases [19, 20]. However p53 mutation rate may be lower than IHC immunoreactivity would suggest. In one series, no p53 mutations were seen [16], also in support of the overestimation of p53 mutation by IHC. In addition, a demonstration of increasing rate

of p53 mutation from AAH to adenocarcinoma in situ and invasive adenocarcinoma with sclerosis has been described along with increasing rate of 17p LOH in this progression, the chromosomal region containing p53.

Epidermal Growth Factor Receptor (EGFR) and Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) Mutations

KRAS

There is conflicting data on KRAS mutations in AAH. In one series of 19 AAH, no KRAS mutations were reported [18]. In an early study of AAH, KRAS mutations were found in 39% of lesions [21], with transversion mutations similar to those seen in cigarette smokers. This was also reported in a separate study of 32 AAH lesions in which 16% harbored KRAS transversion mutations [22].

Some of this data suggests that AAH lesions harboring KRAS mutations are the result of cigarette smoking, so different reported rates could reflect study populations with different smoking rates. Within the lung tissue exposed to cigarette smoke, separate independent KRAS mutations may be induced. For example, in six patients with AAH, 2 of 6 patients with multifocal lesions [23] harbored KRAS mutations, overall in 50% of their AAH lesions. Of note, specific KRAS mutations in AAH did not match those in adenocarcinomas from the same patient [21].

EGFR Mutations

Early studies of EGFR mutation in AAH also showed variable results and included detection in 2 of 7 (28%) AAH [24] in one series and 0 of 5 AAH in another [25]. In a series of 18 AAH, 3 of 18 (17%) showed EGFR mutations with 2 of 18 cases (11%) with KRAS mutations [26]. As experience was gained in larger series, 17 of 54 (32%) AAH showed EGFR mutation [27], with 10 cases of exon 19 deletion and 7 with exon 21 (L858R) mutations. In that set, KRAS mutations were seen in only 1 of 49 AAH (2%). The same authors [28] found a high rate of EGFR mutations in their non-mucinous lepidic pattern containing adenocarcinomas in 64 out of 82 (78%), while KRAS was seen in only 2 of 82 (2.4%). It may be that the low KRAS rate in part reflects an East Asian, nonsmoking population as the explanation for the relatively low KRAS mutation rate.

In an interesting study of adenocarcinoma and their precursors, among 40 AAH lesions, 33% harbored KRAS mutations, while 25% had EGFR mutations, predominantly exon 21 L858R missense point mutations. In the same study, only 12% of adenocarcinoma in situ (AIS) and 12% of invasive adenocarcinoma harbored KRAS mutations, while 36% of AIS and 56% of adenocarcinoma had EGFR mutations, with a relatively balanced distribution of exon 19 and exon 21 mutations [29]. This group noted an increased number of KRAS mutations in smokers. Another series showed a rate of EGFR mutation of 3% in AAH and 11% in AIS, with KRAS mutations in 27% of AAH and 17% of AIS [30]. These observations suggest that KRAS mutations may occur at a higher rate in AAH than adenocarcinoma in these East

Asian populations and that the opposite is true in EGFR mutation. One possibility is that EGFR mutations, when they occur in precursor lesions, are more likely to be obligate precursors to adenocarcinoma or cause more rapid progression to adenocarcinoma, and therefore their rate in precursor lesions appears low.

Looking beyond AAH among AIS preinvasive and early invasive lesions, Soh et al. showed EGFR mutations in 1 of 4 AAH (25%) and a rate of 36, 47, and 50% using the schema of Noguchi A, B, and C, respectively. In the same series, KRAS mutations were seen in 10, 6.7, and 9% in these groups. In a series of 17 AIS lesions (noninvasive BAC) in an East Asian cohort, 88% had EGFR mutations [28] (adenocarcinoma in situ). In a Western series, among 18 cases of non-mucinous AIS and minimally invasive adenocarcinoma, 7 of 18 were associated with EGFR mutation with 1 of 18 harboring KRAS mutation [31].

EGFR Copy Number Gain

Copy number gains in EGFR were seen in AAH with increased rate and level of gains in adenocarcinoma [32]. In the series of Soh et al., EGFR copy number increase occurred during the progression [33] from Noguchi A, B, and C of 5.3, 13, and 32%, respectively. It has been suggested that EGFR amplification could represent a marker of progression.

Measures of Cell Cycle Activity

An examination of cell cycle-related markers in AAH showed decreased CDKN2A (P16) expression with concomitant increase in cyclin D in 55% of cases. However, KI67 was low in these lesions [34]. While proliferation was higher in adenocarcinoma in situ than in AAH, invasive adenocarcinomas showed the highest proliferation index; this observation is somewhat limited by changes in terminology that have occurred since these studies were conducted [14, 20].

The inhibition of p21CIP1 protein results in activation of a pathway of cell cycle initiation and progression, that is, the RB/CDK/cyclin D1 axis is activated when p21 levels are low. However, this may be a later event in carcinogenesis as low p21 is seen in invasive adenocarcinoma [35]. Inhibitors of CDK2 such as p27Kip1 may be actively degraded by JAB1, and higher levels of JAB1 were identified in AAH lesions when compared to normal pneumocytes [36], with increasing frequency of expression from AAH (36%) to adenocarcinoma (54%).

Immunohistochemistry Studies

In an IHC study designed to examine loci related to acquisition of invasion, FHIT was decreased in invasive adenocarcinoma but retained in AAH. CD44v6 and TIMP2 also showed a decrease in the progression from AAH to invasive carcinoma [37]. Destruction of type IV collagen and MMP2 expression have also been

associated with invasion; preservation of type IV collagen was seen in all AAH cases with some cases showing MMP2 without its activating enzyme [38]. MMP2 expression was seen to increase from AAH and adenocarcinoma in situ to invasive adenocarcinoma with central scar formation [39].

Gene Expression Studies

Gene expression studies of lung adenocarcinoma [40, 41] have shown differences between preinvasive adenocarcinomas and invasive adenocarcinoma that contain a lepidic component (in some studies, termed terminal respiratory unit B and A type, respectively). In the series by Takeuchi et al., the noninvasive and focally invasive adenocarcinomas were in the TRU-b group, with EGFR mutations in a subset of these cases, but no p53 or KRAS mutations. Their expression was characterized by gene ontologies related to cellular differentiation. Gene expression studies have also implicated loss of TGFBR2 expression, the receptor for TGF-B, in the acquisition of invasion in adenocarcinoma, and coordinated epithelial cell activation by oncogenic KRAS and loss of TGFBR2 in a mouse model resulted in invasion and metastasis [31].

Other Molecular Alterations

In an examination of DNA methylation in AAH, changes in DNA at loci related to CDKN2a exon 2 and PTPRN2 were seen [42]. Earlier studies also identified CDKN2a promoter hypermethylation in AAH [43]. In the transition from AAH to AIS, further alterations were seen in HOXA1, HOXA11, NEUROD1, NEUROD2, and TMEFF2 and from AIS to adenocarcinoma in CDH13, CDX2, OPCML, SFRP1, TWIST1, and RASSF1.

In one study using mass spectroscopy, differences were noted from AAH to carcinoma, although no specific protein was identified characteristic of that transition [44]. Increase in human telomerase RNA component (hTERC) and telomerase reverse transcriptase (hTERT) mRNA expression by in situ hybridization was described in AAH with increasing rates in lepidic pattern containing adenocarcinomas [45]. In situ hybridization for telomeric repeat-binding factor 1 (TERF1) also showed increase in a similar fashion [46].

Non-AAH Proliferations

In a search for non-AAH precursors of adenocarcinoma, Ullman and colleagues [47] examined airway glandular dysplasia by comparative genomic hybridization and found gain in 1q, 17, 19q, and 20q in these putative precursor lesions, with losses at 3p, 9, 13, and 14. However further study of such lesions remains to be pursued.

Congenital pulmonary airway malformation type 1 can be associated with mucin producing cellular proliferations that resemble adult-type mucinous adenocarcinoma (Fig. 17.4). These can have lepidic mucinous growth and can be both within and outside the cystic lesions [48, 49]. Of interest is the description of oncogenic KRAS mutations in these lesions supporting their neoplastic nature. Additionally, LOH at FHIT, Rb, and CDKN2A have been shown as well as gains in chromosomes 2 and 4. Their biological relationship to adult mucinous adenocarcinoma, while linked by these KRAS mutations and other alterations, is unknown. While more indolent than their adult counterpart, extrapulmonary spread has been reported [50].

Tumors resembling adenocarcinoma in situ, non-mucinous type, have been described in children, adolescents, and young adults, who are nonsmokers and frequently have a history of treatment for an unrelated malignancy. Only seven reported cases were tested for EGFR and KRAS mutations, with 2 of 7 and 1 of 7 positive, respectively. Copy number analysis revealed polysomy 17, as well as other copy number gains [51–53].

Peripheral glandular papillomas, recently renamed ciliated muconodular papillary tumors (Figs. 17.5 and 17.6), have been studied for mutations and found to have BRAF V600E and EGFR exon 19 deletions (specifically E746-T751/S752V mutations) [54]. Recently, two cases were reported with ALK translocations [55, 56]. These alterations are of interest as they are all encountered in lung adenocarcinoma, but to date, malignant transformation of peripheral glandular papillomas or ciliated muconodular papillary tumors has not been reported.

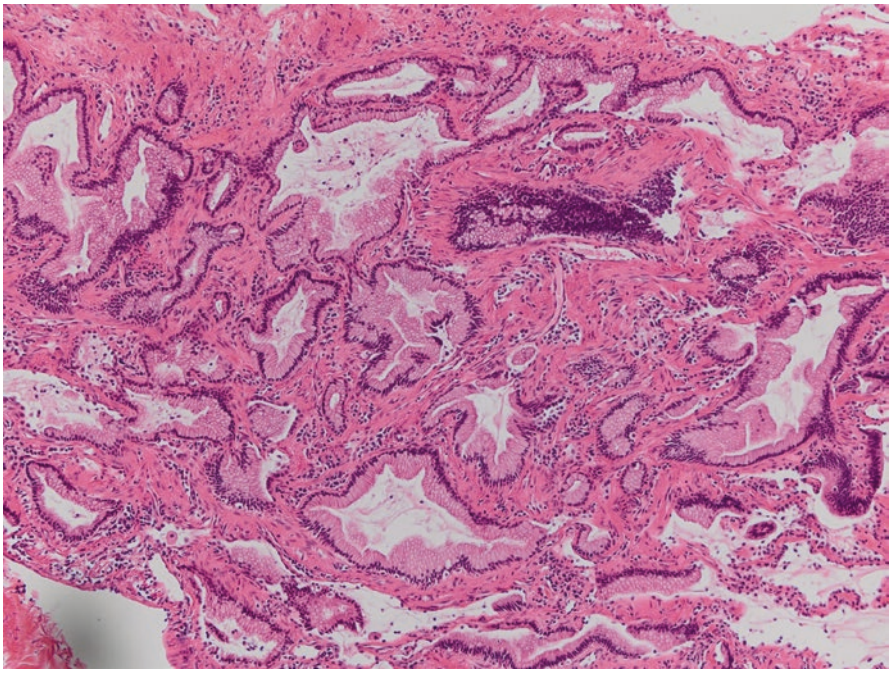

Fig. 17.4 Mucinous proliferation in CPAM. Bland mucinous proliferations morphologically mucinous adenocarcinoma are seen lining pre-existing airways but also with probably stromal invasion

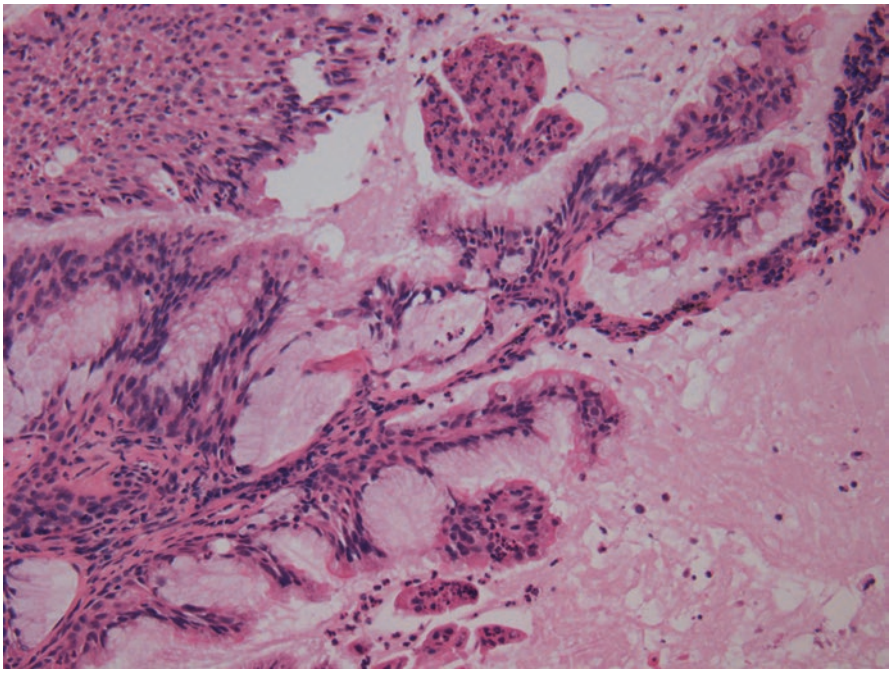

Fig. 17.5 Ciliated muconodular papillary tumor/peripheral glandular papilloma. Mcuin filled airspaces are lined by an admixture of mucinous cells, including goblet cells, and ciliated cells

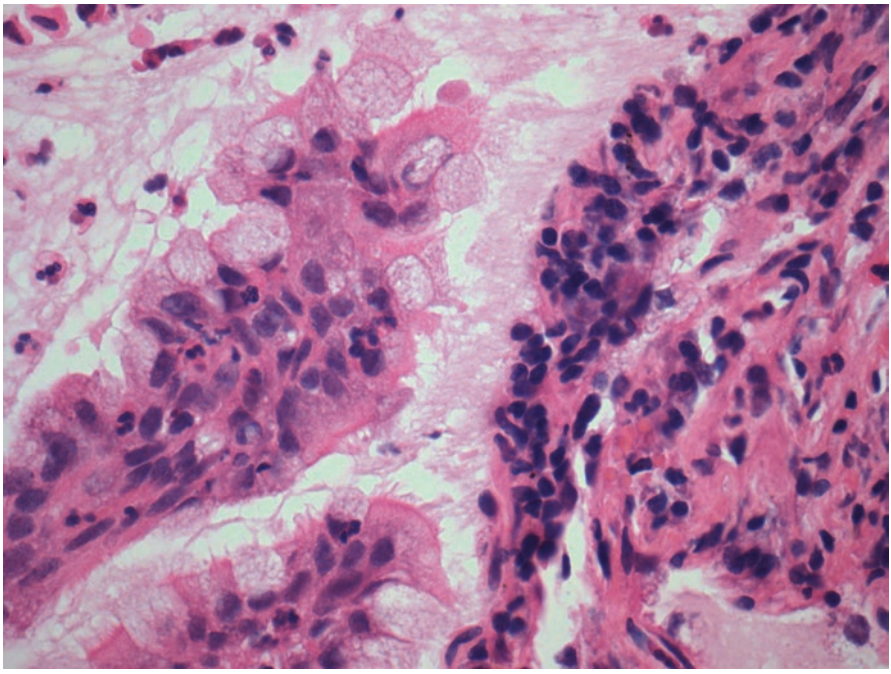

Fig. 17.6 High magnification highlights ciliated cells adjacent to mucinous cells

Squamous Dysplasia and Squamous Carcinoma In Situ

The identification of early preinvasive squamous lesions, ranging from metaplasias, dysplasias (both low and high grades), and carcinoma in situ, has been enhanced by white light bronchoscopic examination, with special tools such as autofluorescence bronchoscopy [57] and narrow band imaging [58] providing enhanced detection. These lesions are associated with cigarette smoking and often heavy tobacco exposure [59]. Lesions can be seen throughout the airways of affected individuals and can be flat or polypoid [60]. They can be erythematous or exhibit leukoplakia. Regression of low-grade dysplasia, the time to progression to invasion, and the frequency by which lesions progress to invasive squamous carcinoma are not known.

