Diagnosis and Molecular Classification of Lung Cancer

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Abstract

Lung cancer is a complex disease composed of diverse histological and molecular types with clinical relevance. The advent of large-scale molecular profiling has been helpful to identify novel molecular targets that can be applied to the treatment of particular lung cancer patients and has helped to reshape the pathological classification of lung cancer. Novel directions include the immunotherapy revolution, which has opened the door for new opportunities for cancer therapy and is also redefining the classification of multiple tumors, including lung cancer. In the present chapter, we will review the main current basis of the pathological diagnosis and classification of lung cancer incorporating the histopathological and molecular dimensions of the disease.

Keywords

Lung cancer · Pathology classification · Molecular targets

Contents

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1 Introduction

Despite a recent decline in the incidence and death rate, lung cancer still remains as the leading cause of death by cancer in the United States, with 158,040 deaths expected to occur in 2015, which represent about 27 % of all cancer deaths [1]. Among the reasons for its high mortality is the fact that 57 % of the cases are diagnosed at a distant stage in which the 1- and 5-year survivals are 26 and 4 %, respectively [1, 2]. Lung cancer is a heterogeneous disease, which comprises several subtypes with pathological and clinical relevance [3–5]. All lung cancer subtypes are strongly associated with exposure to tobacco smoking; however, adenocarcinoma is the most common type in never-smoker patients [1, 2, 6–9]. Based on main histotype, prognostic, and therapeutic implications, lung cancers are divided in two main groups: small-cell carcinoma (SCLC, 13 % of the cases) and non-small-cell carcinoma (NSCLC, 83 % of the cases) [1, 5]. In this chapter, we will focus on the NSCLC group, with special emphasis on the main subtypes of NSCLC and its clinical and molecular importance, while SCLC will be discussed in Chap. XV [5].

2 Histological Classification of NSCLC

To the present day, the gold standard procedure for the diagnosis of lung cancer remains the microscopic evaluation of histological or cytological specimens under the microscope by a pathologist [10]. The biopsy or cytology specimen provides initial key information of clinical relevance including the confirmation of the presence of a tumor and its histotype based on morphological and immunohistochemical (IHC) features [11–13]. In the past, the major focus of the clinical diagnosis was to make the distinction between SCLC and NSCLC, without major therapeutic indications to further classify NSCLC tumors. However, the advent of molecular profiling and targeted therapy renewed interest to the further classify NSCLC into adenocarcinoma (ADC) and its variants, squamous cell carcinoma (SqCC), and large-cell lung carcinoma (LCLC) [2, 4, 6, 14]. Other types, including salivary gland-type tumors, sarcomatoid carcinomas, and others, represent a very minor part of the total NSCLC cases. The first step in the diagnosis of NSCLC is the histological classification based on the evaluation of the tumor morphological features followed by ancillary IHC methodologies. In small biopsies, when the histological features and IHC phenotype are not conclusive to subtype NSCLC, the term "not otherwise specified" (NSCLC-NOS) is used.

2.1 Adenocarcinoma (ADC)

ADC represents the majority of NSCLC, accounting for the 38.5 % of all lung cancer cases [15]. ADC is defined as a malignant epithelial tumor with glandular differentiation, which can show mucin production detectable by mucin staining like mucicarmin, or pneumocyte marker expression like napsin A or thyroid transcription factor 1 (TTF1) [6]. In general, ADC is located at the periphery of the lung [16–18]. ADC can present diverse histological patterns, which can be intermixed in the same tumor including lepidic, acinar, papillary, micropapillary, and solid patterns. While lepidic pattern is associated with a favorable prognosis, micropapillary and solid patterns are associated with a more aggressive behavior [3, 6]. Solid ADC can be confused with SqCC or LCLC; the mucin production and IHC expression of TTF-1 or napsin A can help in the diagnosis of solid ADC in challenging cases [6].

2.2 Squamous Cell Carcinoma (SqCC)

SqCC accounts for nearly 20 % of all lung cancers [15]. SqCC usually presents in a central location, arising in a main or lobar bronchus [6]. Histologically, SqCC is defined by the World Health Organization (WHO) as a malignant epithelial tumor that either shows keratinization and/or intercellular bridges or expresses IHC markers of squamous cell differentiation [6]. Although keratinization is the hallmark of SqCC, many SqCC may not show morphological features of keratinization.

Also, poorly differentiated SqCC can show pseudoglandular appearance, as well as poorly differentiated adenocarcinomas can show pseudosquamous features, making the interpretation of small biopsies or cytological specimens particularly challenging [3, 6, 12]. IHC tests including markers of squamous cell differentiation such as p40 or p63 and cytokeratins 5/6 represent helpful tools in the identification of SqCC in difficult cases. A distinct entity is the basaloid squamous cell carcinoma, a poorly differentiated malignant tumor without morphological features of squamous cell differentiation which can be confused with small-cell lung carcinoma, but it is characteristically positive for immunomarkers of squamous cell differentiation including p40, p63, and cytokeratins 5/6, while TTF-1 is negative [6].

