Molecular Carcinogenesis of Lung Cancer

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Contents

1 Introduction 1
2 Clinically Relevant Molecular Alterations 3
2.1 EGFR 4
2.2 ALK 5
2.3 ROS1 6
2.4 BRAF 7
2.5 HER2 (ERBB2) 7
2.6 MET 8
2.7 RET 8
2.8 NTRK 9
2.9 KRAS 9
2.10 NRG1 10
3 Molecular Testing in Lung Cancer 10
3.1 Immunohistochemistry 13
3.2 Liquid Biopsy Testing in NSCLC 14
3.3 Biomarkers in Immunotherapy 14
4 Tumor Mutational Burden and Immune Microenvironment 15
References 16

1 Introduction

The molecular basis of lung cancer is the progressive accumulation of mutations and epigenetic abnormalities leading to the expression of multiple genes with different functions. These changes lead to the weakening of the DNA structure and its greater susceptibility to subsequent mutations. This is caused by irregularities in cell cycle regulation (mutations of protooncogenes and suppressor genes) and disorders in the repair processes in damaged DNA. Several signaling pathways and specific

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oncogenes are deregulated in lung cancer and are known to influence modifications related to tumorigenesis, including apoptosis, proliferation, cell-cycle progression, and gene expression [1]. These key pathways and oncogenes affect the development and progression of lung cancer as well as prognosis and resistance.

Lung cancer is characterized by a complex molecular background; the clinical and therapeutic managements are driven almost entirely by a smaller group of clinically actionable molecular alterations, the majority of which occur more commonly in lung adenocarcinoma [2, 3]. The number of driver mutations exerting an effect on the carcinogenesis is limited. The majority of somatic mutations are regarded as passenger mutations have no direct effect in terms of selective growth advantage for cancer cells, but they may be associated with a clonal expansion when occurring concomitantly with a driver mutation [1]. Genomic alterations encompass mechanistic rearrangements of DNA, resulting in single nucleotide variants (SNVs) known as point mutations and small-scale deletions/insertions (indels) or copy number variants (CNVs), which reflect large-scale mutations such as amplifications, deletions, inversions, and translocations [4].

Lung adenocarcinoma is the most common subtype of non-small cell carcinoma (NSCLC) and is characterized by a complex genomic landscape defined by multiple types of mutations, a lower level of copy number alterations than squamous cell carcinoma, and some gene rearrangements that provide therapeutic targets when present [2, 4]. Somatic alterations of various types including substitutions, insertions, deletions, and splice site mutations have all been demonstrated to play a role in the carcinogenesis of adenocarcinoma [5, 6]. While TP53 and LRP1B mutations are common in all subtypes of NSCLC, adenocarcinoma appears to have higher rates of somatic mutations in *KRAS*, *EGFR*, *KEAP1*, *STK11*, *MET*, and *BRAF* as compared to lung squamous cell carcinoma. Mutations in these genes tend to primarily disrupt the RAS-MEK-ERK and PIK3CA-MTOR pathways [5–8].

In a population with lung adenocarcinoma, approximately 30% will harbor an oncogenic alteration that predicts response to TKI therapy. While numerous different types of somatic alterations have been described, targetable or potentially targetable mutations and rearrangements include alterations in *EGFR*, *ALK*, *ROS1*, *BRAF*, *HER2*, *RET*, *MET*, and *NTRK* [3, 4, 8, 9]. Oncogenic driver alterations (e.g., *EGFR*, *KRAS* mutations) are mutually exclusive in most cases at least in the untreated setting, suggesting a state of oncogenic addiction that confers susceptibility to targeted inhibition.

Squamous cell carcinoma (SCC) is characterized by a complex molecular background which shares many of the same molecular features as lung adenocarcinoma but retains a group of relatively discrete alterations. One of the most comprehensive studies by The Cancer Genome Atlas (TCGA) of 178 lung SCCs identified numerous recurrent somatic mutations primarily involving *TP53*, *LRP1B*, *CDKN2A*, *PTEN*, *PIK3CA*, *KEAP1*, *MLL2*, *HLA-A*, *NFE2L2*, *NOTCH1*, *RB1*, and *PDYN* [10]. Unlike lung adenocarcinoma, lung SCCs generally do not harbor targetable driver mutations, thus driving treatment algorithms primarily toward immunotherapy and chemotherapy, although recurrent *DDR2* mutations and *FGFR1* amplification have been reported as potential therapeutic targets [11, 12].

Activation of the PI3K/AKT/mTOR pathway is central to cellular proliferation, growth, and apoptosis. Signaling through the PI3K/AKT pathway is negatively regulated by the tumour-suppressor gene PTEN (phosphatase and tensin homologue) [13]. Although aberrations in the PI3K pathway are particularly prevalent in some cancers (including invasive breast cancer, head and neck cancer, colon cancer, cervical and ovarian cancer), they are rare in lung cancer. Alterations in *PIK3CA*, *AKT1*, and *PTEN* occur frequently in SCC and smokers. The most frequent alterations are gain-of-function mutations in *AKT1* and *PIK3CA* and the loss of *PTEN* function (mutations or deletions). *PIK3CA* mutations may promote resistance to EGFR TKIs in mutated NSCLC. Inhibition of *AKT* and *PI3K*inase could be a treatment option in SCC, but often overlap with other molecular changes, which rather represents a "passenger" mutation than a driver. *PI3K* aberrations are associated with an unfavorable prognosis, higher burden of metastatic disease, and higher incidence of brain metastases [14]. The effects of *PIK3CA* mutations on prognosis and response to standard therapies remain unknown.

