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The 2020 update of the recommendations of the Austrian working group on lung pathology and oncology for the diagnostic workup of non-small cell lung cancer with focus on predictive biomarkers

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Summary The knowledge on molecular alterations in lung cancer have increased during the last decade considerably. Almost every year new genes were detected being targetable, and drugs have been developed and provided for those patients being diagnosed with such a lung cancer. Therefore, it was necessary to update previous recommendations to facilitate a uniform handling for the diagnosis and molecular tests of lung cancer specimen all over Austria. Originally mutation of the epidermal growth factor receptor (*EGFR*) was the only actionable molecular alteration, now there are more than 10 driver mutations known, and more are detected, and clinical studies are performed. In addition, the technique to test for these mutations have improved, next generation sequencing has opened the option to test several genes in one test. Immuno-oncology has entered the field, and besides the checkpoint death receptor and ligand molecules PD-1/PD-L1 more molecules have been detected and are also tested in clinical studies.
To provide equal opportunities to our patients the tests have to be implemented in all pathological institutes involved in lung cancer management. Because pathologists as part of the tumor board have to explain the diagnosis and the molecular alterations and suggest possible treatment options, the tests should be performed in-house, which will provide the optimal quality control.

Keywords Lung cancer · Adenocarcinoma · Tests for driver mutation · Resistance testing · Evaluation for immuno-oncologic therapy

Introduction

Because of the growing knowledge regarding molecular pathways driving malignancy and the development of targeted and immunomodulatory drugs, a comprehensive pathological workup of pulmonary carcinomas has become essential for any treatment planning. The Austrian working group on Lung Pathology and Oncology has previously published recommendations for the workup of diagnostic specimen of lung cancer. As numerous new data have been published since, there is a need to update the recommendations. Aiming at all disciplines dealing with lung cancer these general recommendations are restricted to non-small cell lung cancer (NSCLC).

Diagnostic and predictive tests of neuroendocrine and rare pulmonary tumors are not within the scope of these recommendations and should be described in a separate publication.

In addition to the histological typing of lung cancer, targetable molecular alterations and predictive biomarkers regarding immunotherapy have to be tested; they will be discussed in two separate sections below.

Testing predictive biomarkers

Definition

Predictive biomarkers for targeted agents (i.e., tyrosine kinase inhibitors and other small molecules) are somatic genomic alterations in tumor cells, such as single nucleotide variants (SNVs; point mutations), small insertions and deletions (indels), copy number alterations (CNAs) and structural variants (SVs) [1]. Biomarkers used to better predict the response to immune checkpoint inhibitors (CPIs) are the expression of immune checkpoint receptors or their ligands expressed on tumor cells and/or immune cells, and the tumor-mutational burden (TMB), and experimentally the tumor microenvironment and the microbiome. Furthermore, additional biomarkers can be tested to detect resistance mechanisms to these therapies.

Analytical techniques

Sequencing techniques for the analysis of somatic genetic alterations

- Sequencing has primarily been used for *EGFR* mutation analysis. As the sensitivity of Sanger sequencing is low (ideally more than 10% of cells in the specimen should contain the mutation), this technique is not recommended any more.
- Allele-specific testing by polymerase chain reaction (PCR) is a test for prespecified targets, multiplexed in case of *EGFR*, *KRAS* and *BRAF*; it is more sensitive than classical sequencing because of the amplification of the mutated sequence, which can be detected by a fluorescence signal in a real-time PCR assay.
- Reverse transcriptase-PCR (RT-PCR) can detect gene rearrangements, using RNA. Fixation and paraffin embedding can influence the quality of the RNA; however, today's RNA extraction kits usually provide good quality RNA [2].
- Next-generation sequencing (NGS)
  NGS of DNA and RNA (multiplexed PCR [2], amplicon sequencing, for targeted panels [3] and hybrid capture [2] based sequencing for large targeted panels and translocation detection) can evaluate multiple genes in parallel and allows quantitative analysis of alleles and detection of new abnormalities. However, well-engineered automation, computational processing and data storage are essential for valid results [4].
  For diagnostic purposes commercially available targeted NGS panels (for somatic alterations in solid tumors and/or tailored for lung cancer) are recommended. "Lung- or solid tumor-specific" panels usually are designed to cover hot spot regions of certain genes, e.g., exons 18–21 of the *EGFR* gene and the entirety of coding and noncoding sequences [1]; other panels are designed for rearrangements

(fusion products) and/or copy number alterations of genes. Larger panels for determining TMB are available [5].

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) enables detection of amplifications and/or rearrangements (break-apart) of genes/exons using specially designed probes.

Immunohistochemistry

Antibodies are used for the expression analysis and quantification of specific proteins.

Appropriate specimen for molecular testing

Methods of tissue retrieval

For the primary biomarker workup and the evaluation of resistance mechanisms against small molecules, antibodies and CPIs smears (alcohol-fixed) [6], cell pellets, cell blocks, transbronchial needle aspirations (TBNA), endo-, transbronchial and transthoracic biopsies, resection specimen, pleural fluid [7], tissue from cryo-biopsies or frozen sections are equally suited.

The analysis of circulating cell-free tumor DNA (ctDNA) [8] in blood/plasma is established for the detection of *EGFR* resistance mutations. Its use for disease monitoring (early detection of recurrence/progression or residual disease after curative treatment) is promising, but at this moment remains experimental without high level evidence of clinical utility.

Other body fluids such as urine, cerebrospinal fluid and bronchoalveolar lavage (BAL) can in some cases serve as alternative sources for tumor cells or tumor DNA.

Tissue processing

Tissue should be prioritized for *ALK*, *ROS1*, PD-L1, *NTRK* (immunohistochemistry) and *EGFR*, *BRAF*, *RET*, *MET* splice 14 (NGS or equivalent method) testing; thus serial sections at the first cut of the block are recommend (5–8) for diagnostic and predictive testing. If there is classical morphology of squamous or adenocarcinoma in histological or even cytological specimen, immunohistochemistry for the diagnosis is not mandatory [9].

