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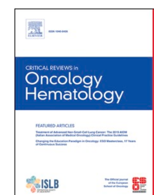

European School of Oncology – Review

Molecular testing and targeted therapy for non-small cell lung cancer: Current status and perspectives

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ABSTRACT

Molecular testing has become a mandatory component of the non-small cell lung cancer (NSCLC) management. The detection of EGFR, BRAF and MET mutations as well as the analysis of ALK, ROS1, RET and NTRK translocations have already been incorporated in the NSCLC diagnostic standards, and the inhibitors of these kinases are in routine clinical use. There are emerging biomarkers, e.g., KRAS G12C substitutions and HER2 activating alterations, which are likely to enter NSCLC guidelines upon the approval of the corresponding drugs. In addition to genetic examination, NSCLCs are usually subjected to the analysis of PD-L1 protein expression in order to direct the use of immune checkpoint inhibitors. Comprehensive NSCLC testing for multiple predictive markers requires the analysis of distinct biological molecules (DNA, RNA, proteins) and, therefore, the involvement of different analytical platforms (PCR, DNA sequencing, immunohistochemistry, FISH). There are ongoing efforts aimed at the integration of multiple NSCLC molecular assays into a single diagnostic pipeline.

1. Introduction

Lung cancer (LC) is the most common oncological disease accounting for more than 2 million cases and causing almost 1.8 million deaths per year worldwide. It is responsible for 11.6 % and 18.4 % of global cancer morbidity and mortality, respectively (Bray et al., 2018). LC is classified for small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is significantly more common than SCLC and is further subdivided for squamous and non-squamous histological types (Duma et al., 2019; Ruiz-Cordero and Devine, 2020). Squamous NSCLC is strongly linked to high-tar tobacco smoking. The genetic profiling of squamous carcinomas did not result in the identification of frequent druggable mutations, so this category of tumors is rarely discussed in the context of targeted therapies (Friedlaender et al., 2019; Relli et al., 2019). Non-squamous NSCLC is a predominant LC entity in the Western world and Asian countries. Lung adenocarcinomas constitute the majority of non-squamous NSCLC cases, while large-cell carcinomas are relatively infrequent. Non-squamous NSCLC is causally related to the Western type of tobacco products, i.e. cigarettes with moderate or low tar content. Dramatic decline of cigarette consumption, which was observed in the past decades in many countries around the world, led to evident decrease in the incidence of smoking-related NSCLC. As a consequence of depletion of tobacco-induced cases, up to a half of lung adenocarcinomas is now represented by non-smokers, at least in some patient series (Nakamura and Saji, 2014; Moiseyenko et al., 2010).

Most NSCLC patients undergo systemic therapy, either being diagnosed at already inoperable stage or experiencing the disease relapse after surgery (Ruiz-Cordero and Devine, 2020; Arbour and Riely, 2019). Potential clinical benefit of cytotoxic therapy did not look obvious as recently as in 1990s, so it took several large clinical trials to demonstrate that conventional anticancer drugs render statistically significant albeit relatively moderate improvement in the disease outcomes (Baxevanos and Mountzios, 2018). Dramatic breakthrough in the NSCLC management occurred in the first decade of the XXI century and was largely attributed to chance discoveries. First-generation EGFR tyrosine kinase inhibitors (TKIs) were initially tested in unselected NSCLC patients, given that virtually all lung carcinomas express increased amount of this receptor. The subsequent analysis of responders to gefitinib and erlotinib led to the identification of EGFR TKI-sensitizing mutations. Another breakthrough drug, crizotinib, was originally developed as a MET inhibitor. Its activity towards ALK kinase turned out to be clinically useful upon the identification of ALK-activating translocations in NSCLCs.

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Further research led to the discovery of a number of additional druggable genetic alterations and mutation-tailored drugs (Sokolenko and Imyanitov, 2018). The incorporation of immune checkpoint inhibitors in the NSCLC management also contributed in the spectacular improvement of disease outcomes, particularly in patients lacking TKI-sensitizing mutations (Nadal et al., 2019). For the time being, there is almost a dozen NSCLC molecular targets requiring clinical testing and associated with treatment decisions. Medical aspects of NSCLC genetic profiling have been comprehensively described in a number of recent reviews (Ruiz-Cordero and Devine, 2020; Arbour and Riely, 2019; Bodor et al., 2020; Camidge et al., 2019a; Herbst et al., 2018; Mascaux et al., 2018; Gregg et al., 2019; Pennell et al., 2019; Lamberti et al., 2020). However, this field is rapidly evolving, with multiple nuances still deserving thorough discussion. This paper is aimed to provide a brief overview on the current status and future development of NSCLC molecular testing.

2. EGFR

2.1. Overview

EGFR TKI-sensitizing intragenic mutations were discovered in the year 2004 during the analysis of results of gefitinib and erlotinib clinical trials. These mutations account for 10–20 % of non-squamous NSCLC in Caucasians and approximately a half of instances of this disease in Asians. The reasons for racial differences in EGFR mutation frequency remain unknown. The incidence of EGFR mutations is approximately three times higher in non-smokers vs. smokers and in women vs. men (Sokolenko and Imyanitov, 2018; Reguart and Remon, 2015; Imyanitov et al., 2016). Although these alterations occur almost exclusively in lung adenocarcinomas and large-cell carcinomas, some investigators observed instances of EGFR mutations in squamous cell carcinomas. Detailed analysis of these cases suggests that the detection of EGFR in presumably squamous NSCLC is rather attributed to histological misdiagnosis than to the true biological phenomenon (Rekhtman et al., 2012).