2.3 Large-Cell Lung Carcinoma (LCLC)

LCLC accounts for the 2.9 % of all lung cancers [15]. LCLC is defined as an undifferentiated NSCLC carcinoma, which does not show histological or IHC evidence of squamous cell, glandular, or small-cell differentiation [6]. The diagnosis of LCLC requires extensive sampling of a surgical resected specimen after ruling out SqCC, ADC, or SCLC, and therefore, it cannot be made on core needle biopsies or cytology samples [6]. Mucin production detected by mucin staining such as mucicarmine is absent. Immunohistochemically, LCLC may be positive for cytokeratins but they are negative for TTF-1 and p40 [6]. LCLC is to be distinguished from large-cell neuroendocrine (usually expressing TTF-1 and neuroendocrine markers), solid pattern of ADC (TTF-1 positive), non-keratinizing SqCC (p40 positive), and rarely adenosquamous carcinoma (showing both ADC and SqCC differentiation) [6]. As stated, in small biopsies, tumors with NSCLC features and null IHC phenotype are named NSCLC-NOS.

3 Molecular Alterations of NSCLC

In the last years, there has been an increasing amount of new molecular alterations identified in NSCLC including oncogenes and tumor suppressor genes, many of them represent novel predictive biomarkers or targets for cancer therapy [5]. A representation of the relative frequencies of molecular targets in NSCLC is shown in Fig. 1. The following molecular alterations represent those which may have clinical relevance as molecular targets for NSCLC.

3.1 Epidermal Growth Factor Receptor (EGFR)

The *EGFR* gene is located on the short arm of chromosome 7 at position 12 [19]. The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily [19]. EGFR is overexpressed in 40–80 % percent of NSCLC and many other epithelial cancers. Approximately 10 % of patients with

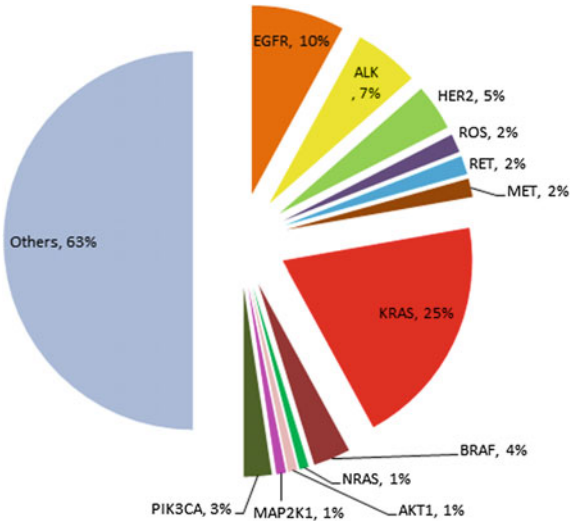

Fig. 1 Representation of the relative frequency of the main molecular targets in NSCLC. Still, the number of novel molecular targets may increase as further research will continue to discover and validate them

NSCLC in the United States and 35 % in East Asia have lung tumors associated with *EGFR* mutations [20–22]. These mutations occur within exons 18–21, which encodes a portion of the EGFR kinase domain. *EGFR* mutations are usually heterozygous, with the mutant allele also showing gene amplification [23]. Approximately 90 % of these mutations are in exon 19, deletions CTG to CGG or exon 21 at nucleotide 2573, that results in substitution of leucine by arginine at codon 858 (L858R) [24]. These mutations increase the kinase activity of EGFR, leading to hyperactivation of downstream prosurvival signaling pathways [25]. *EGFR* mutations are more often found in tumors from female never smokers, defined as less than 100 cigarettes in a patient's lifetime, with adenocarcinoma histology [20–22]. However, *EGFR* mutations can also be found in patients with other clinicopathologic features, including former and current smokers as well as in other histological types. Tumors with *EGFR* mutations are susceptible to be treated highly responsive to treatment with EGFR tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib, erlotinib, and afatinib; however, most patients relapse and develop resistance, most commonly associated with a second mutation in exon 20 (*T790M*, 60 *%0*), *MET* amplification, and *PI3KCA* mutations [5]. Interestingly, "transformation" to SCLC has been described in a subset of lung adenocarcinomas exhibiting resistance to EGFR TKIs [5].