For fixation of histological specimen and cell blocks 10% neutral buffered formalin (4% formaldehyde diluted from 40% stock-solution) should be used [10]. Fixation times between 6 and 72 h [11] for FISH, 12–48, maximum 60 h for sequencing methods are recommended.

Smears should be fixed in alcohol-based solutions.

Cuts (thickness ~4 µm) and smears should be spread on adhesive slides (without addition of proteins in the water bath [12]).

Decalcification is not recommended for mutation [13], testing decalcification with EDTA is possible for FISH analysis [14].

A tumor cell percentage for immunohistochemical methods is not defined, except for PD-L1 with a demand of 100 tumor cells. For FISH analysis at least 50 viable non-overlapping tumor cells are necessary. Depending on the method used, 5–20% cancer cell content is required for molecular methods.

A minimum of 10% tumor cells should be sufficient for the applied methods [9].

The minimal requirements of tumor cell content should be established for each method in the testing laboratory.

Evaluation by a pathologist is mandatory for any technique of analysis, comprising tumor typing, choice of tumor area, definition of tumor cell percentage among all cells in the specimen and exclusion of necrosis.

If necessary, the pathologist or a trained technologist should macro-dissect and in case of very little amount micro-dissect the specimen. Slides marked and prepared in this way should be used within 6 weeks.

Further tests or alteration-specific requests to the specimen will be mentioned in the following sections.

Quality of molecular testing

Quality assurance

Each laboratory should provide external quality assurance by participating in adequate programs [9], offered by professional societies/associations as CAP, ECAT, EMQN, ESP-EQA, QuIP, RCPA QAP, RfB(DGKL), UKNEQUAS, GenQA [15].

Reporting

The report must contain the method used with sensitivity and specificity data and the clone/probes, if applied. All analyzed genes must be included in the report with the kind of genetic alterations detected: mutations (Human Genome Variation Society [HGVS] nomenclature), rearrangements, copy number variations, immunohistochemical expression (including scores), and an interpretation of the potential clinical significance. The targetable genes should be highlighted.

Turnaround time

The used testing methods should provide molecular pathological results in 7, maximum 10 workdays [9, 13].

If the average turnaround time exceeds 10 working days, the laboratory is strongly requested to make a more rapid test available, either in house or through a reference laboratory.

Specimen should arrive at an external molecular laboratory within 3 workdays, at an internal molecular laboratory within 24 h [13].

National availability and standardization

Diagnosis of lung cancer in Austria is not centralized or restricted to cancer centers, but limited to large hospitals, where departments of Thoracic Surgery, Pulmonology, and Oncology are available. Pathologic diagnosis of lung cancer, however, has to be provided in all types of institutes of pathology. The algorithm for diagnostic workup (Fig. 1) is comparable to international standards and should be applied uniformly.

Diagnostic tests by immunohistochemistry should be integrated into the primary diagnosis. Internationally recommended immunohistochemical analyses of PD-L1, ALK, ROS1 and panNTRK can be performed in adequate quality in every institute. Tests for molecular targets for first-line treatment are available in the primary diagnosing pathological laboratory (Fig. 2) and are justified independently of the stage of disease. Most lung carcinomas diagnosed at stage I and II will recur, and therefore, the molecular alterations are already available at time of recurrence. This will also allow better tissue management. Tumor-specific NGS panels should be preferred. Each institute has the option to locally implement NGS or opt for cooperation. At international meetings discussion might be proposed with existing initiatives in neighboring countries, such as the group in the Netherlands, France, or the Netzwerk Genomische Medizin in Germany.

Institutes of pathology should be preferred to commercial laboratories/companies offering molecular tests for the above listed oncogens, in the majority by NGS technology. Commercial tests have limitations: most of the reports of laboratory data do not contain the exact documentation of the selection of tissue and exact laboratory data/curves are not constantly reported, which can be of help for interpretation of test results and which need to be communicated to tumor boards by pathologists, familiar with the tumor morphology. Additionally commercial suppliers tend to charge more than Austrian pathology institutes.

Somatic genomic alterations in lung cancer

Transformation of normal cells to cancer cells is amongst others addicted to mutations of proteins for growth and survival. Oncogenic "driver" mutations (e.g., *EGFR*-, *KRAS*-mutations), which are mutually exclusive in most cases, can be targeted by drugs, mainly TKIs. Blockade of the dysregulated pathway can be very effective, as primarily an alternative signaling is not established in the tumor cell population. In contrast, mutations not essential for the oncogenic phenotype are frequently named "passenger mutations" [4]. Other mechanisms for malignancy development are alterations in tumor suppressor genes like *P53* or *STK11* [12].

Based on the level of evidence and the actual approval of specific drugs our recommendations for testing somatic genetic alterations have been structured in three categories:

1. "Obligatory" or "reflex" tests, which should be performed at the time of diagnosis because of availability of approved drugs. This set of tests should be offered by all institutes diagnosing lung cancer in cytology specimen and/or biopsies and can be performed as single-gene test or part of an approved NGS panel.
2. Tests covering genetic alterations, for which off-label therapies are available [4] and/or are imminent to be approved. Those tests should also be performed and reported without request, either at negativity of *EGFR*, *ALK*, *ROS1*, *BRAF*, *NTRK*, *MET* alterations or primarily as part of a NGS panel.
3. A group of alterations are currently being experimentally targeted with specifically tailored substances in clinical studies to be accorded interdisciplinarily.

As tumors can counteract the blockade of a signaling pathway by additional/alternative genetic alterations, resistance to the drugs develops in nearly every tumor after some time. Therefore, a description of resistance mechanisms and their possible targeted therapies is added to each of the respective genes in the section reflex testing; these tests are however, not performed primarily.

"Reflex" testing of driver genes with approved first-line TKI therapies

Several approved targeted therapies can be used as the first-line therapy in patients with advanced non-small cell lung cancer (NSCLC) either alone or in combination with chemotherapy.

Thus, an analysis of alterations of the biomarkers mentioned below should be performed simultaneously with the diagnosis of adenocarcinoma (immunohistochemistry, if no classical histology: TTF1 positive, p40 negative) and NSCLC with adenocarcinoma component including adenosquamous and pleomorphic carcinoma (in resection specimen), in all small biopsies where an adenocarcinoma component cannot be excluded and in all tumors not otherwise specified (NOS) by the diagnosing pathologist.