There are two major types of EGFR mutations, which account for 85–95 % druggable EGFR alterations and are detected by all diagnostic platforms. In-frame deletions in the exon 19 (ex19del) constitute approximately two thirds of activating EGFR mutations. The nucleotide composition and exact location of these deletions within exon 19 slightly vary, so some allele-specific PCR-based kits are likely to occasionally miss certain rare ex19del alleles. The remaining third is represented by L858R substitution located in the exon 21. Virtually all well-studied EGFR inhibitors, including first-generation (gefitinib and erlotinib), second-generation (afatinib) and third-generation (osimertinib) TKIs demonstrate better responses in tumors carrying ex19del as compared to L858R-mutated NSCLCs (Reguart and Remon, 2015; Soria et al., 2018). This trend was particularly emphasized in the accompanying information for afatinib, given that this drug produced better overall survival as compared to chemotherapy when administered first-line for ex19del- but not for L858R-mutated cancers (Yang et al., 2015). Li et al. (2020a) reported the study, which specifically focused on patients with EGFR L858R mutations by comparing regular and high doses of TKI. They concluded that EGFR L858R-mutated NSCLC patients may benefit from the increased dosage of the first-generation EGFR inhibitor, icotinib. It is of notice that L858R mutation is particularly characteristic for elderly NSCLC patients (Imyanitov et al., 2016). Many elderly subjects may deteriorate during the initial lines of treatment and eventually become illegible for chemotherapy. Overall, it is sometimes advisable to consider ex19del- and L858R-mutated NSCLCs as separate disease entities while discussing the design and the interpretation of clinical trials.

In addition to EGFR ex19del and L858R alleles, there is a number or relatively rare mutations. Systematic analysis of uncommon EGFR mutations is complicated, as different diagnostic platforms have distinct spectrum of analyzed nucleotide hot-spots. The list of rare EGFR mutations includes exon 18 nucleotide alterations (E709X (E709A, E709G, E709K, E709V), delE709-T710insD, G719X (G719S, G719A, G719C and G719D), exon 19 in-frame insertions, exon 20 alterations (A763_Y764insFQEA insertion, other insertions, S768I) and exon 21 mutation L861Q. Interestingly, the EGFR is sometimes affected by paired mutations. In some tumors, the ex19del or L858R is one member of the pair, while the remaining mutation is represented by one of the above uncommon alleles; in other NSCLCs, both alterations forming the doublet belong to the category of "rare" mutations. Consequently, it is difficult to estimate the frequency of rare mutations, as single and complex mutations need to be counted separately. It appears, that approximately 10 % of EGFR-mutated tumors contain uncommon somatic mutations, and their frequencies are more or less evenly distributed among the involved exons (18, 19, 20 and 21) (Harrison et al., 2020; Gristina et al., 2020). It is of notice, that the overall number of NSCLC patients with rare EGFR mutations is comparable to that for, e.g., ROS1-rearranged NSCLC cases; however, while there is a multitude of clinical trials and guidelines related to the management of ROS1-driven NSCLCs, the information on drug sensitivity of NSCLCs carrying uncommon EGFR alterations is limited to a number of case reports and a few small clinical investigations (Harrison et al., 2020; Gristina et al., 2020; Robichaux et al., 2018; Li et al., 2019; Cho et al., 2020; Yang et al., 2020).

All listed above rare EGFR mutations, except non-A763_Y764insFQEA exon 20 insertions, demonstrated some instances of response to conventional EGFR inhibitors, although it seems that they are generally less responsive to TKIs than ex19del and L858R mutants (Harrison et al., 2020; Li et al., 2019; Coleman et al., 2020). Preclinical and clinical data suggest, that distinct mutations may exert distinct sensitivity to various EGFR TKIs, so the choice between gefitinib, erlotinib, afatinib and osimertinib may depend on the type of EGFR lesion. There are also investigational EGFR TKIs, which are capable of targeting gefitinib/erlotinib-resistant EGFR exon 20 insertions (Harrison et al., 2020; Robichaux et al., 2018; Cho et al., 2020; Yang et al., 2020; Baraibar et al., 2020). While the analysis of EGFR ex19del and L858R mutations seemed reasonably sufficient a decade ago, current standards may require explicit EGFR testing, at least in patients, who are negative for other oncogenic driver mutations. There is also a need to specifically consider patients with rare EGFR mutations in clinical trials and in daily practice in order to develop guidelines for the management of this unusual category of NSCLC.

EGFR T790M mutation is often acquired during NSCLC treatment by gefitinib, erlotinib or afatinib and renders escape from the first- or second-generation TKIs. The third-generation inhibitor osimertinib possesses specific activity against this receptor isoform, hence it is recommended for the treatment of EGFR T790M-mutated tumors. Upfront use of osimertinib prevents early acquisition of the T790M substitution and prolongs progression-free survival, therefore osimertinib is also approved for the first-line NSCLC therapy (Recondo et al., 2018). Earlier studies reported that the T790M allele is present in a subset of treatment-naïve tumors harboring EGFR ex19del or L858R mutation and is associated with primary resistance to first-generation TKIs (Sequist et al., 2008). Furthermore, multiple investigations demonstrated that many NSCLCs, being T790M-negative by conventional tests, still contain a small fraction of T790M-mutated cells, and the presence of this "mosaic" mutation is associated with shorter progression-free survival on TKI treatment. However, the detection of "mosaic" EGFR T790M is prone to various artifacts; in particular, archiving of tumor tissues in formalin-fixed paraffin-embedded blocks is accompanied by emergence of false-positive T790M signals. As a consequence, neither threshold for EGFR T790M detection nor its frequency in treatment-naïve tumors are well established. Recent studies demonstrated examples of good response to first-generation TKIs for tumors containing relatively high fraction of T790M-positive cells (Lavdovskaia et al., 2018). Furthermore, there are provocative reports suggesting that "mosaic" T790M mutations are associated with even better NSCLC response to gefitinib or erlotinib as compared to T790M-negative tumors (Vendrell et al., 2019).

The emergence of EGFR T790M substitution may play a role in the natural evolution of NSCLC, irrespectively to TKI treatment, so the analysis of biological significance of this mutation and its interaction with EGFR TKI-sensitizing mutations is of high interest (Lavdovskaia et al., 2018).