2 Clinically Relevant Molecular Alterations

While NSCLC shows a high degree of genomic complexity as described above, current treatment regimens rely primarily on targeted therapies directed at a much smaller group of so-called clinically relevant, actionable, and targetable genetic alterations (Table 1).

Table 1 Biomarkers in NSCLC patients

Gene/protein	Predictive alteration	Methodology
EGFR	Mutation	PCR: sanger, real-time PCR and NGS
ALK	Rearrangement	ıHC, FISH, and NGS
ROS1	Rearrangement	IHC (screening), FISH, and NGS
BRAF	Mutation	PCR: sanger, real-time PCR and NGS
PD-L1	Overexpression	IHC
HER2	Mutation amplification	PCR: sanger, real-time PCR and NGS FISH, NGS, real-time PCR
MET	Mutation Amplification	NGS FISH, NGS, real-time PCR
RET	Rearrangement	FISH and NGS
NTRK	Rearrangement	IHC (screening) and NGS
TMB	Mutations[a]	NGS

EGFR epidermal growth factor receptor, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry, *NGS* next-generation sequencing, *NSCLC* non-small cell lung cancer, *PCR* polymerase chain reaction, *TMB* tumor mutational burden

[a]Measurement of somatic mutations present in tumour cells

2.1 EGFR

The epidermal growth factor receptor (*EGFR*) gene encodes for the epidermal growth factor receptor (*EGFR*) protein, which is a member of the receptor tyrosine kinase (RTK) family and is involved in numerous cellular pathways including the PI3K-AKT-mTOR and RAS-RAF-MEK-ERK. Activating *EGFR* mutations can result in constitutive activation of the receptor, independent of ligand binding, leading to tumor development and growth [15]. *EGFR* mutation represents a major oncogenic driver in NSCLC, in particular in lung adenocarcinomas arising in light and nonsmokers and females with lepidic and/or papillary histology [16]. *EGFR* mutations are present in approximately 15–20% of patients with adenocarcinoma in Western and 25–50% in Asian populations and are largely mutually exclusive with mutations in *KRAS* or *ALK* rearrangements [17]. Most *EGFR* mutations occur in exons 18–21 of the tyrosine kinase domain, with the most common mutations being exon 19 deletions and a L858R point mutation in exon 21 that confer sensitivity to targeted therapies with tyrosine kinase inhibitors (TKIs), while other mutations, such as the *EGFR* T790M point mutation on exon 20 or exon 20 insertions, confer resistance to first-generation EGFR TKIs [18, 19]. Patients with sensitizing mutations may also respond differentially to TKI therapy depending on the specific mutation present [20, 21]. Nevertheless, at least for some of these genetic alterations (mainly exon 18 [21]), TKIs led to remarkable responses and can be considered as valuable treatment options.

EGFR exon 20 insertion occurs in 4–10% of NSCLC and involves the addition of 1–7 amino acids between residues 762 and 774 in the C-helix or regulatory domain of the kinase, leading to constitutive activation and dimerization of *EGFR*. By knowledge about the *EGFR* exon 20 insertion mutants that modify the structure and conformation of *EGFR*, new inhibitors had been designed to specifically target this cohort of *EGFR*-mutant lung cancer [22].

Resistance to a targeted therapy may be primary, resulting from concomitant alterations present at the time of therapy initiation, or acquired, resulting from mutations that manifest after treatment with a particular therapy has occurred.

Resistance to *EGFR* TKIs usually arises with the acquisition of secondary mutations in *EGFR* which prevent the drug from binding to the protein or by activation of bypass tracts which stimulate cell growth independent of *EGFR* [23]. When first- or second-generation *EGFR* TKIs are used initially (gefitinib, erlotinib, afatinib, or dacomitinib), the occurrence of a second *EGFR* mutation in exon 20 T790M mutation represents the most frequent mechanism of resistance. Because a potent drug for this situation, osimertinib, is available, testing for T790M is mandatory in patients developing resistance on TKI therapy [24]. Repeat biopsy or liquid biopsy in this setting detects the resistance mutation T790M in approximately 45–50% of *EGFR*-mutant NSCLC treated with first- and second-generation TKIs [25]. Sensitive methods show that tumor clones harboring the T790M mutation can generally be detected before the initiation of first-generation TKI [26]. Rarely, *EGFR* T790M mutation can occur as a germline mutation and is associated with familial forms of lung adenocarcinoma [19]. Resistance to this mutation was overcome with