If indicated, in young (<50 years) non-smoking patients, molecular analyses should also be performed a priori.

Because clinical information concerning the stage of disease is frequently not available, we recommend reflex testing for carcinomas in all stages to avoid loss of time and to have a basic profile in case of recurrence. This approach is supported by a recruiting study hypothesizing increased diagnostic efficiency and a benefit for more patients from personalized therapy, if stages I–III are also included in primary molecular testing [16].

Because of the rapidly developing availability of targeted drugs and because of the relatively low demand of tissue, NGS panels are a powerful tool for investi-

Table 1 Recommendations for primary *EGFR* testing

Recommendation for primary *EGFR* testing	
Reflex testing (due to practical reasons independent of stage of disease [26])	Limited specimen (cytology, biopsies) in which an adenocarcinoma component cannot be completely excluded, resection specimen with an adenocarcinoma component [13]
Additional settings	With a non- or light-smoking history or young age (below 50 years) also in limited specimen containing squamous- and/or small cell component
Specimen-associated features	Multiple, apparently different primary lung adenocarcinomas should each be tested; no need to test different areas in one tumor
	Primary or metastatic lesions are equally suited for primary mutation testing [13]
Methods (presently used in Austrian laboratories according to the reply to the questions of an unpublished survey; the list does not cover the whole range of products available)	Validated PCR-based method (see sequencing techniques), able to detect all individual mutations reported with a frequency of 1% of *EGFR*-mutated lung cancers
	Allele-specific real-time PCR (IDYLLA® *EGFR* Mutation Test; Therascreen® *EGFR* RCQ PCR Kit Quiagen; Cobas® *EGFR* Mutation Test; *EGFR* XL StripAssay® ViennaLab)
	NGS (FusionPlex™ Lung and Fusion PlexCTL™ Archer; TruSight™ Tumor 15; AmpliSeq™ for Illumina Focus Panel; Oncomine™ Focus Assay, Thermo Fisher; Ion AmpliSeq™ Panels and Oncomine Comprehensive Assay Ion S5 System; GeneRead QIAct Lung UMI Panels®, Qiagen)
Reporting of sequence-tests [27]	Method of test with specificity and sensitivity, including all tested genes
	Specific mutation sequence: Coding DNA: c. (first nucleotide of translation, start codon of the coding reference DNA sequence) and Protein: p. (first nucleotide of genomic reference DNA sequence)—HGVS nomenclature
	Interpretation: according to AMP/ASCO/CAP guideline: I: known sensitizing and resistance mutation II. potential clinical significance III. rare variants can be reported
AMP Association for Molecular Pathology, *ASCO* Amercian Society of Clinical Oncology, *CAP* College of American Pathologists	

Table 2 Recommendations for genetic testing for patients with acquired resistance to *EGFR* TKIs

Recommendations for genetic testing for patients with acquired resistance to *EGFR* TKIs	
Selection	Patients progressing on treatment with 1st- or 2nd-generation *EGFR* TKIs must be tested for T790M before treatment with 3rd generation TKIs
Methods	Assays capable of detection of 5% mutant alleles in tissue – (liquid biopsy) [36] is the preferred primary test for the detection of T790M (sensitivity: 0.1% mutated allele frequency). – allele-specific real-time PCR (Cobas® *EGFR* mutation Test v2) – digital droplet PCR (ddPCR™ Mutation Assay: *EGFR* p.T790M) [36, 37] – NGS (Oncomine™ Lung cfDNA Assay)
Negative for T790M	In case of T790M negativity and when progression occurs during osimertinib treatment, – broad molecular testing (preferable NGS from a tissue rebiopsy) for alternate genetic drivers and – conventional microscopy/immunohistochemistry for recognition of SCLC transformation are highly recommended

gating many regions of the human genome. As standalone assays still are reliable and proven in some institutes, we recommend a list of genes to be tested primarily, which is extended in comparison to recently published recommendations, for all testing laboratories.

EGFR

Primary *EGFR* testing Epidermal growth factor receptor (*EGFR*) activating mutations in exons 18–21 can be found in 15% of Caucasians with pulmonary adenocarcinomas (in a study from Vienna: 12.4% [17]), compared to 60% in Asian patients. Most frequently, deletions in exon 19 and a distinct point mutation in exon 21 (L858R) are detectable (so called "classical EGFR mutations") [18]. Several *EGFR*-TKIs are approved for the first-line therapy of patients in the metastatic setting with any of these mutations [19–23].

The value of TKI treatment of tumors with rare *EGFR* mutations (i.e., mutations in exon 18 or exon 20-insertions) is less well defined. Nevertheless, at least for some of these genetic alterations (mainly exon 18 [24]), TKIs led to remarkable responses and can be considered as valuable treatment options. In the near future new therapeutic options might also become available for tumors with exon 20-insertions (poziotinib [25], TAK 788; Table 1).

EGFR testing at the time of acquired resistance When 1st- or 2nd-generation *EGFR*-TKIs are used initially (gefitinib, erlotinib, afatinib or dacomitinib), the occurrence of a second *EGFR* mutation in exon 20 (T790M) represents the most frequent mechanism of resistance. Because a potent drug for this situation, osimertinib [28], is available, testing for T790M is mandatory in patients developing resistance on TKI therapy. This should initially be done by using a quality-assured liquid biopsy platform [29–31]. If a positive result can be detected, no further tissue testing is necessary. In case the liquid biopsy does not verify the presence of T790M, a tissue biopsy is required, whenever clinically feasible. This is of even