3.2 Anaplastic Lymphoma Kinase (ALK)

Anaplastic lymphoma kinase (*ALK*) was originally discovered from chromosomal translocations leading to the production of fusion proteins consisting of the COOH-terminal kinase domain of *ALK* and the NH_2-terminal portions of different genes [26]. The *ALK* gene is located on the short arm of chromosome 2 at position

23 [27]. Translocation of *ALK* has been identified in approximately 3 to 7 % of lung tumors [28–30]. Nucleophosmin (NPM) is the most common fusion partner of *ALK* accounting for 80 % of *ALK* translocations, but at least six other fusion partners have been identified [30–35]. In NSCLC, the more common *ALK* fusion variants are comprised of portions of the echinoderm microtubule-associated protein-like 4 (EML4) gene with the *ALK* gene. *EML4-ALK* is an aberrant fusion gene that encodes a cytoplasmic chimeric protein with constitutive kinase activity [36]. *EML4-ALK* fusions are more commonly found in younger patients who have never smoked or who have a history of light smoking (<10 pack years) [30, 35] and in patients with adenocarcinomas with acinar histology and with signet-ring cells [28, 34, 37]. Other less common fusion partners for *ALK*, such as *KIF5B* and *TFG,* have also been described in NSCLC [32, 38]. In most cases, *ALK* rearrangements are non-overlapping with other oncogenic mutations found in NSCLC, such as *EGFR* and *KRAS* mutations [28–30, 39]. The most common methods to detect *ALK* fusion genes include break-apart fluorescence in situ hybridization (FISH), IHC, and reverse transcription polymerase chain reaction (RT-PCR). Break-apart FISH has been the standard method for confirmation of *ALK* status in clinical trials [40]. In preclinical analyses, a selective ALK inhibitor (Crizotinib, PF-02341066, Pfizer) reduced the proliferation of cells carrying genetic alterations in *ALK*, supporting the role of ALK in malignant proliferation and crizotinib as a valid therapeutic target [41]. As with all targeted therapies, resistance to crizotinib is a significant issue for therapy, and most patients experience crizotinib resistance as described in a young patient with *EML4–ALK*-positive NSCLC [42]. Two independent mutations were identified cases that developed resistance: a substitution of adenine for guanine at position 4374 of *EML4–ALK*, resulting in replacement of cysteine with tyrosine at position 1156 of *ALK* (C1156Y), and a substitution of adenine for cytosine at *ALK* position 4493, resulting in replacement of leucine with methionine at position 1196 of *ALK* (L1196M) [42]. A third mutation (F1174L) has been identified in a patient with *RANBP2–ALK*-positive inflammatory myofibroblastic tumor, and it was associated with decreased sensitivity of Ba/F3 cells to crizotinib, although this mutation was unlikely to directly prevent binding of crizotinib to ALK [43]. Further investigations to understand the resistance mechanisms to crizotinib are necessary as well as study of potential combination therapies with different intracellular signaling inhibitors to target proliferation and resistance pathways.

3.3 Human Epidermal Growth Factor Receptor 2 (HER2)

HER2, also known as *ERBB2*, *NEU*, or *EGFR2*, is one of the members of the EGFR family and plays an important role in cell growth, differentiation, and survival. *HER2* encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases and is a proto-oncogene located on chromosome 17 at position 12 [44]. The HER2 protein has no ligand-binding domain of its own and therefore cannot bind growth factors. However, HER2 does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing

ligand binding and enhancing kinase-mediated activation of downstream signaling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Activating mutations in the tyrosine kinase domain of HER2 have recently been reported in less than 5 % of NSCLC [45–47]. Studies of *HER2* mutations in lung cancer showed association with Asian ethnicity, female gender, and non-smokers and with adenocarcinoma histology, particularly lepidic pattern [45–47]. However, *HER2* mutations can also be found in other patient subsets of NSCLC, including in former and current smokers [45, 46]. The vast majority of *HER2* mutations are represented by a 12-base pairs duplication/insertion of the amino acid sequence YVMA in exon 20 at codon 776 [48, 49]. The exon 20 insertion results in increased HER2 kinase activity and enhanced signaling through downstream pathways, resulting in increased survival, invasiveness, and tumorigenicity [50].

3.4 ROS Proto-Oncogene 1, Receptor Tyrosine Kinase (ROS)

The *ROS1* proto-oncogene is located on the long arm of chromosome 6 at position 22, and it is part of tyrosine kinase insulin receptor gene family, and is a type I integral membrane protein with tyrosine kinase activity that may function as a growth or differentiation factor receptor. *ROS1* rearrangements lead to constitutively active fusion proteins and are detected in approximately 1–2 % of NSCLC [51, 52]. In NSCLC, *ROS1* gene rearrangements are associated with adenocarcinoma, and more commonly found in light and never smokers and young patients (<50 years) [51], and are most often mutually exclusive from *EGFR* mutations, *KRAS* mutations, and *ALK* rearrangements [53]. Several different *ROS1* rearrangements have been described in NSCLC as *SLC34A2-ROS1*, *CD74-ROS1*, *EZR-ROS1*, *TPM3-ROS1*, and *SDC4-ROS1* [38, 52, 53]. In preclinical studies, patients with advanced NSCLC harboring *ROS1* rearrangements derived great benefit from crizotinib treatment that targets ROS1 in addition to ALK and MET [51, 52]. Furthermore, in a clinical trial published by Shaw et al. [54] on 50 patients with advanced NSCLC who tested positive for *ROS1* rearrangement, crizotinib showed marked antitumor activity with 33 partial responses and 3 complete responses. Interestingly, no correlation between the type of *ROS1* rearrangement and the clinical response to crizonitib was found [54].