The histologic diagnosis of these lesions depends on morphologic assessment of squamous differentiation in metaplasia, characterized by stratified squamous epithelium rather than pseudostratified columnar ciliated epithelium typical of the normal large airway. Within squamous metaplasia, mild nuclear atypia and preservation of surface maturation are typical of low-grade dysplasia, while a thickened epithelium showing dysmaturation with mitotically active cells showing marked nuclear atypia is a characteristic of high-grade dysplasia. Full-thickness nuclear atypia, mitotic activity, disarray, and complete lack of maturation are features of carcinoma in situ (Fig. 17.7). Multiple carcinoma in situ lesions in the same patient harbor the same p53 mutations, suggesting common clonal origin and cellular migration [61].

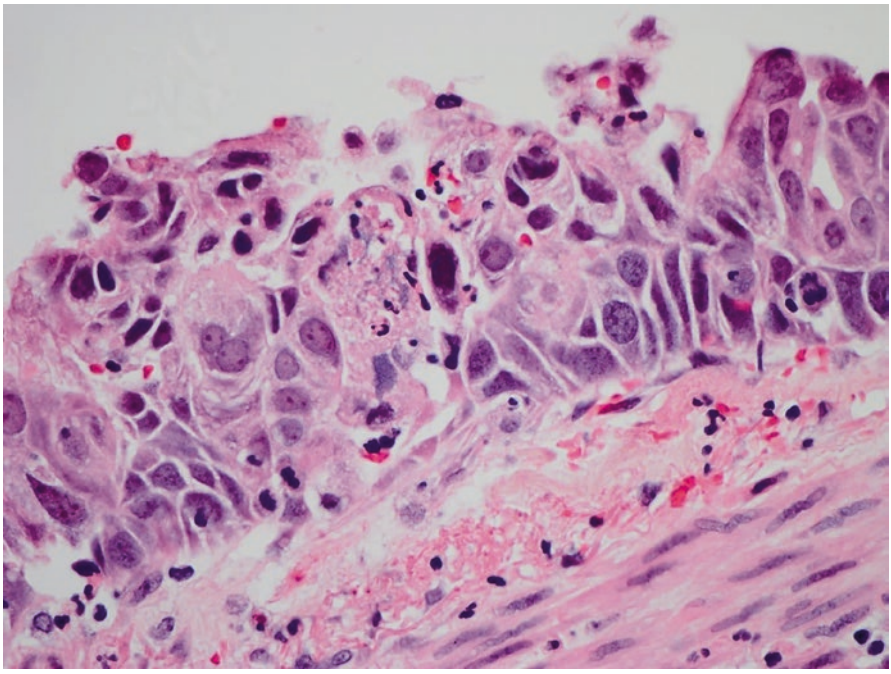

Fig. 17.7 Squamous carcinoma in situ. Squamous epithelium replaces the normal respiratory epithelium, and marked atypia and full thickness dysmaturation are seen. In addition mitotic activity and apoptosis is present

Loss of Heterozygosity and Stepwise Progression of Dysplasia

Allele-specific deletions in 3p were identified in squamous hyperplasia, dysplasia, and squamous carcinoma in situ [62]. Progressive LOH in 3p is seen from low-grade to high-grade dysplasia and is also frequent in carcinoma; both the rate and extent of LOH were seen with increasing grade [63]. LOH was found showing allele-specific losses in 3p, 5q, 9p, 13q, and 17p, with the 3p losses representing earlier events, followed by 9p losses and then 5q, 13q, and 17p [64]. The 3p allele-specific deletions are seen in 76% of hyperplasias [62]. LOH at 9p are also allele specific and start to occur in the squamous hyperplasia histology [62]. This progressive loss includes region 3p21; losses were also described in 5q21 and 9p21 [65]. Progressive LOH at 3p, 5q, 9p, and 17p mutations were seen in high-grade dysplasia [66].

Loss of 3p including the tumor suppressor gene fragile histidine triad (FHIT) was proposed to be important in squamous carcinogenesis [63]. The function of FHIT is complex and includes effects on cell cycle, invasion, and apoptosis. The losses at 9p are likely targeting CDKN2a (p16) and losses at 17p targeting p53.

Acquisition of Invasion

IHC studies have examined MMP levels with decreased MMP and increased MMP3 and 9 from dysplasia to carcinoma in situ [67]. P53 IHC was seen to increase from high-grade dysplasia to carcinoma in situ and microinvasive carcinoma [68]. IHC for EGFR and p53 IHC were seen to increase from dysplasia to carcinoma [69].

Telomerase Activity

Telomerase activity was shown to increase from metaplasia to dysplasia to carcinoma [70]. This was also reported by Lantuejoul and colleagues, and HTERT level was correlated with p53 loss, increased BCL2-to-BAX ratio, and Ki67 increase. Additionally, increased telomere length was seen with carcinoma in situ and squamous carcinoma [71]. Telomere length is shortest at the squamous metaplasia step and increases from dysplasia to squamous carcinoma. This is associated with increase in telomere repeat factors 1 and 3 (TRF1 and TRF2). Other DNA damage response proteins are also increased, including p-ATM, p-CHK2, and p-H2AX.

Cell Cycle Control

Squamous dysplasias were shown to have CDKN2a (p16) loss and gains in cyclin D1 and cyclin E1, but no loss in Rb [72]. This was also reported by Lantuejoul and colleagues, with CDKN2a (p16) loss and cyclin D1 gain [71]. Loss of 9p21 and 1p36 in dysplasia and carcinoma [73] is described. Abnormalities in retinoblastoma pathway were also found [74].

Loss of CDKN2a (p16) by promoter hypermethylation and polycomb complex component BMI-1 overexpression was seen in cell proliferation with severe squamous dysplasia, as measured by MIB-1 [75]. CDKN2a (p16) promoter hypermethylation in smokers was thought to be an early event [76], and CDKN2a (p16) hypermethylation was seen in 17% of hyperplasia, 24% of squamous metaplasia, and 50–75% of carcinoma in situ and invasive squamous carcinoma [77]. The silencing of CDKN2a (p16) and LOH at that locus are mechanisms by which cell cycle control is progressively lost along the RB/CDK4/cyclin D1 axis.

Antiapoptotic Effects

PIK3CA copy number from low-grade to moderate dysplasia to carcinoma in situ was associated with phosphorylated AKT in high-grade dysplasia. It was postulated that prevention of cell death mediated by the phospho-AKT pathway was the result of PIK3CA copy number increase [78]. The antiapoptotic pathway components including p-AKT, p65 RELA, and cIAP-2 were increased in the progression from precursors to bronchial squamous carcinoma [79].

TP53 Loss

Loss of TP53 is a critical event in human malignancy as loss of TP53 has an effect on apoptosis as well as on cell cycle. TP53 mutations, however, increased in frequency from dysplasia to CIS [80]. Mutations in TP53 are seen in high-grade dysplasias [66], and LOH in 17p also targets TP53 as previously noted. The loss of TP53 is likely related to cigarette smoking and may already be detectable in normal-appearing epithelium [81]. The importance of the p53 pathway was further underscored by the work of Masscaux et al. in which it was noted that MDM2 increases were seen from metaplasia to dysplasia. These increases were associated with p14ARF loss or nucleolar localization in lesions with MDM2 increase [82]. The role of miRNA has also been explored with subsequent p53-associated targeting. Reduced Mir32 and Mir34C were seen with progression from normal to dysplasia to carcinoma. Mir15A was downregulated in early dysplasia and is important in regulating apoptosis [83].

EGFR and KRAS Mutations

EGFR and RAS mutations were found to be rare in bronchial squamous dysplasia [84].

Copy Number Alterations and SOX2

Single-nucleotide polymorphism arrays showed multiple regions of chromosomal gains and losses in preneoplastic squamous lesions. These copy number alterations included 3p, 5p, 8p, 9p, and 13q. In addition, loss of RNF20 and SSBP2 and gain of RASGRP3 were also lesions seen in squamous carcinoma, associated with changes in 9q31.1, 2p22.3, and 5q14.1.

Aneuploidy increases from high-grade dysplasia to carcinoma [68] including copy number changes in 5p15.2, chromosome 6, 7p12 (EGFR), and 8p24 (myc) frequent in dysplasia [85]; these changes increased in frequency from low-grade to high-grade dysplasia and to squamous carcinoma.

SOX2 (SRY-BOX 2) is a transcription factor important in maintaining pluripotency [85] but at the same time critical in the commitment of cells toward squamous differentiation [86]. It is important in airway development [87]. Elevated SOX2 and low SOX9 promote squamous non-ciliated differentiation; low SOX2 and high SOX9 encourage regeneration and differentiation toward the ciliated epithelium. In high-grade dysplasia, elevation in SOX2 and PIK3CA is the result of 3q amplification, and SOX9 expression is low [88]. Amplification of SOX2-containing region of chromosome 3q is frequently seen in squamous carcinoma and was studied in earlier lesions. It was not seen in low-grade dysplasia but was identified in all high-grade lesions [89]. This region also contains the gene PIK3CA but in some amplicons only contains SOX2. This was seen in high-grade dysplasias associated with squamous carcinoma [90]. In a three-dimensional organotypic model using bronchial epithelium cells, SOX2 increase, p53 loss, and p-AKT expression all led to transformation to early squamous carcinoma [91].

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Part IV
General and Emerging Fields in Molecular Pathology of Lung Cancer

Chapter 18
Prognostic Biomarkers in Lung Cancer

Sanja Dacic

ALK

The prognostic significance of *ALK* rearrangement in lung adenocarcinoma is controversial. The European Thoracic Oncology Platform Lungscape project has demonstrated better overall survival (OS) in patients with surgically resected lung adenocarcinoma whose tumors were considered ALK positive either by ALK immunohistochemistry or *ALK* FISH [1]. In contrast, study in Asian patients, never smokers, with *ALK*-positive surgically resected lung adenocarcinoma showed worse disease-free survival (DFS) [2]. This sharp difference could be related to the different ethnicity of study population. The prognostic role of *ALK* was also reported in patients with advanced NSCLC who were not candidate for surgical treatment. Patients with *ALK*-positive NSCLC showed improved survival after radiotherapy for brain metastases compared with *EGFR*, *KRAS*, or wild-type tumors. The median OS for *ALK*-positive patients was 26.3 months, while patients with *EGFR*, *KRAS*, or wild-type tumors showed 13.6, 5.7, and 5.5 months of OS, respectively [3]. Subsequent treatment with targeted therapy resulted in further improvement in OS.

BRAF

In contrast to other tumors, non-V600E *BRAF* mutations represent almost 50% of all *BRAF* mutations in lung cancer. The prognostic significance of *BRAF* mutations in lung cancer is still uncertain, because of the limited data. *BRAF* mutations may coexist with other mutations such as mutations in *EGFR, KRAS*, and *PIK3CA* genes.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_18

It has been shown that patients with these coexistent mutations have shorter OS than patients with *BRAF* mutations only [4]. Most of the published studies failed to show any prognostic significance of *BRAF* in NSCLC [5–8].

EGFR

To date, the prognostic value of *EGFR* mutations in NSCLC is controversial. Several studies have shown longer survival in surgically treated patients with *EGFR*-mutated lung carcinomas when compared to *EGFR* wild type, regardless of subsequent treatments [9–12]. Other studies and meta-analysis showed no prognostic value of *EGFR* status in surgically treated lung carcinomas [13–16].

Recently published updates on LUX-Lung3 and LUX-Lung6 trials showed that patients with exon 19 deletion treated with afatinib have a better OS when compared to platinum-chemotherapy subgroup [17]. It has been known from prior retrospective studies and a meta-analysis that all of the EGFR-TKIs are more active in patients with exon 19 deletions than in L858R mutations, but the LUX-Lung studies were the only ones that prospectively showed an OS benefit [18, 19].

The T790M mutations most frequently occur in patients who initially responded to EGFR-TKI treatment but may also occur in EGFR-TKI-naïve patients. The prognostic significance seems to be different depending on the EGFR-TKI treatment status. It has been suggested that patients with pretreatment T790M have shorter PFS when treated with EGFR-TKIs [20–22]. However, other studies showed potential positive prognostic value in post-TKI setting [23, 24].