Table 3 Recommendations for primary ALK rearrangement testing

Recommendations for primary *ALK* rearrangement testing	
Immunohisto-chemistry (IHC) [41]	IHC using antibody-clones 5A4 and D5F3 have been established and are recommended as initial test because of sufficient sensitivity and specificity [10] *Microscopic evaluation* cytoplasmic staining, granular, can be accentuated at membrane scoring systems: – 4 tiered system: 0, 1+ (70% tumor cells faintly) 2+ (100% medium), 3+ (100% strongly dark brown) [42] – H-Score (multiplying percentage of stained tumor cells by intensity 0, 1, 2, 3), highest possible value 300 – negative or positive in tyramide enhancement, which commercially available detection systems apply
	In patients with a strong staining (3+, if scored) for the *ALK* protein in the majority of tumor cells (a homogeneous distribution is usual in specimen with optimal preanalytic conditions), a TKI treatment can be started without molecular analysis *Potential pitfalls*: – in mucin-producing cells membranous staining might be interpreted false negative *Potential false positive*: – membranous staining—can be seen in normal pneumocytes – neuroendocrine cells (LCNEC) can be positive – mucin—extracellular and situated in macrophages can be positive
FISH [14]	Usually for confirmation: – in weak expression (1+ and 2+), if scored and mucin containing tumor-specimen – in addition to less sensitive or specific IC antibodies (ALK1; ALK01; SP8 [43]) preanalytic recommendations: – time to fixation: <1 h, time of fixation: 6–48 h, – section cut-thickness: 5 ± 1 µm counts and cutoffs: – 50 (100) tumor cells: –≥15% positive → report positive for *ALK* rearrangement –<15% positive → report negative for *ALK* rearrangement
NGS [7, 44]	Can be part of primary NGS-based analysis of FFPE samples or as confirmatory test in case of discrepant results in IHC and FISH Enables detection of different ALK fusion-partners with limited clinical relevance at the moment [45] – primary in cytology samples and frozen tissue (Japanese Lung Cancer Society) – detection of rare aberrations (fusion partners) in clinically suspicious, IHC- and FISH-negative cases [46]
Reporting [47]	Tumor assessment: – percentage of tumor cells related to cells of whole section – estimated number of tumor cells, if number is low – extent of necrosis, inflammation – analytical section: FISH: probe set and threshold to define positive result IHC: antibody type RT-PCR: method used, analytical sensitivity of the assay NGS: platform, type of panel, sensitivity of method, results using HGVS mutation nomenclature interpretation: likelihood that tumor will respond to or resist targeted treatment explanation of indeterminate results

Table 4 Genetic testing for patients with acquired resistance to ALK TKIs [51]

Genetic testing for patients with acquired resistance to *ALK* TKIs			
Resistance	Molecular mechanism	Test	Therapy
ALK amplification	*ALK* copy number increase	FISH, copy number variation (NGS)	Not established
ALK mutations (part of known mutations)	L1196M (gatekeeper mutation)	NGS	Other ALK inhibitors
	C1156Y, G1269A, I1171T/N/S and others	NGS	Other ALK inhibitors
	G1202R	NGS	Other ALK inhibitors
Resistance mechanisms with little evidence for possible therapies			
Other mutations	*EGFR* mutations	NGS	*EGFR* TKI
	MET mutations	NGS	MET inhibitor
	CDK4, CDK6 mutations	NGS	CDK4, CDK6 inhibitor
	IGF-1R/*IRS-1* pathway activation	NGS	IGF-1R inhibitor

Table 5 Recommendations for primary ROS1 rearrangement testing

Recommendations for primary *ROS1* rearrangement testing	
IHC [55]	Antibody clones D4D6, also SP384 can be recommended staining pattern according to fusion partner: – granular cytoplasmic pattern or focal or diffuse intensely stained aggregates: *CD74-ROS1* – weak cytoplasmic expression with membranous accentuation*: EZR-ROS1* – solid cytoplasmic staining: *SLC34A2* and *SDC4-ROS1* [56] distribution of positivity almost always diffuse – focal or patchy positivity in false positive cases because of ROS1 expression also in pneumocytes, macrophages and giant cells scoring using different systems – modified H-score [57]: intensity: 3+ (strong: clearly visible ×2 or ×4 objective), 2+ (moderate: ×10 or ×20 objective) 1+ (weak: ×40 objective) 0 (no staining) multiplied by percentage of tumor cells of each staining intensity. – thresholds: H-score ≥100 for positivity, H-score cutoff ≥150, – positive status: ≥2+ intensity in ≥30% of total tumor cells In cases exhibiting a clear negative IHC result no further diagnostic work-up is required. In contrast, every positive staining requires confirmatory assays by FISH or NGS [58, 59]
FISH [60]	– Recommended as confirmation in all immunohistochemically positive cases – scoring: similar to ALK: rearrangement-positive cell rate (%) = ([number of cells with split pattern + number of cells with isolated 3' pattern] / total number of cells evaluated) × 100; at least 50 tumor cells have to be counted, reported positive, if positive-rate 30%, <10%—negative
NGS	As *ALK*

Table 6 Genetic testing for patients with acquired resistance to ROS1 TKIs

Genetic testing for patients with acquired resistance to ROS1 TKIs		
Resistance	Molecular mechanism	Test
ROS1 amplification	Copy or gain	FISH, NGS preferred
ROS1 mutation	G2032R kinase domain mutation	NGS
	L2026M, L1951R and others can co-occur	NGS
Bypass tracks	*KIT* activating mutation	NGS
	Beta catenin mutation	NGS
	GNA11 mutation	NGS

higher importance as transition into SCLC [32–34], which is rare, but a well-described mechanism of resistance, is only detectable by tissue analysis.

Several other mechanisms [35] of acquired resistance have been characterized after initial treatment with *EGFR* TKIs including osimertinib. Several rare additional *EGFR* mutations (C797S as resistance mechanism for osimertinib) can be the cause for secondary resistance. Amplification as another *EGFR* modification, bypass pathway activation as *MET* and *HER2* amplifications, *AXL* and *HGF* overexpression, or downstream pathway activation as *PTEN* loss, mutations of *PI3KCA* and *BRAF* V600. The therapeutic consequences of such genetic events are not clear; however, case reports and small studies suggest that treatment with the respective targeted agents is at least an option. Testing for the respective alterations is therefore suggested wherever available (Table 2).

ALK

Primary *ALK* rearrangement testing *ALK* [38–40] rearrangements are found in about 4–6% of adenocarcinoma patients. The probability of a rearrangement is higher in never-smokers (70–80%), younger patients (40–50 years) and non-squamous and non-neuroendocrine morphology. Testing for ALK protein expression by immunohistochemistry is now recommended in all lung cancers with an adenocarcinoma component. When detected, 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 (Table 3).