3.5 Ret Proto-Oncogene (RET)

The *RET* gene is located on the long arm of chromosome 10 at position 11.2, and it encodes for a tyrosine kinase that is involved in cell proliferation, migration, and differentiation [55]. Although *RET* point mutations and fusions have long been recognized in medullary and papillary thyroid carcinomas, respectively, *RET*

rearrangements in NSCLC were only recently discovered and involve the kinesin family member 5B (*KIF5B*) among other partners [53, 56–58]. Alternative *RET* fusion partners, such as *CCDC6-RET*, *NCOA4-RET,* and *TRIM33-RET* have since been also described [59]. In early studies, *RET* rearrangements were identified in approximately 1–2 % of NSCLC [53, 57, 58]. Like other rearrangements such as *ALK* and *ROS1*, *RET* fusions are associated with specific clinicopathologic features, such as smoking history, younger patients (age $\leq$ 60 years), and adenocarcinoma histology, especially in those with more poorly differentiated tumors [59]. *RET* rearrangements are usually mutually exclusive with genetic alterations in other oncogenic drivers, such as *EGFR*, *KRAS*, *ALK*, and *ROS1*, [53, 57–59] suggesting that *RET* rearrangements define a new, distinct molecular subset of NSCLC. *RET* fusions have been shown to be oncogenic in models, and some in vitro studies have showed evidence that small molecule inhibitors such as vandetanib, sorafenib, and sunitinib can be used as inhibitors of *RET* fusion products [57, 58]. A recent study showed that cabozantinib, a RET inhibitor, represents a promising targeted therapy for *RET* fusion-positive lung adenocarcinoma cases [60].

3.6 NTRK1 (TrkA) Fusions

Neurotrophic tyrosine kinase, receptor, type 1 (NTRK1), also called tropomyosin receptor kinase A (TrkA) or high-affinity nerve growth factor receptor, is a protein encoded by the gene *NTRK1*, which is located on chromosome 1q21-22 [61, 62]. NTRK1 is a receptor tyrosine kinase, which is part of the tropomyosin-related kinases (TRK) superfamily of receptor tyrosine kinases [61, 62]. NTRK1 acts to control of cell growth and differentiation via the MAPK, phosphatidylinositol 3-kinase (PI3K), and PLC-γ pathways when activated by the nerve growth factor (NGF) ligand [63]. *NTRK1* fusions have been reported in colon cancer, thyroid cancer, and glioblastoma multiforme [64–66]. In a study in lung cancer, *NTRK1* fusions have been found in 3.3 % of the cases (3 out of 91 patients) corresponding to ADC histological type [67]. The same study identified two *NTRK1* fusions, *MPRIP-NTRK1* and *CD74-NTRK1*, which can be detected by fluorescence in situ hybridization (FISH) with an *NTRK1* break-apart probe, although the FISH alone cannot discriminate between the two types of fusions [67]. The *NTRK1* fusions trigger constitutive NTRK1 kinase activity via autophosphorilation leading to the oncogenic process [67]. The importance of the *NTRK1* fusions is that they represent a new potential target for therapy, as preclinical tests in cell lines showed evidence of response to *NTRK1* inhibitors [67]. For instance, promising results have been recently reported on case of a patient with metastatic sarcoma harboring LMNA-NTRK1 fusion after treatment with LOXO-101, a highly selective inhibitor for the TRK family of receptors that can be orally administrated [68]. Nevertheless, further studies are still needed to confirm the value of this new target in human patients with tumors harboring *NTRK1* fusions.

3.7 MET

This gene encodes a receptor with tyrosine kinase activity. The primary single-chain precursor protein is post-translationally cleaved to produce the alpha and beta subunits, which are disulfide linked to form the mature receptor [69]. MET transduces signals from the extracellular matrix into the cytoplasm by binding to hepatocyte growth factor/HGF ligand and regulates many physiological processes including proliferation, motility, invasion, and survival [69]. The *MET* gene is located on the long arm of chromosome 7 at position 31 [70] and can have activating mutations, especially in the semaphorin (sema) domain and juxtamembrane (JM) domain, or it can be amplified [71, 72]. In lung cancer, *MET* gene mutations are found both in extracellular and JM domains [73, 74]. The extracellular sema domain, encoded by exon 2, is required for receptor dimerization and activation [75]. The presence of these mutations has been clearly defined in lung cancer; however, because of certain histologic and ethnic variation, their biologic relevance still needs to be further clarified. In NSCLC, overexpression of MET and HGF protein in tumor tissue and in cells lines have been associated with higher pathologic tumor stage and worse prognosis [76–79] and multiple studies have reported primary *MET* amplification in NSCLC adenocarcinoma ranging from 2 to 20 %, particularly in EGFR TKI-naive patients [80–82]. A recent study unveiled a mechanism of activation of MET via diverse exon 14 splicing alterations (METex14) that occurs in multiple tumor types including lung [83]. The same study showed that METex14 mutations are detected most frequently in lung adenocarcinomas (3 %) [83]. Importantly, in vitro tests showed sensitivity to MET inhibitors in cells harboring METex14 alterations and patients whose tumors harbored these alterations derived meaningful clinical benefit from MET inhibitors [83]. Currently, there are a number of clinical trials for MET and HGF that have shown that MET and HGF can be targeted in patients with NSCLC.