the development of newer TKIs such as osimertinib; however it was found that tertiary *EGFR* mutations such as the *EGFR* C797S mutation could then develop that provided additional resistance [27]. The most important problem in disease progression is to obtain a new sample to inform the choice of second-line therapy. Generally, in patients with low performance, biopsy is performed from the most accessible lesion. However, due to tumor heterogeneity, a single sample may be insufficient to accurately represent all existing resistance mechanisms. In treatment, the possibility of subclonal driver genetic changes in more than one disease area should be considered [28]. *EGFR* gene amplification, as detected by NGS or FISH, may play a role in resistance to *EGFR* TKI therapy in patients with a preexisting *EGFR* mutation. *EGFR* amplification on its own, however, is not considered a biomarker of response to any currently approved therapies and should not be used to select patients for *EGFR* TKI therapy [29]. *EGFR* mutations are typically mutually exclusive with KRAS mutations and some other driver alterations, although they can co-occur in some populations, particularly as resistance mechanisms in patients being treated with TKIs [30, 31].

Other mechanisms of primary or secondary resistance include downstream pathway activation as *PTEN* loss; mutations of *PI3KCA*, *KRAS*, and *BRAF* V600; bypass pathway activation as *MET* and *HER2* amplifications; *AXL* and *HGF* overexpression; dysregulation of cell cycle genes; and alterations of some nuclear transcription factors. Another, less common, resistance mechanism that occurs in TKI-treated NSCLC is transformation to different histologies, such as small cell lung carcinoma (SCLC) and squamous cell carcinoma (SCC) morphology that is likely driven by specific underlying genetic alterations [32–34].

2.2 ALK

ALK, a member of the insulin receptor tyrosine kinase family, is encoded by the *ALK* gene on chromosome 2. Rearrangements involving *ALK* are characterized by the control of the kinase domain of *ALK* under the promoter of the fusion partner. The most common is an EML4-ALK rearrangement that leads to an oncogenic fusion protein with the downstream effect of increased tyrosine kinase activity [35]. This results in constitutive activation of the kinase domain that is independent of ligand binding. Subsequently, there is activation of downstream signaling pathways, including the PI3K-AKT, MAPK, and JAK-STAT pathways, promoting uncontrolled proliferation, survival, and migration [36].

ALK rearrangement is found in approximately 3–5% of lung adenocarcinomas, and the patients are of relatively young age and nonsmokers or only light smokers, with no difference in ethnicity or sex [37]. Distinctive histologic patterns including solid growth with focal signet ring cells and mucinous cribriform pattern associated with abundant extracellular mucin occur more frequently in *ALK*-rearranged lung adenocarcinomas [38]. *ALK*-rearranged adenocarcinomas are almost always mutually exclusive with other driver mutations, such as *EGFR* and *KRAS* [39]. These factors could help clinicians to identify high-risk populations that can undergo *ALK*

testing, even if these criteria are not strong enough to select patients based on epidemiological characteristics.

ALK-rearranged tumors are sensitive to multiple generations of TKIs, and sequential treatment with different TKIs, which dock at the kinase domain of the altered ALK protein, is established as the standard therapy of metastatic disease [40, 41]. Secondary resistance mutations occur in the ALK kinase domain. Therefore, primarily the oncogenic fusion has to be proven, when resistance develops and analysis of the *ALK* gene has to be performed. The patients developing secondary *ALK* mutations are of special interest, as the different available ALK inhibitors display different therapeutic activity against some of these fusion proteins. In addition, different TKIs have different activity for brain metastasis [42].

Resistance to ALK-TKI therapy can also be developed by many ALK-independent mechanisms. EGFR pathway activation (via EGFR ligands and/or receptor upregulation), insulin-like growth factor-1 receptor pathway activation, SRC signaling, *c-KIT* amplification, mitogen-activated protein kinase (MAPK) pathway activation (*KRAS* copy number gain or phosphatase *DUSP6* loss), and protein kinase C activation (mediated by P2Y receptors) may occur by several ALK-independent mechanism [43, 44]. Small cell and squamous cell transformation as a resistance mechanism in *ALK*-rearranged NSCLC has also been described [45, 46].

2.3 ROS1

The *ROS1* gene is located at chromosome 6q22 and encodes a receptor tyrosine kinase that is part of the insulin receptor family with downstream signaling pathways, RAS/RAF/MAPK, PI3K/AKT/mTOR, and STAT-3, and leads to cell growth, proliferation, and survival. Gene rearrangements (translocations and deletions) create fusion proteins of ROS1 with a number of different partners in which the kinase domain of *ROS1* turns constitutively activated [47]. Although *ROS1* is a distinct receptor tyrosine kinase, it is structurally similar to the ALK protein due to similarities in their kinase domains and ATP binding sites [48]. *ROS1* gene rearrangements occur approximately 1–2% of NSCLC patients and are associated with younger age, female, and a history of never or light smoking [47, 49]. *ROS1*-rearranged NSCLC shows a similar clinical phenotype to *ALK*-rearranged carcinomas, although some studies have reported lower rates of extrathoracic metastasis, including fewer brain metastases [50]. Similar to *ALK*, *ROS1*-rearranged adenocarcinomas often show solid pattern with signet-ring cell morphology and/or mucinous cribriform pattern [51]. Testing for ROS1 status is important to be able to identify patients who may have a good response to crizotinib and ceritinib and possibly to other *ROS1* therapies [52].