ALK testing at the time of acquired resistance [48, 49] Several mechanisms of acquired resistance after *ALK* TKI treatment have been reported [50]. Therefore, primarily the oncogenic fusion has to be proven, when resistance develops and analysis of the *ALK* gene has to be performed. While the respective therapeutic relevance is still unclear for most of them, 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. Thus, mutation testing influences, at least to a certain extent, the choice of drug or the decision to use a further line of TKI-treatment versus chemotherapy (Table 4).

ROS1

Primary *ROS1* rearrangement testing *ROS1* rearrangements leading to *ROS1* activation and overexpression are found in about 2% of adenocarcinoma patients [52], predominantly in younger never-smokers, more frequent associated with mucin production and signet ring cell adenocarcinoma [53, 54].

Several TKIs are approved for first-line treatment of *ROS1*-rearranged metastatic NSCLC (in alphabetical order): crizotinib and ceritinib, are available to treat these patients.

Entrectinib and lorlatinib (as off-label option in resistance situations) are not yet EMA approved.

Reporting recommendations are similar as in *ALK* (Table 5).

***ROS1* testing at the time of acquired resistance** As in ALK-rearranged tumors, different mechanisms of acquired resistance after TKI treatment have been reported [50, 61, 62]. In 50–60% of these cases, secondary ROS1-mutations seem to be the crucial driver of resistance, with G2032R being the most frequent. As most of the available ROS1-TKIs have no or only moderate efficacy against the G2032R-mutant variant, its detection will influence therapeutic decisions (Table 6).

KRAS

Although drugs targeting mutations of the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) due to the biological heterogeneity of *KRAS*-mutant NSCLC (many downstream activated pathways, different genotypes, mutant allele-copy number gains and co-mutations) [63] are not approved yet, molecular testing is included in primary testing recommendations [64], based on the following reasons: *KRAS* mutations can be indicative of worse prognosis, especially *KRAS* G12C and G12V mutations are associated with poor overall survival (OS), but can also be predictive for poor response to chemotherapy and predictive for immune modulatory therapy. *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, whereas the presence of co-mutation of *STK11* reduces the efficiency of immune-modulatory drugs. A targeting compound for *KRAS* G12C-mutated NSCLC, AMG510 even has already achieved orphan drug designation for metastatic NSCLC [65]. Another reason to test is the frequent (20–25%) occurrence of *KRAS*-mutant NSCLC in Western countries. Hence detection of *KRAS* mutations can be decisive in sequential mutational testing.

BRAF

BRAF is a downstream signaling mediator of Kirsten rat sarcoma viral oncogene homolog (*KRAS*), activating the mitogen-activated protein kinase (*MAPK*) pathway.

Activating *BRAF* mutations, especially the V600 (V600E and V600M) in 59% vs. other genotypes (G469A—22%, D468V—13% and D549G—6%) [66] do occur in 1–2% of pulmonary adenocarcinomas, associated with light- or never-smoking habit in contrast to non-V600 mutations in heavier smokers [4]. Patients with V600 mutations and metastatic disease should be treated with the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib. *BRAF* mutation testing is considered an obligatory analysis.

Analytical methods: PCR (see sequencing techniques) or NGS, which is preferable, because of covering more nucleotides.

Non-V600 mutated tumors tend to be resistant to *BRAF* inhibitors.

NTRK

The neurotrophic tyrosine receptor kinase *(NTRK1-3)* [67] gene family (TRKA, TRKB, TRKC) contribute to central and peripheral nervous system development and function. Activation by gene fusion with different partners, e.g., *CD74* or 6 additional partners to *NTRK1*, *TRIM24* to *NTRK2* in lung cancer [67], in pulmonary adenocarcinomas predominantly *NTRK* 2 and 3 fusions are detected [68, 69] in approximately 1% of adenocarcinomas [70] rand 0.2–3.3% in lung cancer [67], respectively. The estimation of frequency differs and is regarded lower by some authors.

Larotrectinib, EMA- and FDA-approved, and the FDA-approved entrectinib are two NTRK inhibitors for the treatment of advanced tumors (including lung cancer) with documented *NTRK* gene fusions.

Analytical methods:

For tumors with a very low frequency of NTRK fusions (<5%) as in lung cancer [67, 71], the proof of a rearrangement by mRNA-NGS is recommended because long introns are difficult to cover by DNA-based NGS assays. According to the ESMO Translational Research and Precision Medicine Working Group [72] protein expression in NGS-positive tumors should be confirmed by immunohistochemistry.

If NGS is not available in routine diagnostics, immunohistochemistry using pan-TRK monoclonal antibody cocktails (pan TRK clone EPR17341), detecting over-expression of TRKA, B and C proteins should be performed for screening, followed by NGS confirmation of positive IHC results.

Alternatively, a first NTRK1/3 FISH approach would be feasible and only in very rare cases an additional NTRK2 FISH would be required, but analysis by FISH is mainly recommended for tumors with a high frequency of *NTRK*-fusions, mainly rare tumors of childhood, e.g., congenital mesoblastic nephroma, infantile fibrosarcoma, or the mammary secretory carcinoma of adults.

Testing of driver genes with imminent approval to be included in reflex testing

This section is included because approval for compounds to treat tumors with aberrations of these genes are already pending. Analysis of those genes are included in the used NGS panels and should also be reported without clinical request by institutes performing sequential stand-alone molecular tests.