3.8 Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS)

KRAS is an oncogene located on the short arm of chromosome 12 at position 12.1, and it encodes the KRAS protein which is involved primarily in regulating cell division [84]. KRAS is part of a signaling pathway known as the RAS/MAPK pathway, and it relays signals from outside the cell to the cell's nucleus [85]. Activating *KRAS* gene point mutations have been detected in approximately 15 to 25 % of patients with lung adenocarcinoma. *KRAS* mutations are uncommon in lung squamous cell carcinoma [86]. Mutations in the *KRAS* gene have important effects on the process of carcinogenesis, which depend on the cells and tissues involved [87]. The mutations found most frequently in the *KRAS* gene of cancer cells are located at positions 12 and 13 in exon 1, and less frequently in codons 61, 63, 117, 119, and 146 [88]. *KRAS* mutations are associated with tumors from both former/current smokers and never smokers [89]. However, they are less common in never smokers and in East Asian than Western patients [90, 91]. *KRAS* is also one of the most frequently

mutated oncogenes in many cancers, and it is a predictor of resistance to targeted therapy using EGFR TKIs in patients with NSCLC [86, 92, 93]. Molecular analysis revealed that patients who have activating mutations involving exon 1, codons 12, 13, or 61 in the *KRAS* gene with or without increase in *EGFR* copy numbers did not derive benefit from EGFR TKI therapy and had about a 96 % chance of disease progression [94]. Also, *KRAS* mutations may be negative predictors of radiographic response to the EGFR TKIs erlotinib and gefitinib [92, 95].

3.9 B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF)

The *BRAF* gene belongs to a class of genes known as oncogenes. The *BRAF* gene is located on the long arm of chromosome 7 at position 34 [96]. This gene encodes BRAF, a serine/threonine kinase that helps to transmit chemical signals from outside the cell to the cell's nucleus [96]. BRAF is part of a signaling pathway known as the RAS/MAPK pathway, a key molecular cascade that regulates important cell functions such as proliferation, differentiation, migration, and apoptosis. Mutations in *BRAF* are more commonly seen in melanoma (50–70 %) than in lung cancer, where they have been found in 1–4 % [97–103]. In contrast to melanoma, where the majority of *BRAF* mutations occur at valine 600 (V600) within exon 15 of the kinase domain, *BRAF* mutations in lung cancer occur at other positions in exons 11 and 15, within the kinase domain as G469A and D594G, and they are mutually exclusive of *EGFR* and *KRAS* mutations [101]. *BRAF* mutations in NSCLC are most frequently in adenocarcinomas and there are more likely to be found in former and current smokers [101, 102]. Clinically, *BRAF* inhibitors, such as vemurafenib and dabrafenib, have potent and selective activity against the V600-mutant *BRAF* kinases [104, 105]. Agents targeting the BRAF pathway have demonstrated efficacy in NSCLC. For instance, Gautschi et al. [106] have recently documented promising results with BRAF-targeted therapy on BRAF-mutated lung adenocarcinomas. Furthermore, a recent study on multiple non-melanoma tumors BRAF V600-mutated, vemurafenib activity was observed in non-small-cell lung cancer, confirming the potential of BRAF inhibitors for therapy of BRAF-mutated lung cancer [107]. The combination of trametinib and dabrafenib has also demonstrated clinical benefit.

3.10 Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog (NRAS)

The *NRAS* gene is located on the short arm of chromosome at position 13.2, and it encodes a protein called NRAS that is involved primarily in regulating cell division [108]. Although *NRAS* gene mutation might be one of the mechanisms of oncogenesis of lung cancer, this is a very rare event it has been found in $\sim$1 % of all NSCLC [97, 109–111]. *NRAS* mutations are more frequently found in lung cancers

with adenocarcinoma histology and in patients with a history of smoking [81, 112]. In the majority of cases, these mutations are reported at codon 61 and mutations at position 12 have also been described [81]. The result of these mutations is constitutive activation of NRAS signaling pathways. Currently, there are no direct anti-NRAS therapies available, but preclinical models suggest that MEK inhibitors may be effective [81, 113].