2.2. Diagnostic approaches

Initial studies involving EGFR-mutated NSCLCs often relied on conventional DNA sequencing. Sanger sequencing, while being capable of detecting all mutations within a given genomic region, has a risk of obtaining false-negative results when the analyzed sample is not sufficiently enriched by tumor cells. Current guidelines encourage the use of mutation-specific PCR kits, which are usually capable of detecting the mutation even if the proportion of tumor cells in the specimen is as low as 1–5 % (Lindeman et al., 2018). However, none of the currently available PCR kits covers the entire spectrum of EGFR TKI-sensitizing mutations, therefore some potentially druggable EGFR alterations are inevitably to be missed by this approach (Coleman et al., 2020; Iyevleva et al., 2014). EGFR is included in all diagnostic next generation sequencing (NGS) panels; NGS combines comprehension, i.e. the ability to reveal all types of EGFR mutations, and high sensitivity, i.e. the potential of detecting EGFR-mutated copies within a huge excess of normal cells (Lindeman et al., 2018).

The detection of EGFR T790M mutation requires the analysis of genetic event, which was acquired by tumor cells during TKI therapy. If the tumor tissue is easily available for re-biopsy, the detection of the T790M substitution is usually not complicated. Given that re-biopsy is not always feasible in subjects with NSCLC and that this procedure is associated with patient suffering, the analysis of circulating tumor DNA (ctDNA) may be used as a substitute; however, the physician has to be aware about limitations of the "liquid biopsy" (Lindeman et al., 2018). Droplet-digital PCR (ddPCR) has the best performance in identifying EGFR T790M mutation in the plasma when compared to other diagnostic methods (Lavdovskaia et al., 2018; Guo et al., 2019).

3. ALK and ROS1 rearrangements

3.1. Overview

ALK and ROS1 translocations occur in 5–8 % and 1–2 % non-squamous NSCLCs, respectively. As EGFR mutations, they are strongly associated with non-smoking behavior and female gender. In contrast to EGFR, there are no pronounced racial differences in the incidence of ALK and ROS1 fusions. While EGFR ex19del mutations demonstrate a flat age-related distribution and the frequency of EGFR L858R substitutions gradually increases with age, ALK and ROS1 translocations are particularly characteristic for young-onset NSCLCs (Imyanitov et al., 2016; Morris et al., 2019; Rosas et al., 2019).

Rearrangements involving ALK and ROS1 result in the translocation of the kinase-containing portion of the gene towards a "gene-partner", which is under the control of stronger promoter. As a consequence, chimeric ALK and ROS1 demonstrate higher level of expression relative to corresponding normal genes. Most of reported clinical trials utilized break-apart FISH assay for the detection of ALK and ROS1 fusions. It evaluates the distance between 5′ and 3′ portions of the analyzed gene; if the translocation event occurs, these parts of the gene become separated from each other and produce characteristic split signals upon visualization. Many laboratories utilize IHC analysis, which relies on the overexpression of kinase portion of ALK and ROS1 proteins in NSCLCs carrying the corresponding translocation (Lindeman et al., 2018; Thorne-Nuzzo et al., 2017; Conde et al., 2019).

Neither FISH nor IHC, being the most common techniques for ALK and ROS1 analysis, are capable of identifying the variant of the translocation. There is a diversity of ALK and ROS1 rearrangements, which involve different gene-partners and a multitude of break-points. At least some data suggest, that certain translocation variants may have particular biochemical properties and differ from each other with regard to mechanisms of acquiring TKI resistance. Very few clinical studies considered the genetic diversity of ALK and ROS1 translocations. Genotyping of ALK and ROS1 fusion variants may be of some value, especially in studies comparing different ALK and ROS1 inhibitors (Childress et al., 2018; Lin et al., 2018).

Albeit rarely, some NSCLCs demonstrate overexpression of ALK and ROS1 in the absence of gene translocations. There are controversial data on the clinical significance of these events. While almost all ALK translocation-positive tumors demonstrate some sensitivity to corresponding TKIs, the expression of this protein in the absence of rearrangement may or may not be associated with clinical benefit from the TKI administration (Thunnissen et al., 2019).

ALK and ROS1 are highly related kinases, therefore most of relevant TKIs (crizotinib, ceritinib, lorlatinib) have been extensively evaluated both in ALK- and ROS1-rearranged tumors. Alectinib and brigatinib received approval for the treatment of ALK-driven NSCLC, and entrectinib can be considered as an option in ROS1-rerranged tumors (Morris et al., 2019; Rosas et al., 2019; Spagnuolo et al., 2018). In contrast to EGFR, where the spectrum of acquired drugs-resistant mutations is confined to a few hot-spots, there is a significant diversity of secondary mutations in ALK and ROS1 genes. The probability of emergence of particular TKI-resistant substitution may depend on the type of translocation: for example, ALK G1202R substitution is particularly characteristic for tumors carrying EML4-ALK variant 3 gene fusion. These secondary mutations demonstrate distinct sensitivity to different TKIs in preclinical and clinical settings, therefore optimal sequencing of ALK- and ROS1-directed therapies is of utmost importance (Lin et al., 2018). The invention of ALK and ROS1 inhibitors led to unprecedented improvement of long-term outcomes of this category of NSCLC patients. For example, recent alectinib phase III trial registered a median progression-free survival of 34.8 months in ALK-driven NSCLC cases, which were treated by this drug in the first-line therapy (Camidge et al., 2019b). French retrospective study reported median overall survival of 89.6 months in metastatic patients with ALK-rearranged NSCLCs, that is an order of magnitude longer compared to life expectancy in subjects treated by conventional chemotherapy (Duruisseaux et al., 2017).

3.2. Diagnostic approaches

Initial studies on ALK and ROS1 translocations relied on FISH-based detection of these events. The use of FISH in clinical routine is complicated due to high cost, significant requirements to the quality of specimens, need for specific equipment and lack of automatization, therefore there are ongoing efforts to supplement or replace FISH by other methods. ALK and ROS1 IHC assays demonstrate generally high concordance with FISH. It is of concern that IHC identifies a number of tumors, which show increased amount of ALK or ROS1 protein in the absence of the gene fusion. Current guidelines permit the use of ALK IHC as a substitute for FISH, however IHC for ROS1 requires subsequent validation of "positive" samples by an alternative assay (Lindeman et al., 2018; Garrido et al., 2020). PCR-based approaches rely on the identification of the most common translocations or utilize so-called test for 5′/3′-end unbalanced expression; the latter is capable of detecting all gene rearrangements irrespective of their type (Iyevleva et al., 2015). NGS analysis of gene rearrangements is complicated. There are DNA-based assays, which sequence the intronic regions of the analyzed genes; these NGS tests include relatively huge regions of the genome. There are also RNA-based NGS tests, which utilize complementary DNA (cDNA) as a template. These tests are designed specifically for detecting gene fusions and demonstrate good performance in diagnosing actionable gene translocations (Frampton et al., 2013; Cohen et al., 2020; Volckmar et al., 2019).