Table 7 Recommendations for PD-L1 testing

Recommendations for PD-L1 testing	
Selection	NSCLC: reflex testing in parallel to genetic alteration testing: because of frequent missing clinical information concerning the stage of disease
Immunohistochemistry (IHC)	Validated immunohistochemistry test (e.g., clone 22C3, SP263, 28.8 respectively) preanalytic recommendations—similar to general: fixation in 10% neutral buffered formalin, 6–maximum 72 h, thickness of cuts approximately 4 µm positively charged slides, reserve-slides not older than 3 months [12]
Reporting	Type of antibody
	Tumor proportion score (TPS)
	Combined positivity score (CPS) indicating positive tumor cells and certain immune cells, as well as immune cell (IC) score are currently not generally recommended in the context of lung cancer, but should be reported in case of metastasis of other tumors, e.g., urothelial carcinoma or squamous cell carcinoma of head and neck
PCR	Available, e.g., a digital droplet PCR (ddPCR) method can be used for prediction of clinical response using the PD-L1:TIKP3 ratio [93]

Because stand-alone tests for these genetic alterations are not widely offered, a sequential initiation of NGS in another institution is encouraged, without being asked explicitly.

HER2 mutations

HER2-activating mutations [4] (most often insertions and point mutations in exon 20), amplifications and HER2 overexpression [73] occur in 1–3% of adenocarcinomas, predominantly in women and never-smokers. For mutation testing NGS panels are preferred. Anti-*HER2* agents (afatinib, TD-M1 [74]) have shown some activity in small trials and osimertinib could be tested as single agent in *HER2* amplified tumors and as combination therapy in *HER2*-mutated tumors [75].

HER2-amplified NSCLC did not benefit from trastuzumab, so amplification testing (FISH) is not recommended in NSCLC. Immunohistochemically detectable HER2 overexpression is correlated with papillary dominant growth pattern and is a poor prognostic marker in lung adenocarcinoma as well as amplification [73].

MET abnormalities

MET can act as oncogenic driver in adenocarcinoma and squamous cell carcinoma [76] by *MET* exon 14 skipping mutation. This reduces degradation of MET protein and occurs in 3% of lung adenocarcinomas and in up to 20% of pulmonary sarcomatoid carcinomas [4]. *MET* amplification [77] seems to be a negative prognostic marker. In 5–20% of *EGFR*-mutated adenocarcinomas co-mutation of the *MET* gene can be a resistance-mechanism to *EGFR* TKIs.

MET mutations can be detected by NGS, *MET* amplifications by FISH or NGS panels. MET protein overexpression, detectable by immunohistochemistry, can be caused by mutation as well as amplification, but also transcriptional *MET* upregulation of other causes, which are not targeted by MET inhibitors. So MET immunohistochemistry is not predictive for MET inhibition in NSCLC [77, 78].

Crizotinib can be used for *MET* exon 14 skipping mutations and amplification as next-line therapy, but new TKIs (capmatinib, tepotinib, savolitinib) are becoming available and might be more effective.

RET rearrangements

RET is another oncogene created by fusion to other genes (*CCDC6, KIF5B, NCOA4*) in 1–2% of adenocarcinomas, more frequent in younger patients and never-smokers [4].

Detection is possible with FISH break-apart probes and/or NGS gene fusion panels. Immunohistochemistry is discouraged.

Multikinase inhibitors (cabozantinib, vandetanib or alectinib) have led to modest treatment results with response rates in about 30% in this patient subgroup. However, more specific *RET* inhibitors (Loxo 292 = selpercatinib, BLU 667) [79] showed promising efficacy during early clinical development and are emerging options at least for patients progressing after standard treatment.

Tests for genotypes with targeted therapies in development offered in trials only

Neuregulin1 (NRG1)

CD74-*NRG1* gene fusions are activating genomic alterations in mucinous adenocarcinomas [80, 81], promoting *ERBB2–ERBB3* heterodimerization and activation of downstream signaling. Fusion genes are formed with CD74 and SCLA3A2 [82–84]. Drilon et al. [85] demonstrated that GSK2849330 inhibits phosphorylation of ERBB2, a monoclonal anti-HER3 antibody, lumretuzumab in combination with erlotinib [84], and the pan-*ERB-B*-inhibitor afatinib has shown modest efficacy in this patient group, justifying off-label use in advanced treatment lines.

KRAS and/or *KEAP1* combined with *STK11/LKB1* mutations

These combinations 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 [86].

Table 8 CPI immunohistochemistry in relation to antibodies, CPIs, scoring and cutoffs. Adapted from [12]

Checkpoint inhibitor	Clone	Staining platform	Scoring/Cutoffs
Anti PD-1 drugs			
Nivolumab	28-8	Dako Autostainer Link 48	TPS ≥1%, ≥5%, ≥10%
Pembrolizumab	22C3	Dako Autostainer Link 48	**TPS: ≥1%** **TPS: ≥50%** CPS: ?
Anti PD-L1 drugs			
Atezolizumab	SP142	Ventana Benchmark	Tumor- and immune-cells TC/IC1: >1%, TC/IC2: >5%, TC3: >50%, IC3: >10%
Durvalumab	SP263	Ventana Benchmark	**TPS: 1%** [94]