3.11 v-AKT Murine Thymoma Viral Oncogene Homolog 1 (AKT1)

The *AKT1* gene is located on the long arm of chromosome 14 at position 14q32.32. *AKT1* gene encodes AKT1 serine/threonine protein kinase found in various cell types, which plays a critical role in many signaling pathways helping in cellular proliferation, differentiation, and cell survival [114]. AKT1 is a downstream mediator of the PI 3K pathway, and it helps to control apoptosis [114]. Somatic mutations in *AKT1* are rare in lung cancer and they have been found in approximately 1 % of all NSCLC including ADC and SqCC [115, 116]. Mutations in the regulatory domain of *AKT1* lead to structural alteration of the ligand-binding site resulting in cellular transformation in vitro and in vivo [117]. AKT1 is predominantly detected in lung epithelium by IHC, while it is absent in stromal cells. In normal lung tissue, AKT1 is exclusively cytoplasmic but in the tumor tissue, the anti-AKT1 antibodies' stain also reinforces the membrane of those cells [116]. Although *AKT1* mutation is a relatively rare event in NSCLC, it may represent a potential molecular target in a subset of NSCLC.

3.12 Mitogen-Activated Protein Kinase 1 (MAP2K1)

The *MAP2K1* gene provides instructions for making a protein known as MEK1 protein kinase. MEK1 is part of a signaling pathway called the RAS/MAPK pathway, which transmits chemical signals from outside the cell to the cell's nucleus [118]. RAS/MAPK signaling helps control the proliferation, differentiation, and apoptosis of cells. The *MAP2K1* gene is located on the long arm of chromosome 15 between positions 22.1 and 22.33. Somatic mutations in *MAP2K1* have been found in less than 1 % of all NSCLC and are more common in adenocarcinoma than squamous cell carcinoma [119, 120]. In a retrospective study of lung adenocarcinoma cases with *MEK1* gene mutation, these mutations were more strongly associated with former smoking status, and there were no other associations with age, sex, race, or stage [119]. The most frequent mutations of *MAP2K1* were K57N (64 %) and Q56P (19 %), and *MEK1* mutations were mutually exclusive of mutations in *EGFR*, *KRAS*, *BRAF,* and other driver mutations [119].

3.13 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha (PIK3CA)

The *PIK3CA* gene is located on the long arm of chromosome 3 at position 26.3 [121]. The *PIK3CA* gene encodes p110 alpha protein, which is one piece, a subunit, of an enzyme called PI3K [122]. The p110α protein is called the catalytic subunit because it performs the action of PI3K, while the other subunit regulates the enzyme's activity [121]. PI3K signaling is important for many cell activities, including cell proliferation, migration, and cell survival. Mutations of *PIK3CA* occur in many human epithelial cancers, resulting in *PIK3CA* being one of the two most commonly mutated oncogenes, along with *KRAS*, identified in human cancers [121, 123]. However, individual types of epithelial cancers show great variability in their mutational rates, and the rates described in NSCLC are relatively low, 1–3 % [124, 125], and usually affecting the helical binding domain (exon 9, E545K, or E542K) or the catalytic subunit (exon 20, H1047R, or H1047L) [126]. *PIK3CA* mutations appear to be more common in squamous cell histology than in adenocarcinoma [124] and can occur in never and ever smokers. *PIK3CA* mutations can co-occur with *KRAS* and *EGFR* mutations, and it is more frequently seen with *KRAS* than with *EGFR* [91, 127]. *PIK3CA-KRAS* co-mutation is more prevalent in Western countries [128], while *PIK3CA-EGFR* is more prevalent in lung cancer patients from Eastern countries [91, 129].

4 Diagnostic and Molecular Testing of NSCLC

Today the diagnosis of NSCLC usually starts with a small biopsy like a core needle biopsy (CNB) or a cytological like a fine needle aspiration (FNA), in which the pathologist has to make the best effort to not only provide a diagnosis of NSCLC and to further classify it as an ADC or SqCC [5, 6]. The advent of targeted therapy introduced a new challenge to the pathologist in order to maximize the efficiency in the use of small samples for clinical diagnosis and molecular testing. Overall, the diagnostic and molecular testing of NSCLC samples involves the following steps.

4.1 Pathological Diagnosis of NSCLC

As stated before, the pathology classification starts with a hematoxylin–eosin-stained tissue section evaluated under the microscope for morphological changes to identify the presence of a NSCLC and then try to classify it as any of the major subtypes such as ADC, SqCC, LCLC, or special subtypes. However, sometimes, the histology evaluation can be limited, particularly in small biopsies or tumors with poor differentiation, which can make challenging the classification of the neoplasm. In these cases, ancillary diagnostic techniques will help in the pathology classification. Some of the main ancillary diagnostic markers are TTF-1, p40, and mucicarmin.