As in *ALK*-rearranged tumors, different mechanisms of acquired resistance after TKI treatment have been reported [53]. In 50–60% of these cases, the predominant resistance mechanism to crizotinib treatment occurs in the form point mutations in *ROS1*, with the G2032R variant being the most frequent. Newer-generation kinase

inhibitors are becoming available (ceritinib, entrectinib) that will allow for treatment of crizotinib-resistant *ALK*-rearranged NSCLC [54, 55].

2.4 BRAF

BRAF gene encodes a serine-threonine protein kinase promoting cell proliferation and survival through the RAS-RAF-MAPK growth and proliferation signaling MAPK pathway and exerts their effect predominantly through phosphorylation-mediated activation of MEK [56]. *BRAF* mutations are found in 2–4% of NSCLC in which half of the mutations are a V600E substitution and the other mutations occur at different positions within the kinase domain and more frequently found in current or former smokers. Mutations involving codon V600 are activating, whereas mutations involving other codons may be inactivating [57]. This has clinical implications as V600E-mutated NSCLCs are sensitive to inhibitor therapy with dabrafenib and vemurafenib, while non-V600E tumors are resistant [58, 59]. While preclinical evidence suggests that non-V600E mutations may be targetable with alternative therapies, such as MEK inhibitors, there are currently no clear consensus guidelines of the management of non-V600E-mutated NSCLC. Studies have reported conflicting clinicopathological characteristics for *BRAF*-mutated NSCLC that appear to shift depending on the population examined and whether the mutation present is V600E or non-V600E [59, 60]. Also, in contrast to other tumor types where *BRAF* and *KRAS* mutations appear to occur in a mutually exclusive fashion, *BRAF* exon 11 mutations may be found together with *KRAS* and *EGFR* mutations. Comprehensive data on the kinase activity of all known exon 11 mutations are lacking; thus these co-occurring mutations may simply be "passengers" that may have little bearing on the growth/proliferative effects resulting from better-understood upstream pathway alterations [61, 62].

Despite a high rate of initial responses, most patients experience disease relapse due to the development of secondary resistance to *BRAF* inhibition. Resistance mechanisms include on-target mechanism of resistance (e.g., variant form of *BRAF* V600E which dimerizes with regular *BRAF* V600E to induce resistance), paradoxical activation of MAPK pathway resulting in sustained ERK activation, and bypass pathways (e.g., PI3K/Akt/mTOR) [63].

2.5 HER2 (ERBB2)

Human epidermal growth factor receptor 2 (*HER2*) is also known as erbB-2 receptor tyrosine kinase 2 (*ERBB2*) and is a member of the RTK gene family activation signaling through the PI3K-AKT and MEK-ERK pathways [64]. *HER2* mutations are oncogenic and result in constitutive HER2 phosphorylation and activation and EGFR pathway stimulation. The best-defined mechanism of *HER2* activation in lung adenocarcinoma is in the form of small exon 20 in-frame insertion mutations. Copy number changes have not been a component of regular mutation screening in lung

adenocarcinoma, but appear to correlate with the presence of an *HER2*- activating mutation [65]. *HER2* exon 20 mutations have been reported in 1–3% of lung adenocarcinomas, are more common in an older patient population and never smokers, and occur almost exclusively with adenocarcinoma histology and high morphological grade [64–66]. *HER2* mutations occur in a mutually exclusive fashion with other defined oncogenic alterations, although other mutations and amplification events, sometimes in combination with alterations in other genes, have been observed [67]. As with other genes, resistance mechanisms have been described, and *HER2* amplification itself is a reported resistance mechanism in *EGFR*-mutated NSCLC treated with TKI therapy [67, 68].

2.6 MET

MET and its ligand hepatocyte growth factor play a role in cell growth and development and epithelial-mesenchymal transition in cancer cells, through the activation of the downstream cellular pathways including PI3K-AKT-mTOR, STAT, and MAPK which promotes cell proliferation and invasion, blocking apoptosis, and increasing cell motility [69]. *MET* point mutation occurs in 3–4% of metastatic NSCLC, with the most common being exon 14 skip mutations (*MET*ex14), which occur in 2–3% NSCLC [70]. Mutations in the intronic splice donor site alterations, including substitutions and insertion/deletions, affect the normal *MET* gene splicing mechanism and lead to the loss of MET exon 14 (so-called *MET* exon 14 "skipping" mutations), which results in decreased degradation of the MET receptor, accumulation at the cell surface, and increased activation of downstream signaling pathways [70, 71].