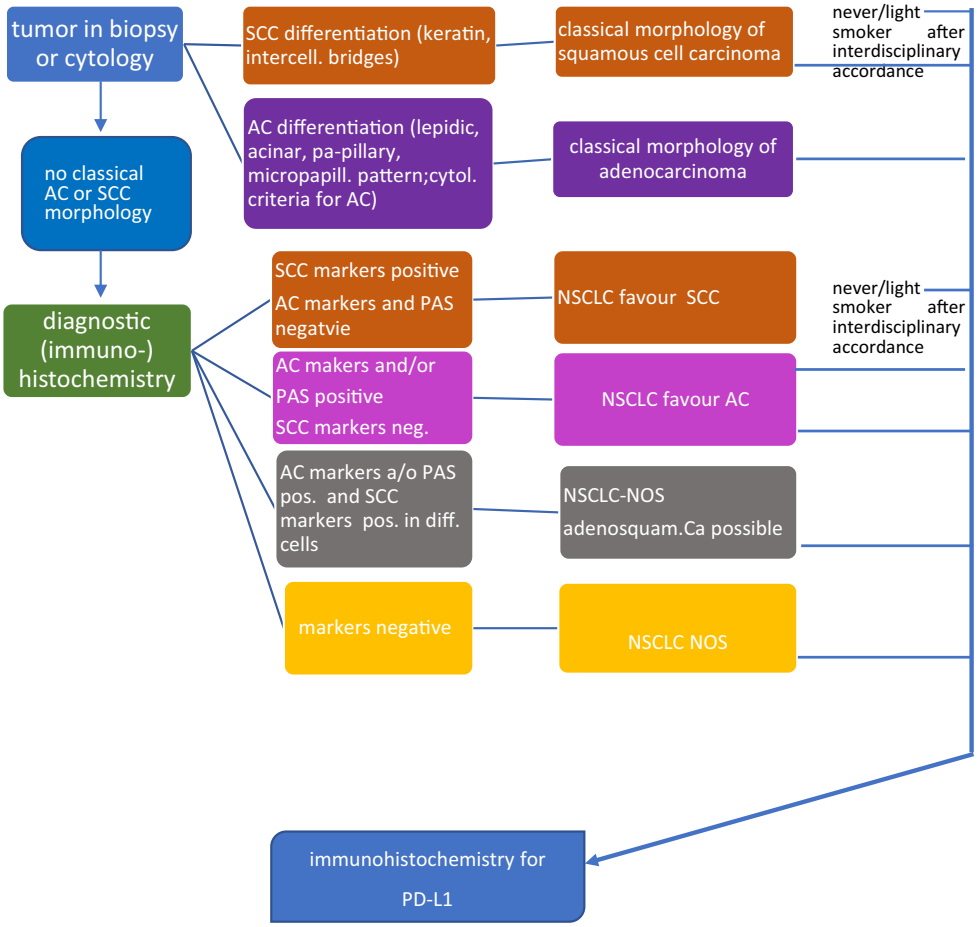

Fig. 1 Diagnostic algorithm for histological diagnosis in biopsies and cytological specimen suspicious for lung cancer. Adapted from [9]. *SCC* squamous cell carcinoma, *AC* adenocarcinoma, *NSCLC* non-small cell lung cancer, *SCC* squamous cell carcinoma, *AC* adenocarcinoma, *PAS* periodic acid schiff reaction, *NSCLC* non-small cell carcinoma, *NSCLC-NOS* NSCLC not otherwise specified, *TKI* Tyrosine kinase inhibitor, *IHC* immunohistochemistry, *WT* wild type

PIK3CA, AKT1, PTEN alterations

Alterations in *PIK3CA, AKT1,* and *PTEN* occur [4] frequently in SCC and smokers. Loss of PTEN function and gain-of-function mutations in *AKT1* and *PIK3CA* can be found. *PIK3CA* mutations may promote resistance to *EGFR* TKIs in mutated NSCLC. Inhibition of AKT and PI3Kinase could be a treatment option in SCC, but often overlap with other molecular changes, which rather represents a "passenger" mutation than a driver. In addition, inhibition of PI3KCA is complicated by the many downstream pathways of this oncogene (*mTOR, RAS, RAL*, etc.).

Tests for immune oncologic treatment—PD-1/PDL-1 axis treatment

PD-1/PD-L1 immune checkpoint inhibitors (CPI) have dramatically changed the therapeutic landscape of advanced NSCLC and are now used as first- or second-line therapy with or without combinations