KRAS

Many retrospective studies reported correlation between *KRAS* mutations and a poor overall survival in patients with resected NSCLC [25]. A meta-analysis of more than 53 retrospective studies identified *KRAS* mutations as a negative prognostic factor [26, 27]. However, a recent pooled analysis including four trials comparing platinum-based adjuvant chemotherapy to observation in early-stage resected NSCLC has shown that *KRAS* mutation status is not significantly prognostic [28].

ROS1

Retrospective studies have shown that ROS1 status has no prognostic impact in Western patients with NSCLC, while study in Asian population suggested a potential negative prognostic value of *ROS1* rearrangement [2, 29].

MET

A high *MET* gene copy number or protein expression has been associated with poor prognosis in patients with surgically resected NSCLC [30, 31].

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Chapter 19
Targeted Therapies for Lung Cancer

Asmita Patel and Eric H. Bernicker

Introduction

For many years, patients with advanced non-small-cell lung cancer (NSCLC) had poor prognoses because the disease did not have screening available and had a minimal response to available anticancer therapies. However, in the last 10 years, these patients' outlook has improved due to two major therapeutic developments: the discovery that immunotherapy could achieve significant clinical responses in some patients and the identification of specific driver mutations that could be targeted by oral agents. This chapter will review clinical advances in the latter.

NSCLC is one of the most genomically diverse cancers, regardless of the histological subtype. This diversity creates immense challenges with respect to therapeutic options. Lung cancer is not a single uniform disease. On the molecular level, NSCLC can be stratified into discrete mutational subtypes that can be treated using different therapeutic approaches. One proof-of-principle example is the identification of a gain-of-function mutation in the tyrosine kinase-activating epidermal growth factor receptor (EGFR). This mutation is the best predictor for tumor response and overall survival following the administration of one of several FDA-approved EGFR inhibitors.

Biology

The perspective on NSCLC treatments has changed because of recent tumor biology studies. NSCLC cases can be subdivided into multiple molecular-level categories with variable prevalences [1]. Recent publications from the Cancer Atlas

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_19

initiative and other groups have revealed significant tumor heterogeneity [2, 3]. The College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology has published guidelines for molecular testing of NSCLC. They recommend testing all advanced adenocarcinomas of the lung for mutations in EGFR, translocations in anaplastic lymphoma kinase (ALK), and gene rearrangements in ROS1 regardless of clinical characteristics [4]. Testing for EGFR mutations and ALK translocations should also be considered for some patients with squamous cell histology, particularly among young patients with a light or nonsmoking history or a biopsy showing mixed histology.

Epidermal Growth Factor Receptor

EGFR is a member of the human epidermal growth factor receptor (HER)/erbB family of growth factor receptors [5]. These receptors are anchored to the cytoplasmic membrane and share a similar structure. They have an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and an intracytoplasmic tyrosine kinase domain. Ligand binding activates receptor dimerization and tyrosine kinase autophosphorylation, which initiates an intracellular signaling cascade that activates multiple downstream receptor pathways. Activation of the RAS/RAF and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways and transcription of their target genes leads to increased cell proliferation, angiogenesis, metastasis, and decreased apoptosis [6].

The presence of EGFR mutations is both a prognostic and predictive indicator [7]. EGFR mutations (detected using gene sequencing) hyperactivate EGFR, rendering the cancer cell dependent on EGFR for survival and progression. In NSCLC, activating mutations mostly occur in exons 18–21 [8]. The classic mutations, which occur in exon 19 and 21, account for 90% of EGFR mutations. Exon 19 mutations are commonly in-frame deletions of amino acids 747–750. Exon 21 mutations typically cause an L858R substitution. EGFR status at diagnosis is often mutually exclusive of aberrations in KRAS and ALK. It is also associated with certain clinicopathologic features, including a never or light smoking history, female sex, and Asian ethnicity.

Anaplastic Lymphoma Kinase

The ALK gene, which encodes a tyrosine kinase, was first identified in a subset of anaplastic large-cell lymphomas. In 2007, Soda and colleagues first identified the echinoderm microtubule-associated protein like 4 (EML4)-ALK fusion from a Japanese patient with lung adenocarcinoma [9]. The fusion is caused by an inversion on the short arm of chromosome 2 (p21p23) that joins exon 1–13 of EML4 to

exons 20–29 of ALK [10]. The fusion activates the tyrosine kinase of ALK and drives downstream pathways that lead to cell proliferation and survival. Estimates indicate that 2–7% of NSCLC cases could harbor this oncogenic driver. ALK can also fuse with other partners, such as tropomyosin receptor kinase or kinesin family member 5B (KIF5B). These fusions have been described in lung cancer but are less common than EML4-ALK [11, 12].

Immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and polymerase chain reaction (PCR) can all be used to identify ALK rearrangements. Of these, FISH is used most frequently in the clinic. However, it is increasingly apparent that IHC produces equivalent results while being cheaper and faster than FISH [13, 14]. EML4-ALK translocations are associated with certain clinicopathologic features, including young, never, or light smoking patients with adenocarcinoma, specifically those with signet ring-subtype histology [15, 16].

ROS1

ROS1 gene rearrangements are oncogenic drivers that are present in approximately 1–2% of NSCLC tumors [17]. ROS1 is a receptor tyrosine kinase of the insulin receptor family. Chromosomal rearrangements involving the *ROS1* gene were originally described for glioblastoma. *ROS1*, located chromosome 6q22, is fused to the adjacent gene, fused in glioblastoma (*FIG*). ROS1 fusions were later identified as potential driver mutations in NSCLC cell lines (i.e., HCC78, SLC34A2-ROS1). These fusions have constitutive kinase activity. Similar to many oncogene-addicted lung cancers, tumors with ROS1 rearrangements commonly occur in young nonsmokers with lung adenocarcinoma histology.

BRAF

BRAF is a proto-oncogene encoding a serine/threonine protein kinase that promotes cell proliferation and survival [18]. BRAF lies downstream of RAS in the RAS-RAF-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway and is a key molecular cascade in cellular growth. BRAF mutations account for 2–4% of NSCLC tumors [19]. Unlike melanoma, in which the majority of BRAF mutations are V600E, only approximately 50% of NSCLC cases harbor a V600E mutation. Other documented mutations include K601 N, L597Q, and G469 V. Similar to EGFR mutations and ALK fusions, BRAF mutations are generally exclusive of other driver mutations. However, unlike EGFR mutation and ALK rearrangements, BRAF mutations are common in patients who are current or former smokers. Clinically, BRAF-mutated lung cancers tend to have a worse prognosis, and many patients with early-stage BRAF-mutated lung cancer develop secondary tumors with KRAS mutations [20].

KRAS

KRAS mutations are among the earliest mutations discovered in human cancers and are the most commonly found mutation in lung cancer adenocarcinomas. They are observed in up to 30% of patients. Unfortunately, there are no KRAS-specific therapies at this time. These mutations are often associated with smoking, although approximately 5% of nonsmokers can have KRAS mutations. Activated KRAS can drive a number of downstream cellular pathways, including the MEK pathway. A number of MEK inhibitors have been studied to see if it is beneficial to block the activated MEK pathway; however, the drug was not effective in a recent clinical trial [21]. It may still be worthwhile to test biopsy specimens for KRAS mutations, because an EGFR-, ALK-, and ROS-negative result with a KRAS-positive result would eliminated the need for further driver mutation testing.

Human Epidermal Growth Factor Receptor-2

Her-2 mutations are seen in approximately 1–2% of lung adenocarcinomas. 97% of the time, when present they are found in female patients with a light or never smoking history [22]. Unlike the Her-2 mutations seen in breast cancers, the activating mutations in lung cancer patients are exon 20 insertion mutations. Gene amplification and overexpression do not appear to be causative factors for lung cancer tumorigenesis, and the exon 20 insertion is usually mutually exclusive of other driver mutations.

MET

MET is an attractive target for lung cancer therapeutic development; however, molecular clues as to which patients would benefit from this therapy have only recently been identified. The *MET* gene is found on chromosome 7. It codes for a receptor tyrosine kinase that binds hepatocyte growth factor [23]. Several mechanisms can lead to MET activation, including ligand binding, amplification/overexpression, mutation, or decreased degradation [24].

Recently, the MET exon 14 skipping mutation was shown to identify patients that are potentially responsive to the oral MET inhibitor crizotinib. It is thought that the deletion of the juxtamembrane domain containing the E3-ubiquitin ligase (CBL)-binding site leads to decreased turnover of aberrant MET protein [25]. MET exon 14 skipping-positive patients tend to be older than patients with EGFR- or KRAS-positive lung cancer. Studies showed that 68% of MET exon 14 skipping-positive patients were woman, and approximately one-third were nonsmokers [26].

RET

The RET proto-oncogene is a tyrosine kinase receptor that binds growth factors of the glial-derived neurotrophic factor family. RET rearrangements constitute a molecular subset of NSCLC [27]. Like ALK, there are several fusion partners that have been identified, including KIF5B, coiled-coil domain containing 6 (CCDC6), tripartite-motif containing 33 (TRIM33), and nuclear receptor coactivator 4 (NCOA4) [28]. Approximately 1–2% of adenocarcinomas have RET translocations; however, the frequency is increased in patients who never smoked, especially when EFGR is wild type [29]. RET rearrangements are often observed in patients with an earlier spread to nodal disease and more aggressive tumors.

Treatment

EGFR Inhibitors

There are two primary approaches for targeting EGFR: tyrosine kinase inhibitors (TKIs) that cross the membrane then bind and inhibit the intracellular tyrosine kinase domain (e.g., erlotinib, gefitinib, afatinib) and monoclonal antibodies that bind the e xtracellular domain and interfere with receptor function and activation. Gefitinib and erlotinib were the first TKIs developed. They are reversible inhibitors that compete with ATP to bind the tyrosine kinase domain of EGFR. Gefitinib was approved by the FDA in 2003 as a monotherapy for the treatment of patients with stage IIIB–IV NSCLC after failure of both platinum-based and docetaxel chemotherapies. Median duration of response was 7.0 months (range, 4.6–18.6 months). Later, a large randomized trial, the ISEL trial, failed to show any survival benefit compared with best supportive care. The median duration of response with gefitinib was 5.6 months compared with 5.1 months for placebo and best supportive care [30]. After approval, gefitinib was studied to evaluate the benefit of adding it to chemotherapy as a first-line treatment for stage IIIB–IV NSCLC [31]. In the INTACT-1 trial, 1093 patients were randomized to placebo, 250 mg gefitinib/day, and 500 mg gefitinib/day combined with up to six cycles of cisplatin and gemcitabine. No differences in objective response rate (ORR), progression-free survival (PFS), or overall survival (OS) were found between the three arms after a median follow-up of 15.9 months. OS was 10.9, 9.9, and 9.9 months for the placebo, 250 mg gefitinib/day, and 500 mg gefitinib/day groups, respectively ($p = 0.46$).

In 2004, Lynch et al. published a study showing that clinical responsiveness to TKIs was correlated with the presence of activating mutations in the EGFR gene [32]. The same year, the FDA approved erlotinib following a study showing a significant survival benefit with drug administration. The BR21 study investigated the use of erlotinib as a second- or third-line treatment following progression on

chemotherapy. The results showed an OS benefit of 6.7 months for the erlotinib group compared with 4.7 months for placebo group ($p < 0.001$) [33]. In the subsequent TRIBUTE study, erlotinib was studied as a first-line treatment in patients with stage IIIB–IV NSCLC. Participants did not show any improvement in survival or overall response rates. OS was 10.6 months for the erlotinib group compared with 10.5 months for the placebo group [34]. Despite the overall negative findings in this study, erlotinib-treated patients who never smoked, were younger, were female, and had adenocarcinomas had a median survival of 22.5 months compared with 10.1 months for placebo-treated patients who were prior/current smokers. Thus, patients with an EGFR mutation and those who never smoked were identified as patients that could significantly benefit from the treatment with EGFR inhibitors.

To further verify these results, the IPASS study was conducted to compare outcomes of treatment with gefitinib versus that of treatment with carboplatin/paclitaxel [35]. The inclusion criteria were East Asian patients who never smoked or were former light smokers and had stage IIIB–IV lung adenocarcinoma. In the gefitinib arm, there was a PFS benefit at 12 months, with a PFS of 24.9% for the gefitinib arm compared with 6.7% for the carboplatin/paclitaxel arm ($p < 0.001$). A better ORR was also associated with gefitinib (43% vs. 32.2% for the carboplatin/paclitaxel arm, $p < 0.001$). Among 437 patients with evaluable EGFR mutation data, 261 had an EGFR mutation. Of these 261 patients, 53.6% had exon 19 deletions, and 42.5% had a missense mutation at exon 21. Among patients with activating EGFR mutations on exon 19 or 21, ORR increased notably in patients who received gefitinib compared with those who received chemotherapy (71.2% vs. 47.3%, respectively, $p < 0.001$).

In 2015, the FDA approved gefitinib as a first-line therapy for metastatic EGFR-mutated NSCLC based on the IFUM study in which chemotherapy-naïve patients with NSCLC were administered gefitinib. The primary end point of that study was objective response [36]. For patients with exon 19 deletions and exon 21 L858R substitutions, ORRs were 50% and 70%, respectively, and the median duration of response was 6 months and 8.3 months, respectively. The IFUM study results were supported by the IPASS study which randomized East Asian light or never smoker patients between gefitinib and chemotherapy with paclitaxel and carboplatin. That trial showed a PFS benefit with gefitinib treatment. As patients who were initially treated with chemotherapy were allowed to cross over to gefitinib upon progression, no overall survival was demonstrable in the gefitniib group. Similar results were observed for erlotinib as a first-line therapy in EGFR-mutated lung cancer [37]. In 2013, the FDA approved erlotinib as a first-line treatment for cases of metastatic NSCLC with EGFR exon 19 deletions or exon 21 L858R substitutions based on the EURTAC trial. In that study, a PFS of 10.4 months was observed for the erlotinib group compared with 5.2 months for the chemotherapy group ($p < 0.001$, hazard ratio [HR] 0.34).

The approved dose of gefitinib is 250 mg daily. The most common adverse events include rash (45% of cases), diarrhea (31%), vomiting (13%), and asthenia and dry cough (10%). In rare cases, interstitial lung disease and pneumonitis have been

observed. The approved dose of erlotinib is 150 mg daily. The most common adverse events are similar to those of gefitinib.