In 5–20% of EGFR-mutated adenocarcinomas, *MET* amplification and increased copy number can be a mechanism for resistance to EGFR TKIs [72]. *MET* alterations are generally mutually exclusive with other driver alterations. Studies examining *MET* alterations have associated them with older patient populations, significant smoking history, and a poor prognosis when *MET* amplification is present [73–75]. These studies have also revealed that *MET* alterations can be identified in nearly all histologic subtypes of NSCLC, but that they appear to be enriched in up to 20% of sarcomatoid carcinomas, particularly *MET* exon 14 "skipping" mutations [76]. Patients with high-level amplification, defined as a *MET:CEP7* ratio $\geq$5.0, may respond to MET inhibitors such as crizotinib [71, 77]. *MET* exon 14 skipping mutation is amenable to therapy with MET inhibitors such as crizotinib and capmatinib, although like other genes, numerous ancillary resistance mechanisms have been documented [78, 79].

2.7 RET

Ret protooncogene tyrosine-protein kinase receptor (*RET*) is a receptor tyrosine kinase that mediates neural crest development; its activation leads to cellular

proliferation, migration, and differentiation. *RET* rearrangement results in ligand-independent dimerization and downstream growth pathway activation, many of which are shared with other genes mutated across NSCLC. Multiple 5′ rearrangement partners have been identified, although the most common appears to be KIF5B [80]. *RET* rearrangements occur in 1–2% of NSCLC and tend to correlate with never or light smokers, younger than 60 years, and are equally common in men and women [81].

Studies have shown *RET*-rearranged NSCLC to be sensitive to a number of inhibitor agents, although it is unclear whether there is variation in treatment response depending on the fusion partner present [82, 83]. Resistance mechanisms to inhibitor therapy in *RET*-rearranged NSCLC consist of various on-target secondary *RET* mutations as well as off-target bypass signaling mechanisms that result in RAS-MAPK pathway activation [84].

2.8 NTRK

The neurotrophic tyrosine receptor kinase (*NTRK*) gene is a member of the tropomyosin-receptor kinase family of RTKs that includes *NTRK1*, *NTRK2*, and *NTRK3* [85]. Binding of the ligands to the respective NTRK activates downstream PI3K/AKT/mTOR, RAS/RAF/MAPK, and PLC-γ, leading to proliferation, growth, and survival of neurons in the peripheral and central nervous system. NTRK dysregulation can result from chromosomal rearrangement, point mutation, overexpression, and alternative splicing [86]. Gene fusion of *NTRK1*, *NTRK2*, and *NTRK3* occurs in 3.5%, 0.2–1%, and <1% of NSCLC, respectively [87]. Studies examining the clinicopathologic characteristics are limited by the small number of reported cases with *NTRK* fusions; however it appears that *NTRK* fusions tend to be mutually exclusive with other driver alterations and may be enriched in minimal or never smoker patient populations [88]. Fusions can occur within the squamous cell and adenocarcinoma subtypes of NSCLC and have also been described in a pulmonary neuroendocrine carcinoma [88, 89]. *NTRK*-rearranged NSCLCs are amenable to targeted therapy with the tyrosine kinase inhibitors entrectinib and larotrectinib [87, 90]. Resistance mechanisms in *NTRK*-rearranged cancers have already begun to be identified and include on-target *NTRK* mutations as well as alternative pathway signaling [91].

2.9 KRAS

KRAS is a member of the RAS protooncogene family and encodes a membrane-bound guanosine triphosphate (GTP) binding protein with intrinsic GTPase activity that is part of the mitogen-activated protein kinase (MAPK) cascade. *KRAS* mutations can be indicative of worse prognosis, especially *KRAS* G12C and G12V mutations that are associated with poor overall survival (OS), but can also be predictive for poor response to chemotherapy and predictive for immune modulatory

therapy [92]. *KRAS*-mutated lung cancer is a rapidly evolving area of research in the search for providing more treatment options for these patients with unmet need. A targeting compound for *KRAS* G12C mutated NSCLC, early-phase trials of two $KRAS^{G12C}$ inhibitors has achieved for treatment option for metastatic NSCLC [93].

KRAS-mutant NSCLCs can be divided into subgroups with potential prognostic and predictive significance, according to the presence of co-occurring tumor suppressor genes alterations, such as *TP53*, cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*), and liver kinase B1 (*STK11/LKB1*) [94, 95]. *KRAS*- (and/or TP53 co-) mutated tumors, associated with high levels of cytotoxic CD-8+ Th1 tumor-infiltrating lymphocytes and frequent PD-L1 expression, tend to respond to immunotherapy [96]. *KRAS* and/or *KEAP1* combined with *STK11/LKB1* mutations are found in up to 25% of adenocarcinoma and should be reported, as they confer a worse prognosis and do not respond to platin-based chemotherapy or immunotherapy [97]. Studies assessing *KRAS* co-mutations have demonstrated lower clinical response rates in *KRAS*-mutant lung adenocarcinomas with *KEAP1* inactivation. This subset of PD-L1 inhibitor-resistant tumors demonstrates lower PD-L1 expression and inactivation of the tumor suppressor *STK11/LKB1*, resulting in the accumulation of tumor-associated neutrophils with suppressive effects on T cells [98]. In lung adenocarcinoma, co-occurrence of *KRAS* and *STK11* gene mutations is associated with a "cold" tumor immune microenvironment, low PD-L1 expression, and poor response to immune checkpoint inhibitors (ICIs) [97].