4. BRAF, MET, RET, NTRK

4.1. Overview

BRAF testing is probably the easiest part of NSCLC molecular analysis, as all druggable BRAF mutations are located in the same hot-spot. BRAF V600E substitution was initially identified in melanomas, and this discovery led to the development of corresponding drugs, vemurafenib and dabrafenib. While BRAF V600E substitutions account for more than a half of skin melanomas, their incidence in non-squamous NSCLC falls within 1–2 %. In addition to V600E, there are some rare mutation types, which affect either codon 600 or neighboring positions and demonstrate either sensitivity or resistance to BRAF-targeted drugs. These rare mutations are often missed by available PCR assays, therefore their thorough clinical evaluation is complicated even for melanoma patients and appears to be virtually impossible for NSCLC. BRAF V600E mutated NSCLCs are responsive to dabrafenib and vemurafenib. Single-agent treatment of BRAF-driven cancers results in rapid acquisition of resistance through re-activation of MEK pathway, therefore the combined use of BRAF V600E and MEK inhibition is a common practice (Lamberti et al., 2020; Planchard et al., 2016, 2017; Mazieres et al., 2020; Malapelle et al., 2020).

In addition to described above BRAF exon 15 alterations, some NSCLCs carry mutations in exon 11 of this gene (Dagogo-Jack et al., 2019). These mutations appear to be non-druggable for the time being (Mazieres et al., 2020; Gautschi et al., 2015).

MET exon 14 skipping mutations result in extended protein half-life and overexpression of this receptor. These mutations occur in approximately 2–3 % of non-squamous NSCLCs, being clearly overrepresented among elderly patients (Mitiushkina et al., 2019). Crizotinib, capmatinib and tepotinib showed high clinical activity towards MET-driven NSCLCs in recent clinical trials (Drilon et al., 2020a; Schuler et al., 2020; Paik et al., 2020).

RET translocations are responsible for 1–2 % non-squamous NSCLCs. Similarly to ALK and ROS1, they are more characteristic for young non-smokers and females. This category of cancers is responsive to RET TKI inhibitors (Hida et al., 2019; Drilon et al., 2019a; Gautschi et al., 2017; Drilon et al., 2020b). Some studies indicate that distinct RET translocation variants demonstrate strikingly different responsiveness to targeted compounds (Drilon et al., 2019a; Tan et al., 2020).

So-called “agnostic” markers, which retain predictive value across all cancer types, are very infrequent in NSCLC. High-level microsatellite instability (MSI-H) is associated with high number of somatic mutations and tumor responsiveness to immune checkpoint blockade. However, the description of MSI-H in NSCLCs is limited by single observations (Warth et al., 2016). NTRK1/2/3 gene fusions, which render sensitivity to TRK inhibitors, occur in NSCLCs at frequency around 0.2 % (Solomon et al., 2020). Despite the rarity of NTRK1/2/3 rearrangements, there are clinical trials which managed to recruit significant number of NTRK1/2/3-driven NSCLC cases and confirm clinical utility of entrectinib and larotrectinib for this category of NSCLC (Drilon et al., 2019b; Paz-Ares et al., 2019).

4.2. Diagnostic approaches

Although BRAF, MET, RET an NTRK markers have been incorporated in the diagnostic standards, the utilization of these tests is complicated in many countries around the world (Smeltzer et al., 2020). BRAF mutation testing is a relatively simple procedure, however this assay was not considered mandatory until very recently, due to the rarity of BRAF activation in NSCLC (Kalemkerian et al., 2018). The analysis of MET exon 14 skipping mutations can be performed by PCR or NGS (Mitiushkina et al., 2019; Wolf et al., 2020). RET rearrangements are usually detected by FISH, NGS or PCR (Drilon et al., 2020b). The analysis of NTRK1/2/3 translocations is complicated, because it involves 3 distinct genes and is compromised by exceptional rarity of these events in NSCLC. Gene-specific NTRK1/2/3 NSCLC testing usually relies on the screening by IHC followed by the validation of the rearrangement by an additional method (Marchiò et al., 2019). It is of notice that BRAF, MET and RET inhibitors have been approved for the clinical use in previously untreated patients, therefore the turn-around time of the corresponding tests is of particular concern. MET exon skipping mutations deserve especial mention, as they are particularly characteristic for NSCLC patients aged above 70 years, i.e., in subjects with expected poor tolerability of standard cytotoxic therapy. While considering sequential NSCLC testing in elderly NSCLC cases, it is advisable to prioritize MET analysis over the ALK, ROS1 and RET testing, given that the probability of detecting actionable gene fusions in geriatric NSCLCs is small (Mitiushkina et al., 2019).

5. Emerging predictive mutations

KRAS mutations occur in approximately 15–30 % of non-squamous NSCLCs. Frequencies of KRAS alterations significantly vary in particular subtypes of cancers. Smoking-induced NSCLCs are characterized by low incidence of other oncogene-activating events (EGFR, MET and BRAF mutations; ALK, ROS1 and RET translocations), while KRAS mutations occur in approximately one third of tobacco-associated tumors. However, at least 30–40 % cancers in non-smokers is attributed to the listed above mutations, and this estimate could be twice higher in Asians due to elevated incidence of EGFR alterations. Accordingly, although KRAS mutations are infrequent in unselected NSCLCs obtained from non-smokers or Asian patients, their share in EGFR/ALK/ROS1/RET/BRAF/MET mutation-negative cancers is comparable with the one in Caucasian smokers. Smoking-related and smoking non-related NSCLCs have distinct pattern of KRAS mutations. Tobacco-induced tumors usually acquire G12C substitution, while G12D is the most prevalent amino acid change in non-smokers (Lindsay et al., 2018; Mitiushkina et al., 2018).