Afatinib is another FDA-approved first-line therapy for metastatic adenocarcinoma of lung. Afatinib is a TKI that irreversibly binds the intracellular domain of EGFR and Her-2. The approval was based on the LUX-Lung 3 study in which patients were randomized to either the 40 mg afatinib daily arm or chemotherapy arm [38]. The primary end point was PFS. The median PFS for the afatinib arm was 11.1 months compared with 6.9 months for the chemotherapy arm ($p < 0.001$, HR 0.58). No OS benefit was observed, but there was a difference in ORR (50.4% for the afatinib arm vs. 19.1% for the chemotherapy arm). The most common adverse events were diarrhea (70% of cases), rash (70%), stomatitis (30%), and decreased appetite (25%). Afatinib can also be used as a second-line agent for metastatic squamous cell carcinoma based on the LUX LUNG 8 study. That study showed a 19% improvement in OS and a significant 18% improvement in PFS in the afatinib arm [39].

Despite the initial dramatic response to EGFR inhibitors among patients who harbor EGFR mutations, drug resistance generally develops within 12–18 months. One mechanism of resistance is the development of an *EGFR* c.2369C > T point mutation (T790 M) that hinders TKI binding and alters ATP handling in approximately 60% of the cases [40, 41]. Secondary resistance can also result from overexpression of c-MET or Her-2. In addition, approximately 5% of patients who experience disease progression after frontline TKIs are used undergo a small-cell transformation. Those transformed cells retain the initial EGFR truncation. Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations and *HER2* amplification are additional mechanisms of resistance [42].

The T790 M mutation is rarely detected at diagnosis in cases of lung cancer. Recently, the third-generation TKI osimertinib was approved for patients with EGFR-mutated adenocarcinoma who develop acquired resistance via a T790 M mutation following first-line targeted therapy. The AURA-2 study showed that the ORR was 51% and the median duration of response was 12.4 months for patients with a T790 M mutation who were treated with osimertinib [43]. In that study, common adverse events were diarrhea (42% of cases), rash (41%), dry skin (31%), nail toxicity (25%), eye disorders (18%), and prolonged QTc. (0.2%).

Mok and colleagues published the results of a randomized study of patients with a confirmed T790 M mutation who had cancer progression following first-line TKI therapy. Patients were assigned in a 2:1 ratio to the osimertinib group or the chemotherapy group, which was treated with either a combination of pemetrexed and carboplatin or cisplatin [44]. The median PFS was higher for the osimertinib group (10.1 months vs. 4.4 months for the chemotherapy group, HR, 0.30). The observed response rate was also higher (71% compared with 31% for the chemotherapy group). The percentage of patients with CNS disease who responded to osimertinib was also higher than that of the chemotherapy group (8.5 months vs. 4.2 months, respectively). The toxicity of osimertinib was generally mild with statistically fewer grade III adverse events compared with that of chemotherapy (23% vs. 47%, respectively).

Given the inevitable acquisition of TKI resistance and the clinical need to develop new active agents targeting resistant mutations, it is very important to perform repeated molecular studies of patients. Many patients with advanced lung cancer are willing to undergo repeat biopsies [45]. However, these procedures are invasive, and there is no guarantee that molecular studies will produce a useable result. Approximately 30% of genomic assays fail. This issue has increased the interest in "liquid biopsies." These tests are advantageous because they are blood- or urine based, are less invasive, and often have a faster turnaround time [46].

Oxnard and colleagues recently compared serum and tissue genotyping of samples acquired from an osimertinib clinical trial [47]. Cell-free serum DNA was assessed and compared with the results of tissue genotyping from a central laboratory. The sensitivity of serum testing was 70%. Among 58 patients who were negative for T790 M based on tissue genotyping, 31% had detectable T790 M mutations upon serum genotyping. The ORRs were similar regardless of whether the T790 M was detected in tissue or blood (62% vs. 63%, respectively). Further validation is needed; however, the success of this cell-free assay suggests that it could be used as an initial test once radiographic progression has been documented. This evaluation scheme would allow clinicians to limit invasive biopsies to those patients with negative cell-free assay results. At this time, there is insufficient evidence to recommend serum monitoring for patients with oncogene-addicted lung cancers in a manner similar to that of patients with chronic myeloid leukemia.

In contrast to EGFR TKIs, the effect of anti-EGFR monoclonal antibodies is not directly associated with any EGFR mutations. Two phase III clinical trials investigating anti-EGFR monoclonal antibodies have been performed. The BMS099 trial randomly assigned patients into two groups: carboplatin/paclitaxel with cetuximab and carboplatin/paclitaxel without cetuximab. In this trial, no significant OS benefit was observed (9.7 months for the with cetuximab group vs. 8.4 months for the without cetuximab group, $p = 0.17$). The FLEX trial investigated the administration of cisplatin/vinorelbine with or without cetuximab in patients who were EGFR mutation positive. A significant difference in OS benefit was observed in the with cetuximab group compared with the without cetuximab group (11.3 months vs. 10.1 months, respectively, $p = 0.04$) [48]. It is unclear whether the different results seen in these trials were related to inclusion criteria or chemotherapy regimen. Another anti-EGFR antibody, necitumumab, showed modest activity in cases of stage IV squamous cell carcinoma. In the randomized SQUIRE trial, patients were administered cisplatin/gemcitabine either with or without necitumumab. There was a modest improvement in OS (11.5 months vs. 9.9 months in the with and without necitumumab groups, respectively; HR 0.84; $p = 0.012$) and PFS (HR 0.85, $p = 0.02$). No difference in ORR was observed (31% vs. 29%) in the with and without necitumumab groups, respectively; $p = 0.4$) [49]. The most common adverse events with cetuximab are infusion reactions (3% of cases), dermatologic toxicity (3%), fever (5%), and diarrhea (6%).

ALK and ROS1 Inhibitors

Crizotinib is a first-generation oral small-molecule inhibitor targeting ALK, ROS, and MET tyrosine kinases. In 2011, the FDA approved crizotinib for ALK-mutated adenocarcinoma of the lung. Crizotinib showed an approximately 60% ORR response rate in phase I and phase II trials [50]. These results were confirmed by the PROFILE 1007 trial investigating crizotinib as a second-line therapy for patients with ALK-positive NSCLC. This phase III trial showed significantly higher response rates for the crizotinib group compared with the chemotherapy group (65% vs. 29%, respectively). There was also significant improvement in PFS (7.7 months vs. 3 months for the crizotinib group and chemotherapy group, respectively, $p < 0.001$) [51]. Moreover, crizotinib was shown to be superior to standard platinum/pemetrexed chemotherapy in the PROFILE 1014 trial in which patients were treated with chemotherapy or crizotinib [52]. In that study, the ORR of the crizotinib arm was 74% compared with 45% for the chemotherapy arm. In addition, PFS was significantly improved in the crizotinib arm compared with the chemotherapy arm (10.9 months vs. 7 months, respectively; $p < 0.001$; HR 0.45). The most common adverse events with crizotinib are visual disturbances (60% of cases), diarrhea (60%), nausea and vomiting (50%), and constipation (40%).

Similar to EGFR TKI therapies, the dramatic treatment response is not sustained due to the development of drug resistance. The resistance mechanism can be ALK dependent (e.g., the development of the L1196M gatekeeper or C1156Y mutation, an increase in the number of ALK fusion copies) or ALK independent (e.g., through the activation of other bypass agents such as EGFR and KRAS) [53]. In addition, recent studies indicate that the spectrum of secondary mutations varies depending on the TKI used. For example, the ALK G1202R mutation is more commonly observed following therapy with a second-generation ALK inhibitor [54]. Thus, repeated sampling of a patient's genetic markers as they progress through various therapies will allow us to better understand the mechanisms underlying resistance and improve treatment planning.

Ceritinib and alectinib are both approved for patients with cancer progression following first-line therapy with crizotinib. There are no companion diagnostics required for their selection. Ceritinib is an oral ATP-competitive TKI that is 20-fold more potent than crizotinib. Unlike crizotinib, ceritinib does not have anti-MET activity. In 2014, the FDA approved ceritinib based on the ASCEND trials, two phase II trials on the use of ceritnib as a second-line therapy for ALK-positive NSCLC. In the trial, there was a response rate (RR) of approximately 58% overall, and the RR in CNS disease was 45%. For patients who progressed on crizotinib, RR was 56%, and PFS was 7 months [55]. Ceritinib has also been studied as a first-line therapy in ALK-rearranged lung cancer, compared against pemetrexed-based combination chemotherapy [56]. The PFS in the ceritinib group was 16.6 months and 8.1 months in the chemotherapy group, and the ORR was 72.5% versus 26.7%. Importantly, the duration of response was much improved with ceritinib: 24 versus 11 months.

The most common adverse events seen with ceritinib are diarrhea (80% of cases) and nausea and vomiting (50%).

Alectinib is another second-generation TKI. It is a highly selective ALK inhibitor. In two phase II trials, alectinib showed an RR of 50% and a PFS of 6.3 months as a second-line treatment in ALK-positive NSCLC [57]. Phase III trials are currently ongoing. In addition, data from the J-Alex trial was presented recently. In that study, alectinib was compared with crizotinib in treatment-naïve Japanese patients [58]. Alectinib showed a significant improvement over crizotinib with respect to PFS (PFS not reached vs. 10 months, respectively) and was less toxic. Encouragingly, alectinib also showed significant intracranial activity and activity in patients who had progressed on crizotinib and ceritinib [59].

Crizotinib is the FDA-approved agent for ROS1 rearrangements. In a phase I study of patients with advanced NSCLC harboring a ROS1 translocation, the ORR of the crizotinib arm was 72% with a median duration of response of 17.6 months and a PFS of 19.2 months [60]. Because this mutation is rare, phase III studies are not feasible. Similar to other targeted therapies for oncogene-addicted lung cancers, acquired resistance is an issue, and the molecular causes of resistance are currently being elucidated. Mutations in the ROS1 kinase, EGFR activation, and epithelial-to-mesenchymal transitions have all been implicated in drug resistance [61]. Some early studies have suggested that cabozantinib can overcome acquired resistance in patients who have progressed following crizotinib therapy [62].

Other Evolving Targets

BRAF Inhibitors

The FDA approved targeted therapy for BRAF V600E mutated lung carcinomas in June 2017, based on emerging data of strong clinical efficacy of combination therapy. Planchard et al. recently published their experience treating BRAF V600E-mutated patients with stage IV lung cancer that progressed following cisplatin-based chemotherapy with oral dabrafenib and trametinib. This treatment regimen was modeled after that used for BRAF V600E-positive melanoma. In patients with BRAF V600E-positive melanoma, this regimen is superior to monotherapy [63]. In the study of BRAF V600E-positive NSCLC, patients with progressive disease following cisplatin-based chemotherapy were enrolled. Patients with brain metastases could be included if the disease was untreated and asymptomatic or the patient had received radiation therapy more than 3 weeks prior to the start of the study. An investigator-assessed response was achieved in 63% of patients, and the median PFS was 9.7 months. Overall, the combination was well tolerated; however, 56% of patients had grade III adverse events, such as neutropenia, hyponatremia, and anemia. This clinical activity is especially noteworthy because the response of BRAF-mutated lung cancer to second-line therapy is very poor, generally 3 months or less.

MET Exon 14 and High-Level MET Amplification

As mentioned above, targeting MET is an attractive therapeutic strategy, but to date, the development of effective therapeutics has been elusive. One trial investigated the addition of the monoclonal antibody onartuzumab, which blocks the extracellular domain of MET, to erlotinib therapy in patients with advanced lung cancer that overexpressed MET. Unfortunately, this trial was stopped for futility [64]. However, the identification of the MET exon 14 skipping mutation, which is often mutually exclusive of other driver mutations, has identified a cohort of patients who seem more likely to respond to targeted MET inhibition and reignited interest in selecting patients for treatment with MET inhibitors. Studies of this therapy are still in early stages; however, several case reports have demonstrated that patients with the MET exon 14 skipping mutation had clinically meaningful responses to crizotinib [26, 65]. Other studies have indicated that patients with high-copy number gain-of-function MET lacking exon 14 skipping and those with high-level MET-amplified tumors could also respond to crizotinib [66, 67]. Clearly, the efficacy of targeted therapies will improve as we better define targets that predict treatment response. Lastly, Gainor et al. reported on a patient with an EGFR mutation who acquired drug resistance due to MET amplification. When crizotinib was added to the treatment regimen, the patient had a significant response [68].

RET

Thus far, therapy responses in patients with RET translocations seem to be lower than those of patients with other driver-mutated lung cancers. However, there is emerging data that many of these patients can achieve meaningful clinical responses with targeted [69] agents. A recent study by Drilon et al. investigated the use of the oral targeted therapy cabozantinib to treat patients with RET-mutated lung cancer [70]. Of the 26 patients treated, 16 had a KIF5B-RET fusion. The ORR of the cabozantinib group was 26%, the median PFS was 5.5 months, and the OS was 9.9 months. The data indicated that the KIF5B fusion was more sensitive to treatment; however, the total number of patients was low. Thus, it is still too early to draw conclusions regarding the differential sensitivity of various fusion partners to targeted therapy. Notably, while cabozantinib is not cytotoxic, nearly 50% of patients had to reduce their dose due to significant adverse events. Yoh et al. treated patients with RET fusions with vandetanib, a multi-targeted kinase inhibitor [71]. In that study, the ORR of vandetanib-treated patients was 53%, and the median PFS was 4.7 months. Further work is needed to determine if different fusion partners have different sensitivities and if combinations of targeted therapies can achieve higher or more prolonged RR.

Her-2

Initial trials using a combination of chemotherapy and trastuzumab, a monoclonal antibody targeting Her-2, were disappointing. Gatzemeier et al. performed a randomized study comparing treatment with cisplatin and gemcitabine either with or without trastuzumab in patients with Her-2-positive lung cancer. There was no apparent benefit to adding trastuzumab [72]. However, those patients were selected based on Her-2 gene amplification and not exon 20 deletion. In a retrospective cohort study, Mazieres et al. reviewed the clinical features of 101 patients identified to have n exon 20 insertion. They reported responses to treatment with trastuzumab and afatinib, an oral pan-Her-2 inhibitor, and prospective trials are now underway [73].