4.1.1 Thyroid Transcription Factor 1 (TTF-1)

Also called NK2 homeobox 1 (NKX2-1), this is a protein encoded by the gene *NKX2-1*. TTF-1 regulates the transcription of genes specific for the thyroid, lung, and diencephalon differentiation. In diagnostic pathology, the IHC detection of TTF-1 in the nucleus of cells is a tool for the identification of thyroid or lung differentiation. In normal lung, TTF-1 labels some of the bronchial epithelial cells, type II pneumocytes, and club cells (Clara cells). In tumors, TTF-1 is expressed on 60–74 % of ADC and between 6 and 32 % of SCC, depending on the study and the antibody used [130]. In diagnostic pathology, TTF-1 expression is considered a marker that favors the diagnosis of ADC [12]. Interestingly, TTF-1 expression has been found to correlate with a better prognosis in ADC [130, 131].

4.1.2 p40

This is an isoform of p63 protein also called ΔNp63-a, encoded by the gene *TP63*. ΔNp63 is involved in multiple functions during skin development and in adult

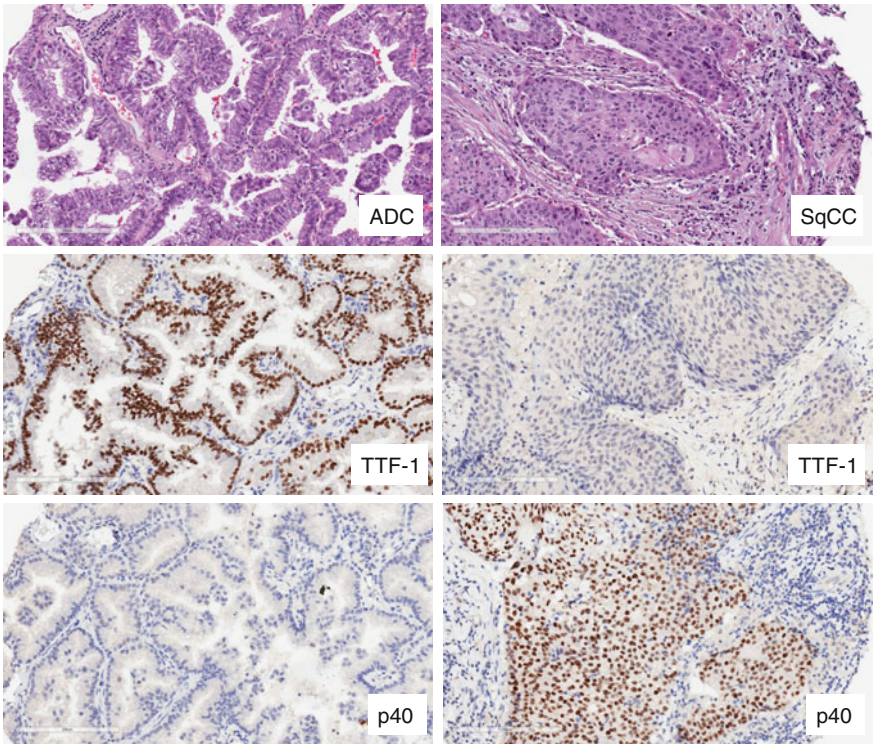

Fig. 2 Example of an adenocarcinoma (*ADC*) and squamous cell carcinoma (*SqCC*). ADC is a tumor showing epithelial cells arranged in glandular like structures, which is usually positive for TTF-1 and negative for p40. Instead, SqCC is composed by tumor epithelial cells arranged in a solid fashion, sometimes showing signs of squamous cell differentiation such as keratinization, which is usually negative for TTF-1 and positive for p40. (Microphotographs taken from Aperio scanned slides using a 20× objective lens)

stem/progenitor cell regulation [132]. In pathology, p40 is expressed in the nucleus of many basal cells (prostate, breast epithelia) and in squamous cells. In diagnostic pathology, p40 is used as a marker for squamous cell differentiation as it labels near 100 % of SCC and up to 3 % of ADC [12]. In general, p40 and TTF-1 are used in combination (Fig. 2) [12].

4.1.3 Mucicarmin

This is an old histochemical stain employed for the detection of mucins, which are high-molecular-weight glycoproteins that are found dispersed throughout several glandular epithelia, including respiratory epithelium. Mucicarmin can also be employed for the differential diagnosis of ADC, particularly in the identification of solid variants, being considered positive when five or more tumor cells are found to show mucin staining in the cytoplasm in two microscopic high-power fields (×400) [6, 14, 133].

4.2 Molecular Testing of NSCLC

The advent of targeted therapy has opened a new door for the discovery and validation of novel biomarkers with prognostic and therapeutic value [5]. Today, the pathologist has to further classify a NSCLC as ADC or SqCC employing the previously mentioned analysis techniques on small samples such as CNB or FNA [5, 6]. The importance of this diagnostic effort is based on the novel therapeutic approaches for ADC. For instance, a tumor classified as ADC or NSCLC favor ADC will undergo routine molecular testing for the currently most important molecular alterations described before, including *EGFR* mutations and *ALK* and *ROS* rearrangement analyses, offering the option of a targeted therapy for these molecular alterations to the cancer patient. Currently, novel multiplexing technologies such as multiplex-PCR platforms and next-generation sequencing (NGS) techniques allow for specific and high-throughput molecular profile of individual tumors, which is necessary for a precision medicine approach for the cancer patient [5].