KRAS mutations are located in hot-spots (mainly in codons 12, 13, 59 and 61) and lead to activation of the protein. The development of inhibitors of mutated KRAS is complicated because of its high affinity to a substrate molecule GTP, so the synthetic compounds cannot efficiently compete with GTP for binding. Breakthrough has been achieved for the most frequent KRAS mutation, G12C, as this protein isoform is accessible to allosteric inhibition (Lindsay et al., 2018; Ferrer et al., 2018). Early clinical trials with KRAS G12C inhibitors, sotorasib (AMG510) and MRTX849, produced very encouraging results (Canon et al., 2019; Hallin et al., 2020; Hong et al., 2020). Interestingly, AMG510 demonstrates significant synergism with the immune checkpoint inhibition both in preclinical and in clinical settings. This interaction may have particular medical relevance: KRAS G12C mutations are strongly linked to smoking history, therefore KRAS G12C-mutated tumors are likely to have high tumor mutation burden and elevated level of antigenicity (Ferrer et al., 2018; Lindsay et al., 2018; Mitiushkina et al., 2018).

KRAS mutations result in MEK upregulation. Selumetinib, an allosteric MEK inhibitor, demonstrated promising results in KRAS-driven NSCLC in a phase II study, however the subsequent phase III trial failed (Jänne et al., 2017). Another inhibitor, trametinib, did not show advantage as compared to docetaxel in previously treated KRAS-mutated NSCLCs (Blumenschein et al., 2015). Different KRAS mutations may exert slightly different biochemical effects and, as exemplified above for smoking-related and smoking-unrelated cancers, arise in distinct genomic background (Mitiushkina et al., 2018; Ihle et al., 2012). It is not clear whether these nuances could have influenced the results of MEK inhibitor trials (Jänne et al., 2017, 2013, 2015). The analysis of KRAS-mutated cells revealed, that the tumor escape from MEK down-regulation may be mediated by autophagy. Excitingly, the addition of the inhibitor of autophagy, hydroxychloroquine, to MEK inhibitor resulted in the shrinkage of KRAS-driven pancreatic cancer and KRAS-mutated colorectal carcinoma (Kinsey et al., 2019; Orlov et al., 2020). Preclinical experiments suggest therapeutic synergy between

MEK inhibition and down-regulation of PD-L1/PD1 immune pathway in determining tumor response (Lee et al., 2019).

None of KRAS inhibitors received approval, therefore KRAS testing is not mandatory in clinical guidelines (Lindeman et al., 2018). However, KRAS analysis is advisable for EGFR/ALK/ROS1/RET/BRAF/MET mutation-negative non-squamous NSCLCs, as the detection of KRAS mutation may serve as internal control for validity of the above tests.

Some NSCLCs demonstrate evidences for HER2 upregulation either by gene amplification and overexpression or by activating mutation (Lamberti et al., 2020; Jebbink et al., 2020; Ekman, 2019). Preclinical and clinical studies revealed, that conjugates of cytotoxic drugs and HER2 antibodies may induce responses in HER2-driven NSCLCs (Baraibar et al., 2020; Jebbink et al., 2020; Li et al., 2018; Peters et al., 2019; Li et al., 2020b). Approximately 2% of NSCLCs carry activating HER2 insertions in exon 20 (Arcila et al., 2012). An irreversible pan-HER inhibitor pyrotinib has shown clinical activity towards NSCLCs carrying HER2 exon 20 insertions (Zhou et al., 2020; Wang et al., 2019).

6. Biomarkers for immune checkpoint inhibitors (ICIs)

The described above mutation-based biomarkers can be conditionally regarded as presumable equivalents for treatment decisions, as their positive and negative predictive value approaches to nearly 100 %. Indeed, almost all patients with appropriate EGFR, ALK, ROS1, BRAF, MET, etc. genetic alterations benefit from corresponding kinase inhibitors by objective tumor response or disease control, while the lack of actionable mutations in the above genes renders virtually null chances for drug-induced tumor shrinkage. These markers are also intuitively attractive because they are either “positive” or “negative”, i.e. there is no any intermediate values or thresholds involved. Consequently, the results of these genetic tests invariably call for the use or non-use of certain targeted compounds, albeit the choice of particular drug within the class and sequencing of various targeted and cytotoxic agents may vary depending on preferences of the patient and his/her physician, availability of relevant clinical trials, allocated budget, etc. (Camidge et al., 2019a).

There is an entirely distinct landscape of predictive markers and tests for immune therapy. Several drugs utilized for NSCLC treatment target the same pathway, being directed either towards PD-L1 (atezolizumab, durvalumab, avelumab) or its receptor PD1 (pembrolizumab, nivolumab). Accordingly, all these drugs were evaluated against the level of expression of PD-L1, as determined by IHC. Unlike for ALK or ROS1, where IHC produces mainly binominal results (strongly positive or no evidence for overexpression), PD-L1 staining is a continuous variable. Each drug is linked to its own companion or complementary test, and these tests utilize different testing platforms, distinct thresholds and distinct scoring systems. Some of these tests produce comparable results and therefore are potentially interchangeable, while others are not. Furthermore, while PD-L1 testing is mandatory for some ICI regimens, e.g. for single-agent pembrolizumab or atezolizumab in the front-line therapy, other schemes, for example, the combined use of pembrolizumab or atezolizumab with cytotoxic drugs in the upfront NSCLC treatment, do not require knowledge on PD-L1 status (Bodor et al., 2020; Camidge et al., 2019a; Gadgeel et al., 2020; Proto et al., 2019; Lantuejoul et al., 2019, 2020; Imyanitov et al., 2020). As with many continuous variables, the mere existence of correlation between the level of PD-L1 staining and the probability of response is beyond the doubt, so the patients with high PD-L1 expression have the highest chances of clinical benefit (Bodor et al., 2020; Camidge et al., 2019a; Imyanitov et al., 2020; Aguilar et al., 2019). However, there are multiple instances of PD-L1 negative tumors responding to ICIs, and, vice versa, of non-responders among strong PD-L1 expressors. There are continuing attempts to develop additional markers for ICI administration.