Targeted Therapy for Non-adenocarcinomas

Compared with adenocarcinomas, squamous lung cancers and small-cell lung cancers are both lacking in identifiable molecular targets that can be exploited. The lack of clinical efficacy led the SWOG network to launch the Lung-MAP master protocol. This clinical trial allows researchers to assemble biomarker-selected subgroups for testing with multiple study drugs and has the flexibility to open or close trials based on signs of efficacy [74].

After years of stalled therapeutic progress, there are indications that small-cell lung cancer could have new agents on the horizon. Rovalpituzumab tesirine is a novel antibody-drug conjugate targeting delta-like protein 3 (DLL3), which is expressed in 80% of small-cell carcinomas [75]. Early dose-finding trials have showed manageable toxicities, such as thrombocytopenia, pleural effusions, and elevated lipase, in a small number of patients. Importantly, 11 of 60 patients with cancer progression after cisplatin-based therapy (18%) responded to second-line therapies. The RR was 38% in patients with greater than 50% DLL3 expression, a group of patients that are notoriously difficult to treat. Thus, this therapy could address an unmet need for therapeutic options in this patient population. Further trials are needed to confirm that the RRs are reproducible and that DLL3 is a viable target for other therapeutic approaches.

Conclusion

The significant advances in our understanding of the genomic drivers behind many lung adenocarcinomas have opened up a new frontier in targeted therapeutics. While not curative, these treatments tend to be well tolerated and can significantly prolong survival with a reasonable quality of life. As we continue to unravel the causes of

acquired drug resistance and develop therapeutics that address KRAS-mutated lung cancer, more advances are within reach. These developments provide options that will benefit patients and their families.

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Chapter 20
Immunotherapy and Lung Cancer: Programmed Death 1 and Its Ligand as a Target for Therapy

Keith M Kerr and Gavin M Laing

Immunotherapy is set to transform the landscape of treatment for lung cancer [1–6]. It is now regarded as an alternative, standard-of-care therapy for patients with advanced stage non-small cell lung cancer (NSCLC), alongside chemotherapy and molecular targeted therapy, the latter a major focus of this book. Radiotherapy may potentiate the effects of chemotherapy, and there are ongoing trials looking at a possible role for immunotherapy in neoadjuvant or adjuvant settings in combination with surgery for early-stage disease.

But this revolution in therapy looked for a long time as if it would never happen. Immunotherapy in lung cancer has a chequered history; global immunostimulation, using BCG or cytokines, and vaccine therapy have not, so far, been successful, which led to a widely held view that immunotherapy in lung cancer would not work [2, 5]. Ipilimumab, a drug targeting an inhibitory immune checkpoint interaction between CTLA4 and CD80 or CD86 [7], and which is a successful treatment in melanoma, has been less successful as a single agent in advanced NSCLC [3, 8]. There may, however, be a role for CTLA4 blockade in lung cancer, in combination with other therapy. Targeting an alternative inhibitory immune checkpoint interaction, between programmed death 1 (PD-1) and its ligand (PD-L1), has been an altogether different story [2, 4, 6].

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_20

Rationale for PD-L1 as a Therapeutic Target

Lung cancer is one of the most highly mutated of solid tumours; many cases have a high mutational burden [9, 10]. This is most likely due to the importance of tobacco carcinogenesis in the development of a majority of lung cancers. It is well known that a genetic 'smoking signature' is very common in squamous cell carcinoma of the lung [11] and that adenocarcinomas which arise in smokers are generally more heavily mutated when compared to those developing in never smokers [12]. It is hypothesised that this high mutational load is likely to mean that there will be many more neoantigens which could potentially be presented on the surface of tumour cells, making these cells appear as 'non-self' to the immune system and therefore the possible target of an immune response [13]. In order to be visible to the immune system, such neoantigens must be presented on the surface of tumour cells in conjunction with MHC molecules. Antigens presented to the immune system by antigen-presenting cells in conjunction with MHC class 1 molecules assist the development of specific CD8+ cytotoxic T cells and with MHC class 2 molecules allow the development of humoral immunity via the formation of CD4+ T-helper cells. Similarly, for specific, primed T cells to recognise neoantigens on the surface of tumour cells, those molecules should be presented in conjunction with MHC class 1 molecules [14, 15]. Perhaps 10% of human tumours, including lung cancers, mutations or other genomic changes, lead to failure of MHC molecule function, and therefore, any neoantigens present are essentially invisible to the immune system [5, 15]. This essentially describes the difference between antigenicity (foreign proteins exist in the tumour cells) and immunogenicity (these proteins are functionally presented on the cell surface and are 'visible' to the immune system). In most cases, however, the neoantigens are appropriately presented; the tumour cells are therefore immunogenic and 'non-self'. This is believed to be a mechanism through which immune surveillance and immune editing take place; immunogenic clones of potentially malignant cells may be recognised and eliminated by the immune system before a clinically evident tumour can develop [16–21].

Clearly, however, for clinically evident tumours to develop, some mechanism, or mechanisms, must be in play to negate or otherwise avoid the immune system, in those cases which are immunogenic. For those clones which are not immunogenic, their ability to escape the immune system is implicit. For immunogenic tumours, there are many potential mechanisms including the presence of immune suppressor cells, such as T regulatory cells (T-regs) or myeloid-derived suppressor cells (MDSC) in the tumour microenvironment, inhibitory cytokines and the complex system of immune regulatory checkpoints—a system of cell membrane-bound receptors and ligands which can up- or downregulate the immune response at various points in the complex series of cell-cell interactions [5, 7, 20, 21]. Immune inhibitory checkpoints are an important physiological mechanism playing a role in the avoidance of autoimmunity. Tumours are thought to adopt some of these mechanisms to effectively induce tolerance to their neoantigen load and thus allow the tumour to grow and develop, untouched by a specific immune response, the capacity

for which has developed, but is prevented from acting on the tumour. One such immune-inhibitory checkpoint, which appears to be important in some lung cancers, as well as in urothelial and some head and neck cancers, is the interaction between PD-1 receptor, found on the surface of many immune effector cells including CD8+ cytotoxic T cells and macrophages, and its ligand PD-L1, which is expressed on some activated immune cells but also on tumour cells [7, 20, 21]. The interaction of PD-1 on the surface of cytotoxic T cells and PD-L1 on tumour cells leads to inhibition of an existing, primed T-cell response to tumour antigens. This involves negative regulation of signalling through the *RAS-RAF-MEK* and *PI3K-AKT-mTOR* pathways, thus leading to inhibition of the various processes leading to T-cell survival, proliferation, activation and cytokine production [22].

Several pieces of evidence appear to support the importance of PD-1-PD-L1 interactions in lung cancer. PD-L1 is commonly expressed on the surface of lung cancer cells; around 25–30% of cases express PD-L1 on the surface membrane of a majority of the tumour cells, about 20% of cases express the marker in a minority of cells and the remainder are negative [23, 24]. Given the vagaries of immunohistochemistry (IHC) techniques used to demonstrate PD-L1 protein expression, these figures are approximate and will vary, depending on the assay used. Although being far from an absolutely exclusive relationship, there is a tendency for tumours with high expression of PD-L1 on tumour cells to have low expression on tumour-infiltrating immune cells and vice versa [25]. Several studies have related PD-L1 expression in tumour cells to prognosis. In a majority of studies published, PD-L1 expression at 'high' levels is associated with relatively poor post-operative survival, in cases where adjuvant therapy was not given. This relationship was confirmed in a recent meta-analysis [26]. This is not, however, a universal finding, and in some studies, no effect has been shown, and in some the opposite effect is found, in which higher PD-L1 expression is associated with a good prognosis [27]. It is very difficult to rationalise these opposing findings. Variation may be partly explained by marked study heterogeneity, and the wide range of anti-PD-L1 IHC clones and assays that were used, with no standardisation. Different definitions of a 'high-expressing' group were used. Follow-up and post-operative survival data will have been assessed differently.

Intuitively, it makes some sense that high PD-L1 expression might be a poor prognostic factor. It is well established that in resected primary lung cancer, an active intra-tumoural immune response, which is associated with tumour cell degradation, is associated with a better-than-average post-operative survival [28–31]. A mechanism which is active in switching off this beneficial immune response might be responsible for perpetuating tumour cell survival and lesion growth and progression. It is much harder to explain why a high PD-L1 expression levels might be a good prognostic factor. In the setting of advanced disease, there is very limited data and no significant effect on prognosis. In this situation, however, outcomes are confounded by a number of issues, including the advanced nature of the disease, possible immune system compromise and the effects of chemotherapy on both the tumour and the host immune system [32]. Finally, the efficacy of therapies targeting PD-1 or PD-L1 also speaks of the likely importance of this molecular mechanism in the development of this disease (see below).

Pathological Associations Between Lung Cancer and PD-L1 Expression

There are some interesting relationships between aspects of lung cancer pathology and PD-L1 expression.

As will be discussed later, a high mutational burden in the tumour is associated with a higher likelihood of response to anti-PD-1 and anti-PD-L1 therapy. It has been speculated that higher mutational burden may be associated with higher PD-L1 expression levels, but there is little evidence to support this. Small cell lung cancer has an extremely high mutational burden, yet PD-L1 expression is generally low or absent [33]. In advanced NSCLC, mutational burden as measured by a Foundation Medicine mutation platform showed only a weak association with tumour cell PD-L1 expression and no association with PD-L1 expression in tumour-infiltrating immune cells [34]. Kadara et al. found that a number of immune-related markers expressed on tumour cells, including PD-L1, were increased as mutational burden increased [35].

A history of tobacco smoking is also associated with response to PD-1-axis inhibitors, and most of the literature supports the finding that smoking histories are also associated with higher levels of PD-L1 expression [36–40]. This is not, however, a universal finding as studies have shown no relationship [41] or found the opposite that a positive smoking history was associated with lower PD-L1 expression [42]. Once again these studies are confounded by variable IHC techniques and scoring methods.

Regarding the association between PD-L1 expression and single driver mutations of clinical significance, the data are few and mixed. The only really consistent finding is that *EGFR*-mutated adenocarcinomas tend to have less PD-L1 expression [38, 39, 43]; these tumours will also have a lower overall mutational burden and tend to show less responses to PD-1 axis targeted therapy. Whilst *KRAS*-mutated tumours tend to show more response, and tend to have a higher mutational burden, data in PD-L1 expression are not consistent. Studies have shown higher PD-L1 expression in *KRAS*-mutated adenocarcinomas, but most show no association [36, 37, 44, 45]. *TP53* mutation has been associated with higher PD-L1 expression in NSCLC [35, 45]. In a study comparing NSCLC cases with no driver mutation, versus cases with one driver, versus cases with multiple drivers, PD-L1 expression was positively associated with the number of drivers [46].

Although there are no consistently applied definitions of tumour grade or tumour cell differentiation in lung adenocarcinoma, there is consistent reporting of higher PD-L1 expression and high tumour cytological grade or poor differentiation [38–40, 44]. This is despite the above variations and marked heterogeneity of PD-L1 assessment. A better defined way of grading lung adenocarcinomas is to describe the predominant histological pattern [47–50]. Surgically resected cases which are predominantly of a micropapillary or solid pattern have a more aggressive postoperative course. These cases are regarded as high-grade tumours, and there is a fairly consistent finding of higher PD-L1 expression in cases that are solid or

micropapillary pattern predominant [37, 38, 40, 51–53]. This trend is further supported by the observation that pleomorphic and sarcomatoid carcinomas of the lung [54], NSCLC which show molecular evidence of epithelial-mesenchymal transformation (EMT) [55, 56], and cases associated with upregulation of the MET gene or expression of MET protein, which drives EMT [51], consistently show higher levels of PD-L1 expression. Lymphoepithelioma-like carcinomas of the lung are undifferentiated, Epstein-Barr virus associated tumours with a heavy lymphoid infiltrate, and one study has reported high PD-L1 expression in these tumours [57].

The relationship between PD-L1 expression on tumour cells and the presence of an immune cell infiltrate (ICI) in the tumour is extremely complex. There is no simple and consistent relationship between PD-L1 expression in tumour cells and the presence or absence of an infiltrate. Although there is a tendency for a positive association between PD-L1 expression and increased ICI, this is not consistent [53, 58, 59]. Parra et al. further found that the infiltrates in adenocarcinomas when PD-L1 expression was higher tended to be macrophage poor [53]. Furthermore, this study recapitulated the observations made in a number of tumour types that there are four possible categories of tumour microenvironment:

(Type 1) Adaptive immune resistance—tumour cells PD-L1+; tumour-infiltrating lymphocytes (TIL) PD-L1+

(Type 2) Immunological ignorance—tumour cells PD-L1-; TIL PD-L1-

(Type 3) Intrinsic induction—tumour cells PD-L1+; TIL PD-L1-

(Type 4) Tolerance (other suppressors active)—tumour cells PD-L1-; TIL PD-L1+ [22, 60]

It is suggested that type 1 cases are most likely to be responsive to PD-1 axis inhibitors (see below). Parra et al. found that, in a surgically resected population, 19 and 24% of adenocarcinoma and squamous cell carcinomas, respectively, fell into this category [53]. Type 3 tumours are thought to have high PD-L1 expression due to intracellular oncogenic events upregulating mechanisms such as EGFR signalling, the KRAS-BRAF-MEK pathway, the PI3K-AKT-mTOR pathway through KRAS mutation or PTEN loss, PD-L1 gene amplification or mechanisms increasing JAK2/STAT pathway signalling, such as JAK2 amplification [61, 62].

It is difficult to understand what is important from all these findings. Many of the above factors, high mutational burden, smoking history, poorly differentiated, high-grade tumour and EMT, are biologically related. Despite the huge technical heterogeneity in studies using different PD-L1 IHC and different definitions of ‘positive’ or ‘high’ expression, the relatively consistent associations described above, which are intuitively related to a central theme, speak of a significant biological effect. Some tumour types that may be more highly mutated, or likely to have more neoantigens through nuclear aberration, may adopt high PD-L1 expression as a defence mechanism against a primed immune response in order to promote clonal survival. This high expression of PD-L1 may be an intrinsic, genomically driven feature of the tumour due to dysregulation of PD-L1 gene function or overexpression related to gene amplification. More often, however, it is probably an adaptive response, driven by cytokines such as interferon gamma. These four microenvironment scenarios described above add a layer of complexity to the PD-L1 expression story and

add weight to the idea that the prediction to response to PD-1 axis inhibitors by assessment of PD-L1 expression alone may be enhanced by additional assessment of immune cell infiltrates (see below).