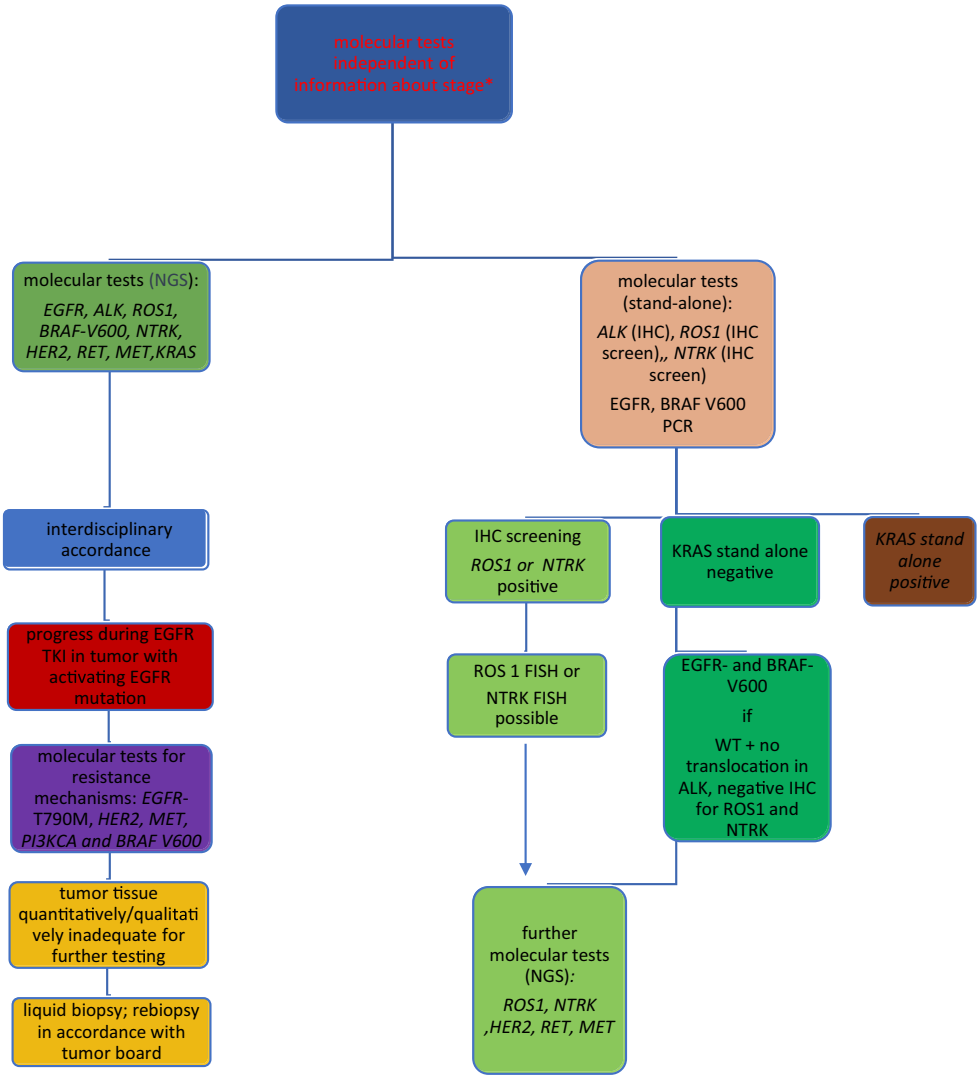

Fig. 2 Algorithm for molecular testing in non-small cell lung cancer (NSCLC) specimen either by NGS: all mutations and rearrangements in panels or consecutive testing—stand-alone tests: immunohistochemistry, PCR and FISH. Third group: tests according to interdisciplinary agreement at progress during *EGFR* TKI therapy. *NGS* next generation sequencing, *FISH* fluorescence in situ hybridization, *PCR* polymerase chain reaction. *Asterisk* Specific Austrian procedure, *SCC* squamous cell carcinoma, *AC* adenocarcinoma, *PAS* periodic acid schiff reaction, *NSCLC* non-small cell carcinoma, *NOS* not otherwise specified, *TKI* Tyrosine kinase inhibitor, *IHC* immunohistochemistry, *WT* wild type

for the majority of patients with advanced non-oncogene-driven tumors.

The expression of PD-L1 on tumor cells and/or immune cells is used as main predictive marker for the efficiency of CPI therapy, but up to 60% of those patients will not benefit from this treatment [87]. In contrast to gene mutations as predictive markers the expression of the programmed death protein 1 (PD-1) and/or PD-L1 is related to multiple factors influencing the immune system, and not "the" one quantifiable marker predicting therapy response. There are also additional predictive markers for CPI therapy.

Tumor-related factors are the following: tumor mutational burden (TMB)/load (TML); mismatch repair and DNA replication genes; the tumor microenvironment (TME) consisting of immune cell infiltrate [88] (T- and B-lymphocytes, granulocytes-PMN, macrophages, dendritic cells, natural killer cells and others), fibroblasts, vascular and lymphatic endothelial cells and signaling molecules; immune gene and IFN-γ related mRNA-based signatures [87].

Furthermore, genetic aberrations of *STK11(LKB1)* can cause resistance to CPI.

Biomarkers related to the host include the following: peripheral blood cell counts; myeloid-derived suppressor cells; lactate dehydrogenase (LDH); regulation of immune-related genes and single nucleotide polymorphisms; the microbiome of the gut [87].

Some of those factors can already be evaluated for prediction in NSCLC.

PD-L1 testing is established in the clinical workflow and recommended as a primary test for all NSCLC. Determination of TMB/TML and negative predictive mutations could be compared to groups 2 and 3 in the somatic mutation test recommendations.

PD-L1 testing

As the probability of a durable clinical benefit from PD1-/PD-L1 inhibitors increases with incremental PD-L1 expression on tumor cells and as the approval of at least some antibodies as monotherapy is restricted to certain expression cut-offs [89–91], we rec-

ommend to perform PD-L1 immunohistochemistry as a reflex test in all newly diagnosed NSCLC.

Together with the development of different CPIs, several immunohistochemical antibodies have become available as companion diagnostics or independently (Table 8).

Several studies have shown a high concordance of the clones 22C3, SP263 and 28.8 suggesting that they can be used interchangeably for tumor cell scoring (Blueprint Study/Astra Zeneca Study). In house validation and participation in international ring trials is highly recommended, especially when other clones or platforms are used.

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. For monotherapy in first line in metastasized NSCLC with pembrolizumab a TPS of at least 50% has to be reached, for second line therapy of NSCLC with pembrolizumab or durvalumab after radiation therapy in locally advanced NSCLC the cutoff is a TPS of at least 1%.

Other scores [92], including the combined positivity score (CPS) = (stained TC + stained MIC[1]) / TC and the IC score: percentage of positive immune cells per tumor area, are mainly used in other cancer types (Tables 7 and 8).

Tumor mutational burden (TMB)

TMB has been evaluated as another predictor of immunotherapy efficacy following the hypothesis that mutations of genes can result in neoantigens, increasing the likelihood that T-lymphocytes will detect those proteins, presented on MHC complexes, as foreign and therefore attack these cells. Despite not all mutations giving rise to neoantigens, a high number of somatic mutations can be an indicator for the neoantigenic load. TMB is evaluated by molecular methods—one possibility is whole exome sequencing, which sequences concomitant tumor and normal tissue and can filter real germline variants, but it is currently not compatible with the diagnostic workflow [5]. Panels for analysis of TMB/TML which interrogate approximately 300 to 22,000 genes and cover 0.8 to 30 Mb of DNA determine mutations per Mb of the tumor genome [95] are already commercially available. The trial Checkmate 227 used TMB high-status for randomization of patients to ipilimumab and nivolumab versus chemotherapy, demonstrating significant improvement in progression-free survival and response rate for the combination arm [96]. However, the methodology is not standardized and TMBs relevance for treatment decisions and the optimal cut-offs of mutations per Mb are not clear yet. As a consequence, we acknowledge that TMB analysis might be done in certain situations on request [97].

STK11 (LKB1) aberrations

Mutations in the serine/threonine kinase *STK11(LKB1)* gene, the second most commonly altered tumor suppressor in NSCLC, might induce primary resistance against PD-L1 blockade [98] and are frequently associated with prognostically adverse genetic alteration such as *KEAP1* and/or *KRAS* mutations. Whether patients with this kind of mutations should be treated differently is, however, still not established.

Algorithms for diagnosis and molecular testing

Shown in Figs. 1 and 2 are algorithms for histological diagnosis and molecular testing.

Conclusion

Molecular testing for targeted therapy and immune-oncologic treatment of advanced NSCLC should be performed according to available therapeutics. Genetic alterations, for which approved drugs are available or in process of approval, should be tested upfront (reflex test), initiated by the diagnosing pathologist. Analysis of alterations, for which off-label therapy is available should be tested at request of coordinators of clinical trials.

Testing methods must be evaluated and quality assurance is mandatory.

Reporting of the results should be standardized and contain explanations and comments according to the current scientific knowledge.

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[1] MIC = mononuclear immune cells: macrophages, lymphocytes, dendritic cells

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