2.10 NRG1

Genetic rearrangements of neuregulin-1 (*NRG1*) represent an emerging oncogene-addicted subgroup of NSCLC, with a reported frequency of ~0.2–0.5% in unselected patients [99]. Neuregulin-1 (*NRG1*) encodes a ligand for HER2 and HER3 tyrosine kinase receptors; and in normal tissues its expression is restricted to neurons. In NSCLC, multiple 5′ fusion partners have been identified and lead to an aberrant HER2/HER3 signaling pathway and subsequent activation of the PI3K-AKTmTOR and RAS/MAPK cascades. *NRG1* rearrangements in lung cancer samples are found without other known driver mutations from females and nonsmokers with a median age of 70 years and occur most commonly in invasive mucinous adenocarcinoma. These rearrangements cause PI3K-AKT pathway activation, which induces tumorigenesis [100].

3 Molecular Testing in Lung Cancer

Over the past decades, the discovery that a significant percentage of lung carcinomas harbor specific genetic alterations that are amenable to targeted therapeutic agents has increased the importance of molecular diagnostic testing in routine clinical practice.

Accurate tumor genotyping is essential for optimal treatment of patients with metastatic non-small cell lung cancer. Differentiation of lung adenocarcinoma and squamous cell carcinomas according to morphological and immunophenotypic features is necessary to recognize different oncogenic alterations as well as distinct responses to chemotherapeutic agents. Given the established clinical efficacy in the first-line setting for specific TKI therapies or immune checkpoint inhibitors for NSCLC patients with advanced disease, tumor molecular profiling should be completed before patients begin any targeted systemic therapies [101].

Current evidence-based consensus guidelines on molecular testing in lung cancer patients from the College of American Pathologists, the International Association of Lung Cancer, and the Association for Molecular Pathologists recommend that all patients with advanced-stage lung adenocarcinoma (or with an adenocarcinoma component), regardless of clinical features, should undergo biomarker testing for *EGFR* mutations and *ALK* and *ROS1* rearrangements. Testing for *BRAF*, *RET*, *ERBB2* (HER2), *KRAS*, *MET*, and *NTRK* alterations were not recommended as single gene testing but were considered appropriate as part of larger multiplex panels in order to identify treatment options beyond the minimal recommendations [102]. Subsequently, the American Society of Clinical Oncology (ASCO) stated that *BRAF* should be offered as a stand-alone test on all lung adenocarcinoma patients, irrespective of clinical characteristics [103]. In the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology for NSCLC, the list of recommended testing targets has grown to include *EGFR*, *ALK*, *ROS1*, *BRAF*, *MET* exon 14 skipping mutations, *RET*, and PD-L1. In addition, *EGFR* exon 20 insertions, *ERBB2* mutations, *NRG1* fusions, *KRAS* G12C, and tumor mutational burden (TMB) are evolving targets/biomarkers and may be included in the test panel. *EGFR* T790M testing was the only strong recommendation for patients with targetable mutations who relapsed on targeted therapy. For squamous cell carcinomas, to date, only PD-L1 immunohistochemistry is required, but the landscape is changing [104].

Tumor types to be tested are adenocarcinoma, non-small cell carcinoma with adenocarcinoma component including adenosquamous and pleomorphic carcinoma, large cell carcinoma, and NSCLC-NOS. Other rare NSCLC subtypes, such as large cell neuroendocrine carcinoma and sarcomatoid carcinoma, should also be subjected to molecular testing when clinically indicated because of the possibility of underlying targetable oncogenic alterations. Targetable oncogenic alterations are enriched in never or light smokers; when a never/light smoker develops lung cancer, it is most likely to be an adenocarcinoma. However, at a population level, the majority of adenocarcinomas of the lung occur in patients with a smoking history. Although squamous cell carcinoma of the lung is strongly associated with smoking, a small proportion of cases arise in light or never smokers. These may represent undersampled adenosquamous carcinomas and, rarely, pure squamous carcinomas with adenocarcinoma-type oncogenic driver mutations. In case of high clinical suspicion deemed appropriate by the clinician (in patients where adenocarcinoma component cannot be excluded due to low tumor tissue, young age, never or light smoking), it can also be applied to any non-small cell histology of tumors other than

adenocarcinoma. Molecular testing guidelines therefore recommend that any NSCLC patient with a light or never smoking history undergo tumor mutational profiling [102].

The accuracy and sensitivity of molecular testing are highly dependent on the quality of the specimens received for testing. This quality is dictated by intrinsic properties of the sample (size, extent of tumor necrosis, inflammatory infiltration, and posttreatment changes) and preanalytic variables (cold ischemia time, type of sample fixation, duration of fixation, and storage). Preanalytical steps such as sampling, verification of sample adequacy by the pathologist, and nucleic acid extraction account for most of the workflow time required to complete tumor tissue molecular profiling for certain genes [105].