PD-1 and PD-L1 as Therapeutic Targets in Lung Cancer

It is beyond the scope of this chapter to provide an extensive review of this topic; several reviews have been published although the data on this subject are numerous and constantly changing, such that almost all published accounts are incomplete by the time they are read [1–6]. Generally speaking, therapeutic agents targeting the PD-1 axis in patients with advanced NSCLC who have received at least one line of prior therapy perform well, if not better, than standard-of-care docetaxel cytotoxic chemotherapy. This matching of, or modest superiority to, the rather poor response to docetaxel, allied to a better toxicity profile [24, 63–66], has led to three of the five drugs, most advanced in terms of their clinical development (see Table 20.1), receiving regulatory approval for this indication. In an all-comers population, objective response rates (ORR), which translate into progression-free (PFS) and, especially, overall survival (OS) benefit, are around 15–20% in most trials. Therefore, most patients do not benefit from the treatment. PD-L1 IHC has emerged from many trials as a biomarker capable of improving the average, overall response rates to around 45–50% for a treated group, selected by higher levels of PD-L1 expression. The selection criteria used to select patients vary [23, 67–71]. As each drug was taken through phase 1 and then later trials, a different, independent PD-L1 IHC biomarker assay was also developed, by each pharmaceutical company, for each drug [71–73]. The complications of trial design and regulatory authority requirements probably drove this practice, which has now become a problem (see below) [23, 67–69]. There is recurrent evidence from these trials that as the degree of PD-L1 expression increases, in terms of the proportion of tumour cells expressing PD-L1 (tumour proportion score or TPS), so does the probability of a benefit from therapy. This matter is discussed in more detail later. The anti-PD-1 agent pembrolizumab has been approved for use in second line in advanced NSCLC with a companion PD-L1 IHC diagnostic assay which must show at least 1% of tumour cells expressing PD-L1 (Table 20.1). Nivolumab (anti-PD-1) and atezolizumab (anti-PD-L1) have been approved without a mandatory test; their associated assays are described as ‘complementary’—testing may be useful in making a therapy decision, but it is not required, in the drug label, for prescription [74]. Pembrolizumab now has regulatory approval for use in first-line therapy for advanced NSCLC without an *EGFR* mutation or *ALK* rearrangement, as an alternative to standard chemotherapy, as a result of superiority of pembrolizumab over chemotherapy as shown in the CheckMate 026 trial [75]. This superiority was shown in a group of patients who had tumour expressing PD-L1 in at least 50% of the tumour cells in tested samples. This extremely significant, practice-changing finding has major implications for PD-L1 testing in diagnostic practice [76].

Table 20.1 Five leading anti-PD-1 axis inhibitors and their trial-validated assays

Drug	Drug specificity	Cut-offs used in trials[a] Unless stated, these represent % tumour cells stained: cell membrane staining	PD-L1 immunohistochemistry primary antibody clone	Complementary or companion diagnostic test	Staining platform required by assay
Nivolumab	Anti-PD-1	≥1%, ≥5%, ≥10%, ≥50%	28-8	Complementary	Dako[b] link 4800
Pembrolizumab	Anti-PD-1	≥1%, ≥50%	22C3	Companion	Dako[b] link 4800
Atezolizumab	Anti-PD-L1	TC: ≥1%, ≥5%, ≥50% IC: ≥1%, ≥5%, ≥10%[c]	SP142	Complementary	Ventana[d] benchmark or ultra
Durvalumab	Anti-PD-L1	≥25%	SP263	Not known	Ventana[d] benchmark or ultra
Avelumab	Anti-PD-L1	≥1%, ≥50%, ≥80%[e]	73-10	Not known	Dako[b], specifics not known

TC tumour cells by % tumour cells stained, *IC* % area of tumour infiltrated by PD-L1-positive immune cells

See also reference [71]

TC0—<1% tumour cells stained; TC1—≥1%– < 5%; TC2—≥5%– < 50%; TC3—≥50%

IC0—<1% tumour area infiltrated by PD-L1-positive immune cells; IC1—≥1%– < 5%; IC2—≥5%– < 10%; IC3—≥10%

[a]It is important to note that these cut-offs are relevant to the drug and indication and not to the assay used

[b]Dako, Carpenteria, CA, USA

[c]In the trials using atezolizumab, patients were placed into a treatment group based on several grades of tumour cell or immune cell staining. These grades are defined as follows, based upon a minimum of 50 tumour cells assessed

[d]Ventana, Roche Diagnostics, Tucson, AZ, USA

[e]As avelumab is still at a relatively early stage in its development, there are limited details of which cut-offs will be used as and when the drug gains regulatory approval

PD-L1 Immunohistochemistry as a Biomarker

PD-L1 IHC and its use as a biomarker for selecting patients for anti-PD-1 or anti-PD-L1 drugs has been a controversial issue [23, 67–69]. This is far from being a perfect biomarker, but no biomarker is perfect. PD-L1 IHC represents a biological

continuum of protein expression in different tumours, from nil, through very low, through moderate, to very high numbers of tumour cells expressing the protein. There is evidence or inference from many trials, of a relationship between the amount of PD-L1 in a tumour and the likelihood of response. It is difficult to be certain, but this relationship may not be linear; a certain amount of PD-L1 may be needed, in order to be predictive, but that level is not known. All clinical trials using the biomarker to select a group of patients enriched for chance of response have used a threshold or cut-off level somewhere along the continuum of tumour proportion score. This threshold has been set at 1, 5, 10, 25, 50 or 80% in various trials with various drugs. For atezolizumab, the IHC assay validated in trials also considered the percentage area of tumour infiltrated by PD-L1-expressing immune cells at thresholds of 1, 5 and 10% as an alternative to tumour cell expression, in order to qualify for treatment [65]. These thresholds or cut-offs try to deal with the biological continuum of PD-L1 expression which is not a clear 'present vs absent' binary situation (Fig. 20.1). This is quite unlike the other, well-known, addictive oncogene biomarkers such as *EGFR* mutation or *ALK* rearrangement, which do represent a clearer selection of patients who will or will not benefit from treatment, biomarkers with a much better performance in terms of predictive power. The use of a cut-off to create the group selected for therapy does not select patients with the same probability of response. The lower the cut point used, the more

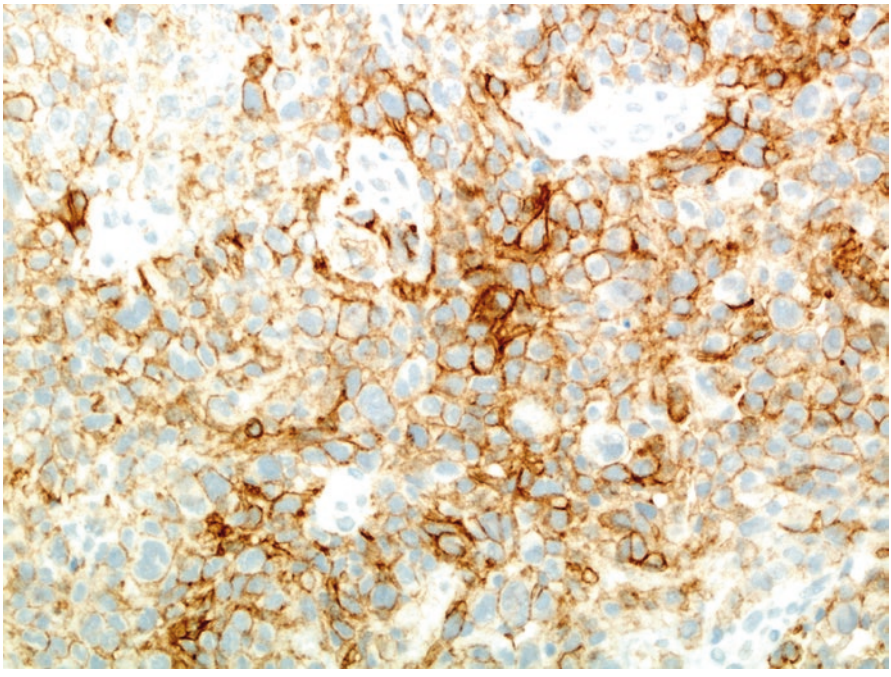

Fig. 20.1 An example of poorly differentiated non-small cell carcinoma stained using the Dako 22C3 anti-PD-L1 immunohistochemistry assay. In this particular field, a majority of tumour cells express PD-L1 on their cell membrane; if this is true for the entire sample assessed, the tumour proportion score would be over 50%

heterogeneous this responsiveness will be in the treatment group. There is evidence that the average, 'class' effect for treatment benefit is largely driven by the high PD-L1 expressing patients in the group. With lower thresholds, there is a risk that patients who have low levels of PD-L1 expression, just above the threshold, will have a significantly less-than-average chance of responding to the therapy. PD-L1 expression can be very heterogeneous in NSCLC, so there is a definite probability of sampling error, in that biopsy samples may not reflect the overall expression in the patient's disease burden. This error risk is more likely at lower levels of expression. It does mean that some patients will be inappropriately allocated to the wrong side of any threshold being considered. This is probably a major contributing factor to why response rates in groups above threshold are lower than they might otherwise be and why we do see patients who benefit, even if their PD-L1 assessment was considered below threshold or even completely negative. Fortunately, for any given threshold or cut point, most patients' samples appear clearly above or below the mark, but for those cases close to the cut point, great care needs to be taken to ensure as accurate and consistent an assessment as possible.

There are currently five drugs targeting the PD-1 axis, either approved or in advanced stages of study, each with their own validated PD-L1 IHC assay (Table 20.1). As multiple drugs get approved, and used in various indications in NSCLC, the pathology community is faced with considerable challenges [23, 67–69]. Each drug-associated assay is different, and there are stringent equipment requirements for each [71]. It would be impractical for most pathology laboratories to offer all five tests, and it may well be impossible, and undesirable, to test the patient's tumour with all five assays. Instead, can we use any one of the trial-validated assays to stain the sample, but then read the sample according to any number of thresholds that might be required by the oncologist for the patient? Several studies have been undertaken to compare the technical performance in NSCLC samples stained with various trial-validated assays [77–82]. Some of these studies also included laboratory developed tests (LDTs) using a range of anti-PD-L1 IHC clones, some using the same clones as in the trials, and some using clones that have never been used to select any patient in a clinical trial. The findings from these studies may be summarised as follows:

- The trail-validated commercially produced assays using the 28-8, 22C3 and SP263 clones show technical equivalence.
- When slides stained with these assays were read according to cut points other than the ones used with their associated drug, the concordance for allocating the cases on the same side of the cut points was anywhere between 81 and 95%.
- The SP142 assay seems to consistently stain fewer tumour cells compared to the other three assays assessed, leading to lower tumour proportion scores.
- All four assays behave similarly in their staining of immune cells.
- Some of the LDTs used in the comparisons did match the performance of the comparable trial-validated assays. In one study, however, where several LDTs were developed and compared with the validated assays, about half of these LDTs failed to show adequate concordant staining, even when using the primary IHC clones used in the validated assays [82].

The question for the oncology community is whether this apparent loss of staining performance when using an alternative assay is acceptable. If laboratories choose to use an LDT, there should be a very extensive and rigorous process of validation, in order to ensure that patients are not compromised by being allocated to the wrong treatment group. It is not clear what this validation process should be. Although the College of American Pathologists has some guidelines for the development of IHC diagnostic LDTs, it is unlikely that these will be stringent enough for the adequate validation of a PD-L1 biomarker assay that will be required to perform adequately over the dynamic range of staining and cover several different cut-offs [83, 84]. External quality assurance programmes will help laboratories achieve and maintain adequate performance.

Although there is considerable variation around the world, in the proportion of patients with advanced NSCLC for whom only cytology-type samples are available for diagnosis, it is not unusual for this proportion to be 50% or more. Currently, cytology samples are not trial validated for PD-L1 testing, but work is ongoing, trying to prove that these samples are adequate for testing [85]. There is some anecdotal evidence that PD-L1 staining is diminished by alcohol-based fixation, a common step in the preparation of many cytology-type samples.

Training is important in achieving good performance in reading PD-L1 IHC stains. The estimation of tumour proportion score can be quite challenging in some samples, particularly when immune cells, and especially macrophages, also express PD-L1. The sample used for scoring should have a minimum of 100 tumour cells, and all of the tumour present in the sample section should be used in the assessment. Studies of interobserver variability that have so far been reported describe moderate agreement, but this is often on a background of limited training and experience [77].

How should PD-L1 staining outcomes be reported? Although there might be a temptation to simply report cases as above or below a particular cut-off, as ‘positive’ or ‘negative’, it will surely be much better to give an actual figure for estimated tumour proportion score, as a percentage. Realistically this will be to the nearest 10% for cases showing more staining, but a more specific figure should be given if staining is under 10%. The report should indicate the details of the assay used and give some indication that the tumour cell number was adequate. If the SP142 assay is used, it is recommended to first assess the tumour proportion score. If this is below 1%, an immune cell score, expressed as the percentage area of tumour infiltrated by PD-L1 positive immune cells, is given. It is not yet clear what value there would be in attempting this assessment, or any other measure of immune cell staining, when reading any other assay.

Other Possible Biomarker Strategies for Immunotherapy in Lung Cancer

A number of other biomarker approaches are being investigated, in the search for a strategy that might either be better than PD-L1 IHC or, perhaps more likely, be used in conjunction with PD-L1 IHC to improve the efficiency of patient selection.