Tumor mutation burden (TMB) correlates with overall tumor antigenicity and was shown to be predictive for immune therapy response across multiple cancer types. TMB is also a continuous variable requiring next-generation sequencing of the entire exome or of multiple representative genomic loci. High TMB has been shown to correlate with the efficacy of immune therapy for NSCLCs irrespectively of PD-L1 status in some studies (Pennell et al., 2019; Proto et al., 2019; Fang et al., 2019). TMB is a novel marker, and its daily clinical use is compromised by high cost, poor turnaround time, lack of validated thresholds and some methodological issues (Bodor et al., 2020; Camidge et al., 2019a; Hendriks et al., 2018; Pacheco et al., 2019; Heeke et al., 2020; Sholl et al., 2020). High TMB is characteristic for smoking-related NSCLC, and one may suggest that smoking history or the presence of smoking-related KRAS mutations can be considered as a surrogate for TMB and spare from this expensive test (Camidge et al., 2019a; Ferrer et al., 2018; Mhanna et al., 2019).

The diversity of HLA class I peptides may influence outcomes of immune-directed therapy (Chowell et al., 2018). However, NSCLC studies demonstrated that HLA genotype is not predictive for tumor responsiveness to immune checkpoint inhibitors (Negrao et al., 2019).

7. Choice of diagnostic platforms: David or Goliath?

Proper genetic examination of non-squamous NSCLC requires some mandatory tests (EGFR, ALK, ROS1, BRAF, MET, RET), the analysis of some emerging markers (KRAS, HER2, etc.) and the consideration of some agnostic drug indications (NTRK1/2/3). In addition, PD-L1 IHC analysis may be needed for mutation/translocation-negative tumors in order to assist the choice of immune therapy (Table 1).

Nowadays many laboratories utilize sequential approach (Fig. 1). EGFR and BRAF mutations are usually determined by PCR-based tests. The analysis of gene rearrangements is mainly done in morphological laboratories using IHC and/or FISH. PD-L1 analysis is also based on the use of diagnostic antibodies. The need for multiple tests, which often require distinct diagnostic platforms, is logistically complicated and poorly compatible with the amount of available tumor tissue. Indeed, many diagnostic lung cancer samples are represented by tiny biopsies, given that the majority of NSCLC patients are diagnosed already at advanced stage and do not undergo surgery (Pennell et al., 2019; Lindeman et al., 2018; Liam et al., 2020).

Next generation sequencing (NGS) provides an opportunity to analyze all medically relevant genes within the same run. It is already used for NSCLC diagnosis in many advanced hospitals and is likely to replace conventional techniques of gene analysis in the mid- or long-term perspective (Aggarwal et al., 2019). NGS is capable of detecting small mutations across all regions of the analyzed genes; therefore, the use of NGS is expected to increase the number of identified EGFR or BRAF mutations, which are not detectable by conventional PCR assays. The detection of more complex alterations, e.g. ALK, ROS1, RET, NTRK1/2/3 rearrangements and exon MET exon 14 skipping mutations, is more complicated and may require NGS analysis of both genomic DNA and RNA (Cohen et al., 2020; Benayed et al., 2019; Davies et al., 2019; Li et al., 2020c). Evaluation of gene copy number variations, e.g. MET and HER2 amplifications, can be achieved by comparative analysis of the number of reads and still requires some additional validation. Overall, the available NGS assays are sufficiently well adjusted to the need of genetic testing of NSCLC (Pennell et al., 2019). However, clinical analysis of expression markers, e.g. PD-L1, still cannot be achieved by NGS and requires an additional technique, IHC (Fig. 1).

Despite obvious advantages, the use of NGS is impractical in many circumstances. NGS requires highly expensive and sophisticated equipment and reagents, so this technique is not yet easily accessible for all cancer centers around the world. All druggable mutations in NSCLC are mutually exclusive, therefore simultaneous testing for the entire spectrum of mutations is not always justified (Cohen et al., 2020). For example, the frequency of EGFR mutations in Caucasian females or non-smokers approaches 30 %, and this estimate in Asians may exceed 50–70 %. Therefore, it is advisable to start testing for these categories of subjects with a relatively simple and non-expensive EGFR test, and to

Table 1
Predictive molecular markers in NSCLCs.