Histological or cytological samples may be used for testing if there is adequate tumor cellularity, and primary or metastatic sites are appropriate for biomarker assessment. In the majority of the cases, cytology and formalin-fixed paraffin-embedded (FFPE) tissue specimens are suitable for biomarker testing, but in some instances (e.g., when tissue is not available or for assessment of resistant/recurrent disease), testing of plasma from peripheral blood can serve as a useful alternative [106].

The pathologist provides an estimate of the tumor amount and percentage of tumor cells, in order to select the optimal specimen for subsequent analyses. It is important to evaluate the total amount of tumor cells and the percentage of tumor cells with respect to the total number of cells. Current molecular testing guidelines recommend that the minimum analytic sensitivity of a clinical mutation detection assay must be such that it is able to detect mutations in a sample with $\geq$20% tumor cell content [102].

In general, testing can be accomplished either by comprehensive molecular panels (usually sequencing-based panels) or with sequential testing using smaller panels (including smaller targeted sequencing assays, fluorescence in situ hybridization [FISH], and/or polymerase chain reaction-based assays). PCR-based assays require comparatively low DNA input and allow the investigation of a sizeable number of genes and mutations, including point mutations and small insertions/deletions. The advantage of larger comprehensive sequencing panels allows for the detection of copy number variants (CNVs) and gene fusions in addition to single nucleotide variants and small, in-frame insertions and deletions [107]. These assays have the ability to identify fewer common alterations not tested for by smaller directed panels, but which have available therapies, such as NTRK rearrangements. Other advantages to more comprehensive molecular profiling testing include the assessment of emerging biomarkers including microsatellite instability (MSI) and tumor mutational burden (TMB). The majority of studies have reported an association between high TMB and progression-free survival and the objective response rate when treated with immune checkpoint inhibitor therapy. In addition, broader NGS-type panel testing can provide valuable information regarding the tumor's co-mutational profile, which may help to direct therapeutic choices in the future, such as the identification of inactivating STK11/LKB1 mutations in KRAS-mutated

lung adenocarcinomas, which are predictive of primary resistance to immune checkpoint inhibitor therapy via PD-1 and/or PD-L1 blockade.

However, these tests require a higher tumor cell content depending on the test and are associated with longer turnaround times. The advantage of smaller directed panels is that they tend to be more rapid and allow for faster clinical decision-making. In addition, a select biomarker testing using predictive IHC and FISH may provide a more rapid analysis and help supplement NGS testing, especially in settings where broad-NGS-based testing may not be readily available or in those cases for which available NGS panels cannot reliably detect gene fusions and CNVs. Break-apart probes are ideal for genes with multiple fusion partners, whereas a locus-specific probe is more appropriate for detection of CNVs.

According to current guidelines, the turnaround time for these molecular testing should be within 10 working days of receiving the specimen in the testing laboratory. In the new-generation sequencing study, this period takes 2–3 weeks [102].

3.1 Immunohistochemistry

The current World Health Organization (WHO) classification criteria for those cases of NSCLC where specific subtyping by morphology alone is not straightforward recommend a targeted panel of IHC to determine the correct histologic subtype. Multiple diagnostic modalities are required to arrive at a complete diagnosis of advanced NSCLC including histological assessment, primary IHC testing for the diagnosis of NSCLC where specific subtyping. Secondary IHC testing including PD-L1, and ALK and ROS1 in the appropriate setting, serves as a reliable tool in the assessment of predictive biomarkers with the advantage of faster and cheaper test results and less tissue consumption than any DNA or RNA extraction-based method [108].

Expression of the gene assessed by IHC can be used as a surrogate for fusion testing, depending on the specific antibody used and the robustness of performance. ALK IHC has now been incorporated in the recommendations as an equivalent alternative to fluorescence in situ hybridization for routine marker assessment. Similar to ALK testing, FISH has been used as standard test to identify ROS1 rearrangements in lung adenocarcinoma [109]. Immunohistochemistry may be used to screen for tumors with potential ROS1 fusion at high sensitivity but low specificity, although FISH or other sequencing-based confirmatory testing is still required [110].

NTRK fusion detection has gained importance as NTRK inhibitors have received regulatory approval for patients with NTRK fusions [87]. Studies have shown that pan-TRK IHC, detecting all three TRK proteins (TRKA, TRKB, TRKC) originating from fusions involving NTRK1, NTRK2, and NTRK3, respectively, has utility for the rapid assessment of solid tumors with NTRK fusions. However, to date, IHC is only used as pre-screening tool or confirmatory tool for sequencing-based methods. The proof of a rearrangement by RNA-based NGS is recommended because long

introns are difficult to cover by DNA-based NGS assays. Alternatively, NTRK1/2/3 FISH approach can be performed [111].

3.2 Liquid Biopsy Testing in NSCLC

Although tissue remains the gold standard for molecular diagnostics, tissue-based testing can be limited by inadequate or unavailable biopsies or tumor inaccessibility. Plasma-based assays have many advantages over tissue-based tests, being noninvasive, fast, and easily repeatable over time, but they may be less sensitive than tissue-based assays and therefore cannot serve as stand-alone testing for patients with NSCLC [112].