The importance of mutation burden, leading to neoantigenicity and possible immunogenicity, was discussed earlier in this chapter. A relatively high mutational burden, measured by whole-exome, next-generation sequencing, has been associated with superior response to pembrolizumab [86]. McGranahan and colleagues showed that it was important that the high mutation burden was a clonal phenomenon and not restricted to a subclone of cells in the tumour [87]. *EGFR* mutation is associated with poorer response, whilst *KRAS* mutation favoured response to nivolumab in non-squamous NSCLC in second or greater line therapy [64]. This probably reflects the fact that *EGFR* mutation is found in tumours not related to tobacco carcinogenesis and which have a lower overall mutation burden, whilst *KRAS* mutations are associated with smoking and tumours with a higher overall mutation burden. Similarly, smokers respond better than non-smokers to immunotherapy. Using whole-exome sequencing to deliver a routine, high demand biomarker would be challenging. One possible approach may be to find a smaller panel of mutations, perhaps including *KRAS* and *TP53*, which might be used as a surrogate for overall tumour mutation burden. There are interesting data beginning to emerge, relating different immune profiles in tumours, in association with different coexisting mutations in *KRAS*-mutated lung adenocarcinoma [88, 89]. High microsatellite instability (MSI high) similarly predicts for response in colorectal, endometrial and gastric carcinoma [90, 91], whilst polymerase E (*POLE*) mutations, also associated with DNA repair deficiency and high levels of genomic disarray, have been investigated in this context in other tumours [91]. These abnormalities are rare in lung cancer and unlikely to be useful.

For immunomodulatory therapy to work, a potential inflammatory response must exist. The immune system must be primed to recognise the immunogenic tumour cells. The therapy works by 'releasing the brakes' on the existing immune infiltrate, allowing specific cytotoxic T-cell action. As discussed above, an immune infiltrate in NSCLC may be prognostic, but could it be predictive of response to these drugs? None of the published trials of immunotherapy in lung cancer using drugs against the PD-1 axis present any data on the value of immune cell infiltrates, either in general or assessing particular cell types, as a predictive biomarker. In colorectal carcinoma and melanoma, however, there is some evidence that these infiltrates may help predict response [92]; as mentioned above, it is likely to be the 'inflamed' tumour with the adaptive immune response which is most likely to respond to immunomodulatory therapy [15, 92, 93].

Upregulation of genes related to the immune response and in particular gamma interferon function assessed in a tumour sample may well be an alternative way of identifying tumours with an immune infiltrate [94]. Immune gene expression signatures have been shown to select for a group of patients more responsive to atezolizumab in the Poplar trial [65]. A panel of around 14 serum cytokine levels was assessed in nivolumab-treated advanced NSCLC, where two, somewhat different, panels, when highly expressed in serum, were associated with better responses in squamous and adenocarcinoma, respectively [95]. High levels of these cytokines were also, however, associated with better outcomes in the chemotherapy arms of the trials, so there is the possibility that this biomarker is prognostic rather than predictive.

Other factors are related, or potentially related, to response to inhibitors of the PD-1 axis, but it is uncertain what role, if any, they may play in selecting patients for treatment [15]. Setting aside generic issues around performance status and other metabolic indicators like serum LDH, and a positive smoking history which is associated with responsiveness, the patient's microbiome presents an intriguing matter worthy of exploration. The latter concerns the observation that immune responsiveness in general, and responses to immunomodulatory therapy in particular, may be influenced by the composition of the gut flora [96].

Currently, virtually all the biomarker data from clinical trials, published on NSCLC, concerns PD-L1 IHC. Trials are being designed with pre-specified selection of patients based upon PD-L1 expression, although many will have secondary analysis of other biomarkers. The introduction of pembrolizumab into first-line therapy for advanced NSCLC, with a PD-L1 IHC companion diagnostic, will consolidate the practice of PD-L1 IHC assessment in NSCLC [75, 76]. From the available evidence, it seems unlikely that an alternative biomarker approach will be found to replace PD-L1 IHC, in the context of selecting patients for PD-1 axis inhibitors; PD-L1 is, after all, either the target of these drugs or functionally inhibited by them. Most of the evidence suggests that, when PD-L1 is *truly* absent or at low levels in the tumour, these agents do not benefit the patient. It seems more likely that PD-L1 IHC will remain at the core of the testing process, but that additional biomarker approaches, such as some measure of mutational load and/or 'inflammation', may improve the overall selection process. How much additional improvement in ORR these strategies will achieve remains to be seen. How much improvement in ORR will be considered enough to justify an increasingly complex and costly testing process is also open to question. Some of the approaches mentioned above are certainly promising, but published tumour gene expression profiles are based upon a fresh, frozen tumour, a difficult medium for widespread, routine biomarker testing. Next-generation sequencing technology is still not a mainstream diagnostic tool, though things are improving, and this will undoubtedly become a reality in a majority of centres. Serum-based biomarkers are always attractive, given the ease of sample acquisition, but blood-based immune parameters are readily confounded by immune reactions unrelated to a cancer-specific response.

Conclusion

After many years of failed trials and major issues with toxicity, immunotherapy for advanced NSCLC has certainly come of age. Most of the failed approaches involved some attempt at stimulating the immune system, usually in a rather non-specific manner, and with toxic consequences for patients. Although a more specific, antigen-based, tailored approach involving the ex vivo production of tumour-specific T cells is being trialled, it seems that targeting immune-inhibitory mechanisms, to release an existing immune response, is a more successful strategy. Of course, this will not work for all patients with advanced NSCLC; for a minority their tumour will be

non-immunogenic, and there is no effective immune response to reactivate. Increased understanding of the molecular mechanisms regulating the immune system, and how these processes interact with tumour development, not only in terms of cellular interactions but also at a molecular level, has been crucial in helping advance the cause of immunotherapy in lung cancer. An important driver of future developments, in both immunotherapy and the selection of patients for these treatments using biomarkers, will be our increasing understanding of the molecular pathology of lung cancer.

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Chapter 21
Liquid Biopsies

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Lung cancer is the leading cause of cancer mortality and an international public health issue [1]. After many years of very slow and disappointing clinical progress over the past decade, the advent of targeted therapies and immunotherapies has significantly expanded our available armamentarium against advanced lung cancer as well as other metastatic solid tumors. Similarly, the revolution in genomics has provided a much better understanding of what drives cancer initiation and growth and allowed improved drug development against oncogene-addicted tumors. However, in many cases of metastatic solid tumors, tissue biopsy offers scarce material and hence next-generation sequencing is impossible. Patients suffering with advanced lung cancer often pose a significant challenge for pathologists and treating clinicians as current treatments necessitate reliable genomic information. However, they frequently have disease that is only accessible through invasive biopsies and, in addition, often have significant comorbidities such as chronic obstructive pulmonary disease or coronary artery disease that increase their risk of complications from invasive biopsies. In the past when treating a patient required only a simple "yes" to the question of is it lung cancer, a small biopsy was adequate. However, with the veritable explosion of discovered driver mutations that now have available and well-tolerated therapies, coupled with the need to correlate tumor histology and the microenvironment as the field of immune-oncology advances, small biopsies are no longer acceptable to assess and plan state-of-the-art treatment for patients. Thus, the zeal that many oncologists are showing toward adopting the developing technologies of "liquid biopsies," whether circulating tumor cells (CTCs), circulating tumor DNA fragments (ctDNA), or exosomes and tumor-educated platelets, is understandable. This chapter will review the emerging science behind these tests and discuss the potential clinical benefit that is starting to emerge.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4_21

While the description "liquid biopsy" has been disputed by pathologists, the term has entered the vernacular and will undoubtedly stick. These "biopsies" are tests that are performed on the blood or other body fluids and do not require a needle put into a visceral organ or lymph node nor do they require a surgeon or pulmonologist to obtain it. There is no histologic or architectural information obtained. Obviously the target of interest—either whole cells in the case of CTCs or DNA fragments for ctDNA—is not liquid. Many potential roles can be envisioned should the technologies continue to develop and be shown to have meaningful clinical uses: diagnostic purposes when patients first present with a radiographic abnormality, monitoring of response to therapy, analysis of driver mutations for selection of targeted therapy, and further analysis when patients on targeted therapy develop acquired resistance. There will probably also eventually be interest in using it as a screening test in completely healthy patients (most likely even before evidence even exists to support that use). Clearly much work remains to be done in order to fully assess the strength and weaknesses of the various assays and how to best utilize them in the care of patients. Importantly, assessing the benefit will require multidisciplinary evaluation and consensus between oncologists, pathologists, and bench researchers.

Circulating Tumor Cells

The fact that cells could break off from the tumor colony and spread through the blood or lymphatics has been noticed for over a century, but it took a long time to develop techniques to reliably capture and analyze these cells. Even in many patients with advanced cancers, CTCs are rare compared to the normal blood cells that they travel among. Thus strategies were employed that tried to separate tumor cells out of the blood using cell capture techniques that relied on epithelial adhesion molecules such as EpCAM. The CellSearch system (Veridex) is approved by the FDA for monitoring some tumors, such as breast and colon cancer, however, not for lung cancer. It has been demonstrated in metastatic breast cancer that levels of CTCs correlate with progression-free survival as well as overall survival [2]. However, obviously not all pathogenic cells express EpCAM; cells that have undergone epithelial to mesenchymal transition will not be detected by this method, and this has led to the development of assays that do not depend on cell surface markers but rather on cell size. Krebs et al. compared two strategies for detecting CTCs in patients with advanced lung cancer: a cell surface marker-dependent method (CellSearch for EpCAM + cells) and a surface marker-independent method that relied on isolation by the size of the epithelial tumor cells (ISET) [3]. In a small study of 40 patients with advanced lung cancer, 23% had CTCs detected with the surface-marker approach while 83% of the ISET tests were positive. Still, there remains little consensus on how CTCs compare with cfDNA, the methods of collection are not standardized, and there is difficulty in controlling the pre-analytic phase [4]. Attempts have been made to try to enhance the enrichment phase in order to increase yield. Pallier and colleagues combined blood filtration and FISH assay optimized for CTC

characterization to detect ALK-positive CTCs in the blood of patients known to harbor the mutation and found 18/18 had greater than four ALK-positive CTCs and negative controls had 1 or 0 CTCs [5]. Still, even with the ability to isolate and sequence single cells in patients with lung cancer, the primary information that has been derived from studying CTCs in patients with advanced lung cancer has been prognostic. Yields of CTCs have been higher in patients with small-cell lung cancer (SCLC) rather than NSCLC, and some studies have suggested that CTCs better predict survival than stage and that a reduction in the number of CTCs detected after the first cycle of chemotherapy has been associated with better outcomes [6]. While the data is intriguing, there is as of yet no routine clinical role for utilizing CTCs to monitor response in small-cell lung cancer, which also has much to do with the paucity of effective salvage therapies for patients who fail to respond to initial therapy or rapidly relapse [7]. The success of meaningful diagnostic information is always tethered to available therapeutic options.

CTCs have been studied in some of the other driver mutations that cause oncogene-addicted lung cancers. CTCs were looked at in four patients being treated with crizotinib for ROS-1-mutated lung cancers as well as in four ROS-1-negative patients [8]. All four ROS-1 patients with tissue-proven mutations also had CTCs harboring the mutation. Patients progressing on therapy had significant increase in ROS-1 copy number in their CTCs.

One of the appealing possible strategies of CTCs as opposed to ctDNA would be the development of technologies to culture patients' CTCs ex vivo and allow assays of drug sensitivity [9]. Hodgkinson recently reported that CTCs from patients with small-cell lung cancer, either chemosensitive or chemo-refractory, are tumorigenic in immune-compromised mice [10]. Furthermore, the CTC-derived explants mirrored the donor patients' response to cytotoxic chemotherapy. Similar results have been reported with CTCs from patients with colon cancer and prostate cancer [11, 12].

So while the techniques to isolate and study CTCs in lung cancer patients have improved upon prior results, the main explosion over the past few years in clinical application has not been utilizing CTCs but rather using ctDNA.

Circulating Tumor DNA

Circulating tumor DNA was noted 40 years ago to be detectable in the blood of patients, and even at that time, levels were noted to be higher in patients with metastatic disease and the levels dropped in patients who responded to therapy [13]. However it was not until the genomic revolution, ushered in by the Cancer Genome Project, that the ability to sequence and analyze circulating DNA fragments began to reliably provide meaningful, actionable data.

Healthy humans have free DNA fragments that circulate in plasma and can be detected using quantitative PCR techniques. In patients with cancer, a percentage of the circulating DNA is derived from the tumor cells, generally between 0.1% and

10% of the total circulating DNA [14]. ctDNA is thought to be released from tumor cells both from passive mechanisms, such as apoptosis and necrosis, and therapy-induced destruction such as from radiation and chemotherapy. Recent data looking at cfDNA from cultured cells suggest that the process is an active one of secretion and not release through apoptosis or necrosis [15]. cfDNA is enriched in fragments of 150–180 bp as one would expect from apoptotic fragmentation [16]. Initially cfDNA levels were noted to be quantitatively higher in cancer patients than normal controls; Yoon et al. found that the median plasma DNA concentration in lung cancer patients was 22.6 ng/ml compared to 10.4 ng/ml in healthy volunteers [17]. The blood specimen must be centrifuged within 2 h as cfDNA begins to degrade quickly.

The real breakthrough with the technology was when not just the circulating levels of DNA could be assayed but the somatic mutations present in the tumor could be detected. Coming at a time when multiple new targeted therapies for lung cancer were being developed and validated in trials, the technology seemed to open the door on following patients and detecting new acquired resistance mutations without having to subject the patients to repeated biopsies.

Bettegowda and colleagues reported their results using digital PCR in 640 patients with early stage and advanced malignancies [18]. In patients with metastatic pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers, ctDNA was detectable in over 75% of patients. In patients with advanced primary brain, renal, prostate, or thyroid cancers, the rate was under 50%. In patients with localized disease, ctDNA was detectable in 73% of patients with colorectal cancer, 57% of gastroesophageal cancer, 50% of breast cancers, and 48% of pancreatic cancers. Importantly, ctDNA was often detectable in patients when CTCs not found, indicating that these are different markers.

One of the questions that arises when discussing ctDNA is how well it correlates with the genomic landscape of the primary tumor. Of course, the issue of tumor heterogeneity and the resulting clinical implications remain a pressing and vexing one. Even when tissue is biopsied, there can be intra-tumoral variation in detected mutations, raising the question of the validity and actionable yield of single-site tumor biopsies [19]. Proponents of using ctDNA state that the value of blood-based assays is that it captures a better snapshot of the various mutations within the heterogeneous tumor colonies. Critics state that correlative studies between tissue and the primary site are still ongoing and that it is possible that the mutations detected in the blood come from necrotic cells and might not be driver mutations.