5 Future Directions: Immunotherapy Revolution and Its Integration with Lung Cancer Diagnostics and Therapy

The development and application of high-throughput molecular profiling techniques for the cancer patient allows for further identification and validation of novel biomarkers of cancer, which can help to define a precision medicine approach for cancer therapy [134]. For instance, precision medicine requires a detailed molecular profile of the tumor for an individual patient that will allow the design of a specific targeted therapy strategy for the particular tumor.

A novel area of cancer therapy is represented by the development of immunotherapy for cancer. The specific blocking of immune checkpoints such as programmed death ligand 1 (PD-L1, also known as B7-H1 or CD274) and programmed cell death protein 1 (PD1, or CD279) can unleash the immunological system, particularly T-cell lymphocytes, to attack the cancer cells [135, 136]. Immunotherapy has shown promising results in several solid tumors, including melanoma, kidney cancer, and lung cancer, however, in lung cancer, further studies and clinical trials are needed [137–139]. Currently, IHC markers for the expression of key immune checkpoints such as PD-L1, and also others like PD-L2, VISTA, B7-H3, and B7-H3, are being added to the pathological analysis of NSCLC [140]. Also, particular attention is being paid to the amount and composition of the inflammatory cells present in the tumor region, which includes different subpopulations of tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) [141]. For instance, a novel immunological classification of tumors has been proposed based on the T-cell infiltration and the PD-L1 expression, classifying them into four categories: type I or adaptive immune resistance (TIL+/PDL1+), type II or immunological ignorance (TIL−/PDL1−), type III or immunological tolerance (TIL+/PDL1−), and type IV or intrinsic induction (TIL−/PDL1+) [142]. The demonstration of the clinical value of such immunological classification of cancer for immunotherapy will enforce pathologists to incorporate immunological markers into their clinical practice.

One of the challenges for the pathologists is the evaluation of PD-L1 expression in IHC assays on biopsies. Currently, there are several antibodies and clones available, employing property staining platforms and particular scoring systems, and some of them are not fully validated [143]. One of the validated and FDA approved PD-L1 antibodies is clone 22C3 (Dako); its cell membrane expression on at least 50 % of tumor cells has a positive correlation with improved efficacy of pembrolizumab, a monoclonal therapeutic antibody targeting PD1 [144]. Another FDA approved assay for PD-L1 IHC is antibody clone 28-8 (Dako and Abcam), a complimentary test for nivolumab, a human IgG4 PD-1 inhibitor. Interestingly, a recent study comparing nivolumab and docetaxel in patients with advanced, previously treated lung squamous cell carcinomas, the overall survival, response rate, and progression-free survival were significantly better with nivolumab than with docetaxel, regardless of PD-L1 expression level evaluated with the PD-L1 antibody clone 28-8 [145]. A similar study in advanced non-squamous cell carcinomas of the lung comparing nivolumab and docetaxel also showed longer overall survival with nivolumab than with docetaxel in patients with tumors expressing PD-L1 [146]. In this study, there was a trend toward a greater response rate as the PD-L1 expression level increased (>1, 5, and 10 % of membranous positive tumor cells); however, a meaningful separation of the overall survival curves was observed across all expression levels [146]. Interestingly, both studies seem to suggest that nivolumab is a reasonable therapy for advanced NSCLC regardless of the PD-L1 IHC expression level [145, 146]. Another challenge in the evaluation of PD-L1 is the fact that PD-L1 is expressed not only in the tumor cells but also in the tumor inflammatory infiltrate component, including macrophages,

dendritic cells, and T cells [147]. For instance, PD-L1-positive tumor-infiltrating inflammatory cells have been found to be more common than PD-L1-positive tumor epithelial cells [147]. A study by Herbst et al. [147] showed evidence that the evaluation of PD-L1 in both cell compartments, tumor cells and the inflammatory cell infiltrate, can have clinical relevance. Furthermore, as the tumor-infiltrating inflammatory cell component may have relevance for the clinical response to immunotherapy, also the mutation burden in tumors may be relevant. In a study by Rizvi et al. [148] on NSCLC, higher non-synonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and progression-free survival. Taking this information together, it is likely that a new classification of lung cancer will include the immunoprofiling status integrated with the mutation status of key genes.

6 Conclusions

Lung cancer is a complex and heterogeneous group of diseases in which a multidisciplinary approach for diagnosis, classification, and therapy is needed. The advent of large-scale molecular profiling and targeted therapy represent the main future direction for personalized and efficient cancer therapy. In this regard, the ongoing cancer immunotherapy revolution is already redefining the classification and treatment of cancer, offering promising therapeutic windows to lung cancer patients. However, further research is still needed to integrate the complex information from genomics and immunology into a new classification of lung cancer with clinical relevance for therapy and improved outcomes.

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