Currently, plasma cfDNA testing is increasingly used to genotype lung cancer patients with advanced disease. Liquid biopsy provides a global perspective by assessing tumor DNA shed into plasma from all tumor sites, thus potentially abrogating the issue of tissue heterogeneity. Liquid biopsy is proficient in detection of both tumor-associated genomic abnormalities at the time of initial diagnosis and at the time of acquired resistance [113].

In most cases, the detection of an actionable mutation from cfDNA is highly specific, and therapy based on this finding results in clinical responses. However, a negative result must be interpreted with caution and confirmed on tissue given the variable sensitivity of cfDNA methods [114]. In addition, there are emerging applications including early cancer detection and minimal residual disease testing that are currently under investigation.

3.3 Biomarkers in Immunotherapy

3.3.1 PD-L1 Testing

Programmed death receptor-1 (PD-1) is a type 1 membrane protein of the immunoglobulin superfamily that has an important role in restricting immune-mediated tissue damage secondary to inflammation and/or infection. PD-L1 in NSCLC is expressed on the membrane of tumor cells and/or on immune-infiltrating cells, dendritic cells, antigen-presenting cells, and T lymphocyte. PD-1, the PD-L1 receptor, is expressed on tumor-infiltrating lymphocytes, mainly CD4 T cells, T and B regulatory, NK, monocytes, and DC. Concerning PD-L1 binding, PD-1 inhibits kinases involved in T-cell activation [115, 116].

For interpretation of PD-L1 immunohistochemistry, different scoring systems are available. The tumor proportion score (TPS), the percentage of membranous positive tumor cells, is recommended for NSCLC evaluation. There are several PD-L1 clones available for IHC testing [117]. The four most widely used are DAKO 22C3, DAKO 28–8, Ventana SP263, and Ventana SP142. For routine diagnostics, the most frequently used clones are any of the first three, since these have shown good expression correlation between them in several studies. With respect to the other clones, SP142 stains a lower proportion of tumour cells [118].

4 Tumor Mutational Burden and Immune Microenvironment

PD-L1 expression by immunohistochemistry (IHC) is currently the only FDA-approved biomarker for immune checkpoint inhibitor (ICI) therapy. However, there are multiple issues associated with PD-L1 IHC including the presence of multiple IHC platforms, each anti-PD-1/PD-L1 agent coupled to the specific PD-L1 IHC assay and spatial and temporal heterogeneity of PD-L1 expression in the tumor. Importantly, PD-L1 expression is not the best biomarker to predict response to PD-1/PD-L1 blockade given the relatively low specificity [119]. To improve the prediction of response to ICI, several biomarkers have been studied. Of those, tumor mutational burden (TMB) and immune microenvironment are emerging as predictive biomarkers.

TMB is commonly defined as the total number of somatic coding mutations and is considered a surrogate for the amount of neoantigens based on the notion that the greater the number of nonsynonymous mutations in a given tumor, the more probable it is that some of the mutations will be immunogenic, providing targets for T-cell attack [115]. Rizvi and colleagues have shown higher synonymous mutational burden in tumors associated with improved objective response, durable clinical benefit, and progression-free survival (PFS) in two independent NSCLC patient cohorts treated with pembrolizumab [120]. Subsequently, the efficacy of TMB predicting response to ICI has been reported in several clinical trials [121].

Unfortunately, similar to PD-L1 IHC, the TMB assessment has not been standardized yet. Although conducting comprehensive whole-exome sequencing WES is ideal to assess TMB, it is currently not feasible in daily practice given the high costs, long turnaround time, and suboptimal quality and quantity of tissue available from advanced lung cancer patients. Now, targeted panel sequencing or next-generation sequencing (NGS) has become a part of clinical practice; thus, it would be desirable to leverage it to assess TMB. However, size and composition of panels and read depth and coverage are diverse between NGS platforms; thus, the cutoff for high TMB is platform dependent. Importantly, TMB values represent a continuum rather than distinct clusters, NGS platforms with small gene panels may be difficult to predict TMB comparably to WES, while larger panels could [120, 122].

As for the immune microenvironment, there are many types of immune cells, including T cells, B cells, myeloid-derived suppressor cells, tumor-associated macrophages, and neutrophils. Of those, CD8+ cytotoxic T cells have been recognized as an important player, and their density and location have been reported in association with response to ICI [123]. However, not all CD8+ T cells are in the same functional status. For instance, they may be activated characterized by the production of granzyme B and perforin and high Ki-67 proliferative index, and they may be exhausted characterized by the expression of LAG3, TIM3, and/or B7H3, among others. Interestingly, Gettinger and colleagues using multiplexed quantitative immunofluorescence have shown that a dormant tumor-infiltrating T lymphocyte (TIL) phenotype, characterized by elevated CD3+ TILs with low activation (granzyme B in CD3) and low proliferation (Ki-67 in CD3), is associated with survival benefit in patients treated with ICI [124]. Evaluation of multiple immune markers at the

same time to understand the immune microenvironment, preferably using a multiplex platform, is recommended.

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