Gene	Genetic alteration	Examples of associated drugs	Methods of detection	References
EGFR	ex19del, L858R and “uncommon” TKI-sensitizing mutations	gefitinib, erlotinib, afatinib, dacomitinib, osimertinib	Mutation-specific PCR; NGS	(Reguart and Remon, 2015; Soria et al., 2018; Harrison et al., 2020; Gristina et al., 2020)
	T790M (acquired TKI resistance)	osimertinib	Mutation-specific PCR (re-biopsy of the tumor tissue or liquid biopsy)	(Recondo et al., 2018)
	exon 20 insertions	poziotinib	Not standardized yet (PCR, NGS)	(Robichaux et al., 2018; Baraibar et al., 2020)
ALK	rearrangement	crizotinib, alectinib, brigatinib, ceritinib	IHC; FISH; various modifications of PCR; DNA- or RNA-based NGS	(Spagnuolo et al., 2018)
ROS1	rearrangement	crizotinib, lorlatinib, entrectinib	IHC as a screening assay followed by a validation test; FISH; various modifications of PCR; DNA- or RNA-based NGS	(Morris et al., 2019)
BRAF	V600E	dabrafenib or vemurafenib (in combination with MEK inhibitors)	Mutation-specific PCR; NGS	(Planchard et al., 2016, 2017; Mazieres et al., 2020)
MET	exon 14 skipping	crizotinib, capmatinib, tepotinib	NGS; various modifications of PCR	(Mitiushkina et al., 2019; Drilon et al., 2020a; Schuler et al., 2020; Paik et al., 2020; Wolf et al., 2020; Salgia et al., 2020)
	amplification	capmatinib	FISH; NGS assays capable of measuring gene copy number	(Schuler et al., 2020; Wolf et al., 2020)
RET	rearrangement	lenvatinib, selpercatinib, RXDX-105	FISH; various modifications of PCR; DNA- or RNA-based NGS	(Hida et al., 2019; Drilon et al., 2019a, Drilon et al., 2020b; Markham, 2020)
NTRK1/2/3	rearrangement	entrectinib, larotrectinib	IHC as a screening assay followed by a validation test; DNA- or RNA-based NGS; various modifications of PCR; FISH	(Drilon et al., 2019b; Paz-Ares et al., 2019; Marchiò et al., 2019)
KRAS	G12C	Sotorasib (AMG510), MRTX849	Mutation-specific PCR; NGS	(Canon et al., 2019; Hallin et al., 2020; Hong et al., 2020)
HER2	activation by mutation or amplification/ overexpression	T-DM1	Not standardized yet (PCR or NGS for mutations; FISH/IHC for amplification/ overexpression)	(Baraibar et al., 2020; Jebbink et al., 2020; Li et al., 2018; Peters et al., 2019; Li et al., 2020b)
	exon 20 insertions or other mutations	pyrotinib	Not standardized yet (PCR, NGS)	(Zhou et al., 2020; Wang et al., 2019)
PD-L1	expression	immune checkpoint inhibitors	IHC	(Bodor et al., 2020; Camidge et al., 2019a; Cheung et al., 2019)
Tumor mutation burden	high number of coding mutations	immune checkpoint inhibitors	NGS	(Bodor et al., 2020; Camidge et al., 2019a)

See also diagnostic guidelines described in (Lindeman et al., 2018; Garrido et al., 2020; Kalemkerian et al., 2018).

look for other NSCLC molecular targets only in EGFR mutation-negative cases (Rajadurai et al., 2019). Turnaround time for NGS may be longer than for conventional tests, as NGS assays are usually done in centralized laboratories and/or require accumulation of several samples for a single run. However, even if a given hospital or laboratory performs thorough sequential testing for all druggable targets, it is essential to consider NGS analysis for “mutation-negative” cases, at least for NSCLCs diagnosed in females and non-smokers. Indeed, the vast majority of tobacco-unrelated and/or female NSCLCs carry mutation in one of the above genes (EGFR, ALK, ROS1, BRAF, MET, KRAS, RET, HER2, NTRK1/2/3, etc.), therefore failure to detect actionable mutation may indicate either a technical error or the presence of rare but still potentially relevant genetic alteration (e.g., in NRAS, NF1 or other MAPK pathway genes).

There are some PCR-based diagnostic pipe-lines, which may combine comprehension and low cost. As already stated above, the detection of hot-spot mutations in EGFR, BRAF and KRAS genes is usually achieved by allele-specific PCR and does not require extraordinary infrastructure or labor resources. Some laboratories supplement allele-specific PCR tests by high-resolution melting (HRM) and Sanger sequencing or pyrosequencing in order to detect rare mutations, which are not included in allele-specific PCR assays. The detection of gene rearrangements by PCR is problematic, as gene fusions are characterized by almost indefinite diversity of breakpoints and involved gene-partners. Consequently, variant-specific PCR tests are limited by the number of translocations included in the panel and are destined to miss rare types of fusions. However, there is an elegant alternative to the variant-specific PCR, which is based on the comparison of the level of expression of 5′ and 3′ portions of the analyzed kinase gene. It is assumed that in the absence of translocations all parts of the gene will have identical expression, as assessed by RNA analysis. However, if the rearrangement event occurs, the kinase-specific portion of the involved receptor fuses with another gene, which has significantly higher level of transcription. Consequently, rearranged kinases will have so-called unbalanced 5′/3′-end expression, which can be easily detected by the real-time PCR (Iyevleva et al., 2015). This test shares the advantages of IHC or FISH, as it is capable of detecting both common and rare variants of NSCLC-specific rearrangements. Reliable PCR-based methodology has also been reported for MET exon 14 skipping mutations (Mitiushkina et al., 2019).

The only current drawback of PCR technology is the lack of established assays for PD-L1 expression determination. Some studies demonstrated a correlation between PD-L1 transcript expression, as determined by PCR, and protein level, as measured by IHC (Vannitamby et al., 2019) However, PD-L1 RNA expression tests still need to undergo some adjustment and clinical validation. Atezolizumab studies utilized 3-gene RNA expression assay (PD-L1, CXCL9, IFN-gamma) as a potential predictive marker, however its technical aspects and scoring algorithm are not disclosed due to intellectual property restrictions (Socinski et al., 2018). It is of notice, that RNA expression tests, which always have an in-build standard (i.e., gene-referee), are generally more reproducible and better suitable for quantitation as compared to IHC assays. Consequently, there are attempts to supplement or even replace some IHC techniques by corresponding PCR expression assays. For example, Oncotype Dx breast cancer multigene expression platform includes estrogen receptor, progesterone receptor and HER2 oncogene, so the results of conventional IHC analysis are subjected to validation (McVeigh and Kerin, 2017). Some studies demonstrated that PCR-based evaluation of Ki-67 expression has better clinical utility as compared to conventional morphological scoring (Yamamoto et al., 2013; Gao et al., 2019).