To try to answer this question, Zill et al. looked at somatic genomic profiling of 15,000 patients with advanced cancer utilizing a 70-gene panel run on ctDNA [20]. The researchers compared the results with the incidence of mutations found in tissue biopsy specimens from the TCGA. Over half of the patients had lung, colon, or breast cancer. The specific frequency of mutations by cancer type as well as the mutual exclusivity among driver mutations matched well with the results from tissue. The overall accuracy of ctDNA analysis was 336/386 (87%) with matched tissue and that increased to 98% when the tissue and blood were acquired within 6 months of each other. While many additional details need to be worked out, at

least the current data would suggest that the mutations detected in cfDNA numerically correlate with what has been documented in the tumor.

It is important to point out that many of the early genomic-based basket trials, such as BATTLE 1 and BATTLE 2 performed at MD Anderson, utilized tissue biopsies, not ctDNA, and that current large-scale basket trials, such as NCI Match and the American Society of Clinical Oncology's Targeted Agent and Profiling Utilization Registry (TAPUR), also are looking at tissue-based sequencing [21, 22]. Much published data thus far, such as the SHIVA trial as reported by Le Tourneau, suggests that when trying to use tissue genomics to identify targets to exploit with targeted treatment, the response rates are low [23]. SHIVA was a genomic matching trial comparing molecularly targeted therapies with physician's choice; 741 patients were screened to randomize 26%, and the progression-free survival was 2.3 months against 2.0 months, leading the authors to caution against treating patients outside of a clinical trial based on molecular profiling of the tumor. It is certainly possible that should plasma-based genomic profiling of patients with advanced cancers achieve further validation, then the number of patients who would potentially benefit would expand more easily in the community, especially in later-stage patients where repeat tissue biopsy could be problematic. Wheeler et al. recently reported on a single-institution study of 500 enrolled patients where the plan was to do comprehensive genomic profiling [24]. Three-hundred and thirty nine patients were successfully profiled, and patients with a higher score of genomic aberrations and medication matches had significantly better 6-month survival than patients with low scores. While this data needs to be confirmed, it does suggest that a significant minority of patients with advanced disease can potentially benefit from genomic profiling, and thus improving the ease and accessibility of acquiring such data would greatly expand the number of patients eligible for these treatments. To move the field forward, we will need not just refinements to show that we have the capacity to quickly obtain data; we will need to demonstrate that we can use the data to effect meaningful treatment that changes the course of the disease in patients.

The success of tyrosine kinase inhibitors (TKIs) for certain oncogene-addicted metastatic lung cancer has profoundly changed the management of patients and made biomarker testing imperative for formulating the most appropriate treatment plan for individual patients [25]. While blood-based diagnostics have not yet replaced tissue biopsy as part of the initial workup of patients, there might be situations when patients have undergone a biopsy and the tissue is insufficient to run specific mutation testing. While clinical characteristics are suggestive, there are no absolute historical features (smoking status, ethnicity) that fully predict for the presence or absence of targetable mutations. In that case, the treating clinician might elect to re-biopsy, or they might decide to run ctDNA studies to look for actionable mutations.

Reck and colleagues looked at the ability of ctDNA to discover EGFR mutations in advanced lung cancer patients [26]. In the ASSESS study, patients with newly diagnosed lung cancer had tissue and ctDNA assays run. In 1162 matched samples, the mutational concordance was 89% (sensitivity 46%, specificity 97%, positive predictive value 78%, and negative predictive value 90%). The EGFR mutation

frequency was 16% on tissue and 9% on plasma. Patients with extra-thoracic metastases were more likely to have mutations detected in plasma (13%) versus intrathoracic metastases (7%). This study confirmed that detection of ctDNA for EGFR mutations was possible in real time across many centers in different countries.

Despite the initial success of most targeted therapies to achieve clinical response, the majority of patients develop acquired resistance and subsequent radiographic or clinical progression. Many centers began repeating biopsies in patients who progressed in order to look at the mutation spectrum and see what was driving therapeutic resistance [27]. While many patients agree to re-biopsy, which initially struck many oncologists as counterintuitive, the yield was not 100%. Drilon et al. at MSKCC presented data on patients with adenocarcinoma of the lung with initial negative NGS, and they underwent hybrid capture and almost a third had actionable mutations [28]. Interestingly, many of those patients had between two and four biopsies in order to get successful next-generation sequencing, a strategy that is helpful for generating hypotheses but not one that is practical across a large population.

The development of TKIs that were active in cases of acquired resistance made re-biopsy an urgent issue in patients with advanced lung cancer progressing on initial therapy. Recently, a number of studies have confirmed that ctDNA can play a major role in detecting resistance mutations and help guide selection of therapy. Oxnard et al. recently reported their results using plasma genotyping using BEAMing in patients on a trial of a third-generation TKI osimertinib in patients with EGFR-mutated lung cancer [29]. They compared the plasma results to tissue genotyping run at a central laboratory. The sensitivity of plasma genotyping was 70%.

Regardless of whether the patient was T790 positive in tissue or blood, osimertinib was effective and response rates were similar. Obviously since the sensitivity is only 70%, patients negative on blood would require a biopsy as clinicians do not want to deny patients the chance to remain on targeted therapy should the tissue biopsy be positive.

Sacher and collegaues utilized digital droplet PCR in looking at two groups of patients: advanced lung cancer patients at the time of initial diagnosis and patients who were found to be EGFR mutation positive and progressed on initial TKI therapy [30]. All patients underwent tissue biopsy to use as the reference sample to correlate with the blood specimens, and the blood was analyzed for the presence of *EGFR* exon 19 del, L858R, T790 M, and/or *KRAS* G12X. The median turnaround time for ddPCr at initial diagnosis was 3 days (range 1–7), and the tissue genotyping median TAT was 12 days (range 1–54) for newly diagnosed disease and 27 days (range 1–146) for acquired resistance. Plasma ddPCR had a positive predictive value of 100% for EGFR 19 deletion mutations, L858R, and KRAS and a 79% for T790. The sensitivity of the assay was 82% for EGFR 19, 74% for L858R, and 77% for T790. The sensitivity for EGFR and KRAS mutations was better in the setting of multiple metastatic sites and especially with extra-thoracic metastases to the liver or bone. The study shows that rapid TAT was possible for a defined molecular target that could significantly decrease the need in many patients to undergo a repeat biopsy.

Besides the practical issue of immediate relevance in the clinic—identifying patients with acquired resistance who would benefit from an available medication—ctDNA has the potential to better elucidate the mechanisms driving resistance and allowing researchers to develop therapeutic strategies to combat them. Patients being treated with rociletinib, a third-generation TKI against mutated EGFR, had their ctDNA analyzed [31]. Fourty-six percent had various resistance mechanisms noted after first-line therapy, such as MET, KRAS, and RB1. In addition, different mutations were noted in patients depending on whether they received osimertinib (33% had C797S) or rociletinib (<3%), underscoring the potential for this technology to greatly enhance our understanding of the biology behind advancing resistant disease.

The use of ctDNA in managing other driver-mutated lung cancers in the clinic remains under study. Currently, selection of specific ALK inhibitors for patients with ALK translocated lung cancers is not dependent on particular fusion partners, although it is possible that it could potentially change [32]. Still, studies of ctDNA to detect ALK fusions in peripheral blood confirm that it is possible with a capture-based targeted sequencing panel [33]. In 24 patients with tissue-confirmed ALK mutations, 79% (19 of 24) had detectable ctDNA giving a sensitivity of 79.2% and a specificity of 100%. The investigators were also able to detect some ALK resistance mutations, and in longitudinal measurements, they found that ctDNA levels corresponded to response. While further work needs to be done, it is encouraging that this technology might emerge as a significant tool in the managing of these patients.

Other molecular targets in addition to EGFR and ALK have been identified and have active targeted therapies available. Studies are beginning to look at ctDNA in these situations as well. It has been demonstrated that BRAF mutations occur in 3–4% of patients with lung cancer; 50% are V600E [34]. Unlike a number of the other driver mutations seen in adenocarcinoma, these mutations often arise in heavy smokers [35].

Planchard et al. looked at a combination of trametinib and dabrafenib in patients with advanced BRAF-mutated lung cancer and found a response rate of 63.2% [36]. Guibert and colleagues reported on droplet digital ctDNA analysis of six patients with a BRAF v600 mutation being treated with targeted therapies [37]. The mutation was only detected in one patient using CTCs, but the ctDNA was positive in all six patients.

MET 14 skipping has also emerged as a targetable driver mutation that can be exploited in treating patients. This abnormality occurs in 3–5% of patients and is often seen in older patients who tend to be female; 36% were never smokers [38]. This mutation can also be present in squamous carcinomas as well as adenocarcinomas. It confers sensitivity to the MET inhibitor crizotinib [39]. Given the recognition of a targetable driver mutation, blood-based studies are being reported. Dong et al. have reported on a patient with a MET 14 skipping activated lung cancer that initially responded to crizotinib [40]. The mutation was detected in plasma, and upon progression three acquired mutations were detected in the MET kinase domain.

Besides the obvious appeal in advanced lung cancer, ctDNA technology is also starting to be evaluated in early-stage disease as well. Chen and colleagues looked at ctDNA in 58 early-stage (IA, IB, and IIA) patients with NSCLC who underwent surgery [41]. Frequent driver mutations were discovered in KRAS, EGFR, PIK3CA, and p53; the overall study concordance was 50.4%, and the sensitivity and specificity between serum and tissue were 53.8% and 47.3%, respectively. The authors also found that patients with ground-glass opacity majority tumors had ctDNA concentrations 10× lower than patients with solid histologies and that stage II patients had higher concentrations than stage I patients, average 14.28 ng/ml vs. 4.57 ng/ml, respectively.

Exosomes

Exosomes are small vesicles that contain cargo of lipids, proteins, RNA, and DNA that bud off of parent cells through exocytosis. They are small and have a 30–100 mm diameter, essentially similar to a herpes viral particle. These vessels can function as a type of intracellular communication, leading to a reprogramming of recipient cells [42]. They are more difficult to detect than other soluble factors; however, they are much more abundant in blood than CTCs, and technologies are beginning to be developed where exosomes can be isolated and then their contents characterized by western blot or other modalities.

Sandfeld-Paulson et al. looked at exosomes from 431 patients with lung cancer and 150 controls [43]. They performed a feasibility study using an extracellular vesicle array to profile exosomes in advanced-stage lung cancer. CD151, TSPAN8, and CD171 were highly expressed in patients with lung cancer compared to patients without. Exosomal protein profiles were able to detect patients with lung cancer with an accuracy of 68% and adenocarcinoma, more so than patients with squamous carcinoma, 72–64%. Interestingly, the majority of the exosomal proteins did not differ between stages.

With the explosion of interest in immunotherapy, there is increasing interest in exosomes to understand their effect on the tumor microenvironment. Some data suggests that tumor-derived exosomes act on the tumor microenvironment in an immunosuppressive fashion, keeping some tumors “cold” or with a suppressed immune infiltrate [44]. While not ready yet to serve as a tumor marker, the relative stability of exosomes as well as the ability to assess various microRNA and DNA mutations makes this an attractive area for further study.

Tumor-Educated Platelets

TEPs have also emerged as a potential liquid biopsy of the tumor microenvironment. Tumor cells can transfect (?) mutated DNA into platelets, and these in turn can be isolated and sequenced [45]. Platelets taken from patients with glioma and

prostate cancer show mutations consistent with the cancer of origin biomarker. In a study that looked at 228 samples from cancer patients and compared them with 55 healthy controls, RNA sequencing could distinguish between the groups with a 71% accuracy [46]. How this strategy will compare with exosomes or ctDNA remains to be seen.

Soluble PD-L1

It would be difficult to understate the effect that the burgeoning field of cancer immunotherapy has had on the treatment of advanced cancer. The treatment of advanced lung cancer has been greatly impacted by the development of immune checkpoint inhibitors that interfere with the PD-1/PD-L1 axis [47, 48] and now is used in the frontline setting as a monotherapy in those patients who are high expressors of PD-L1 on tissue biopsy [49]. While not technically yet a tissue biopsy, there is growing interest in measuring circulating PD-L1 in cancer patients. Zhang and colleagues used an enzyme-linked immunoabsorbent assay to test 109 patients with advanced NSCLC and 65 healthy controls [50]. The expression of PD-L1 was significantly higher in the lung cancer patients compared with healthy controls ($p = -0.001$), and patients with high PD-L1 had a significantly worse survival than patients with low expression (18.7 months versus 26.8 months, $p = 0.001$). Okuma and colleagues found similar results when they looked at 96 patients with advanced lung cancer, and in multivariate analysis, patients with high levels of sPD-L1 had significantly worse survival [51]. Whether measurement of soluble PD-L1 will identify patients who need different strategies or allow us the ability to more quickly identify patients who are benefitting or not—to immune checkpoint therapies—will be studied in future trials.

Future Directions

In many ways, regardless of the developing clinical data, liquid biopsies have rapidly entered clinical practice in a classic example of technology advancing prior to fully confirming the clinical applications. Certainly in the treatment of advanced lung cancer, where a substantial minority of patients harbor driver mutations and where adequate tissue always seems to be an ongoing issue, it is hard to imagine that the use of this technology will decrease. The issue for patients and physicians will be how to best incorporate this data into routine care in the clinic. Many questions still need to be answered regarding the optimum schedule of testing and how to interpret the often chaotic results. Patients who have been resected with curative intent should not have their blood sent off for testing unless they are on a clinical trial. With the hope for expansion of lung cancer screening in high-risk individuals, hopefully further research will reveal a blood-based signature that can help

discriminate low risk from high-risk pulmonary nodules. And lastly, with the inevitable growth of immunotherapeutic combinations, it is hoped that blood-based assessments of the immunosome will allow us to determine who is going to benefit and who won't from these enormously expensive and potentially toxic therapies.

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P.T. Cagle et al. (eds.), *Precision Molecular Pathology of Lung Cancer*,
Molecular Pathology Library, https://doi.org/10.1007/978-3-319-62941-4