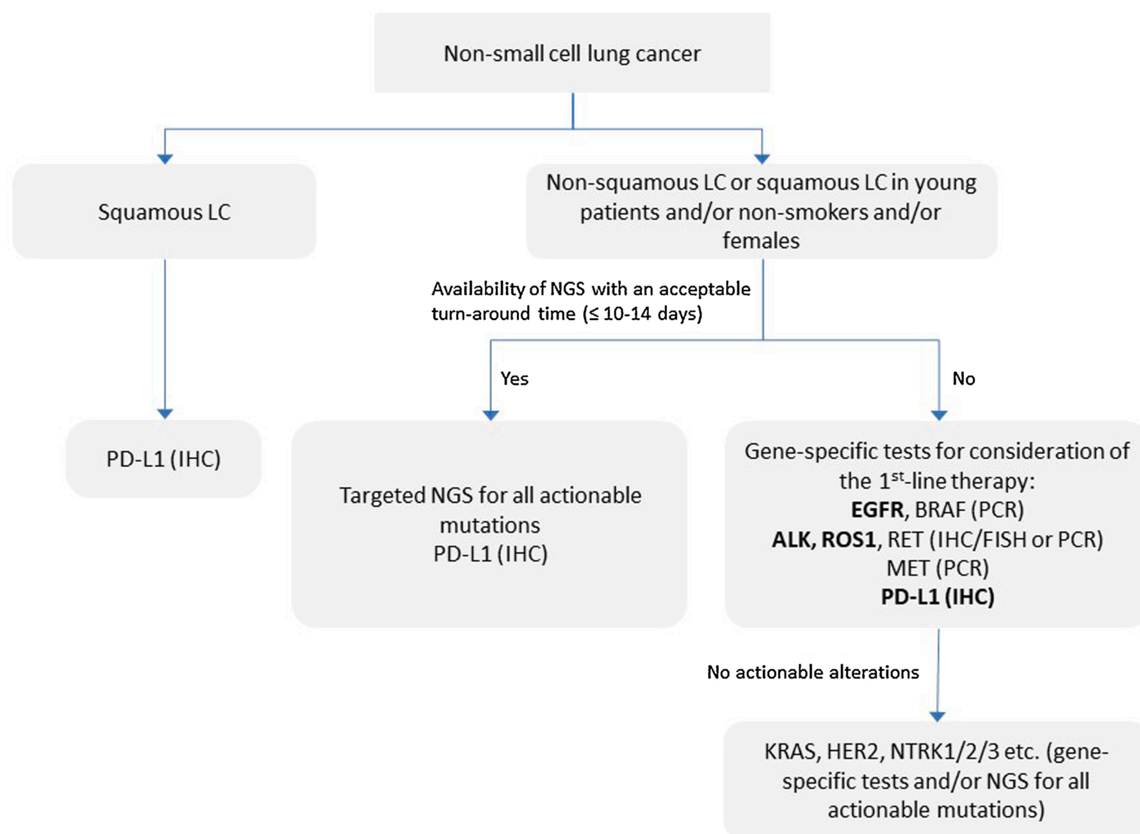

Fig. 1. NSCLC molecular testing. The choice of testing algorithm largely depends on the availability of various molecular tests, turn-around time, financial considerations and the approval/reimbursement status of corresponding targeted drugs. EGFR, ALK, ROS1 inhibitors and ICIs are commonly used in the first-line treatment, therefore it is particularly important to arrange rapid assessment of the status of the relevant targets (marked in bold). BRAF analysis gained the status of mandatory procedure relatively recently (Kalemkerian et al., 2018) and unfortunately not all NSCLC patients receive this test in the regular clinical setting (Smeltzer et al., 2020). MET and RET inhibitors received FDA approval in the year 2020 and may not be available yet in all countries around the world. It is desirable to know MET and RET status at the time of NSCLC treatment planning, however some clinics may have difficulties in arranging rapid MET/RET testing (Smeltzer et al., 2020). Other molecular tests are currently relevant to the second-line therapy or clinical trials, therefore their utilization is less time-sensitive.

RNA expression measurement is compatible not only with PCR but also with NGS technology (Haynes et al., 2019). It is likely that further development of NGS clinical testing will consider the inclusion of some expression-based markers. There are also some attempts to improve the throughput of IHC-based assays by multiplexing several markers in the same platform (Hofman et al., 2019).

Real-world studies demonstrate, that many NSCLC patients do not receive proper molecular testing (Smeltzer et al., 2020). As one would expect, some difficulties in the access to achievements of personalized medicine are related to socio-economic inequalities (Norris et al., 2020). It is important to encourage reasonable flexibility in the methodology of tumor analysis in order to stimulate the uptake of NSCLC molecular testing (Fig. 1).

8. Re-biopsy and liquid biopsy

The molecular profile of tumors may significantly change over the treatment course, as drug-sensitive cells are rapidly eliminated during therapy and the remaining clones become adapted to therapeutic pressure. There are general mechanisms of acquired drug resistance, which are relevant for all types of drugs. For example, many tumors contain so-called stem cells, which have the ability to survive in unfavorable circumstances and rapidly repopulate the tumor mass. Escape from the therapy often involves activation of drug efflux, down-regulation of apoptotic pathways and epithelial-mesenchymal transition. Administration of targeted drugs is usually associated with pathway-specific emergence of drug resistance. Some targets acquire secondary mutations and become insensitive to the drug. In addition, tumors may lose the addiction to the target by activation of down-stream molecules or collateral signaling pathways (Aleksakhina et al., 2019).

The detection of newly acquired molecular events is instrumental for guiding the subsequent lines of therapy. For example, approximately 50 % of NSCLC treated by first-generation EGFR inhibitors develop resistance due to secondary mutation EGFR T790M, and only these tumors are sensitive to osimertinib treatment. Consequently, the detection of T790M mutation in TKI-resistant tumors is included in the drug label as a mandatory test for TKI-pretreated NSCLC (Hotta et al., 2019). In contrast to EGFR, TKI-resistant mutations in ALK and ROS1 genes are characterized by significant diversity, and there are sophisticated algorithms for choosing the ALK/ROS1 TKI according to the identity of the secondary amino acid substitution (Recondo et al., 2018). Druggable tyrosine kinase receptors, such as EGFR, HER2, ALK, ROS1, MET, etc., seem to be interchangeable in certain circumstances, so some NSCLCs acquire the TKI resistance by activation of neighboring signaling molecule. Consequently, change of therapy for the corresponding TKI may down-regulate newly activated receptor and render clinical benefit (Asao et al., 2019).

While chasing the molecular characteristics of TKI-related signaling pathways is a rapidly evolving concept, which is already incorporated in NSCLC management either in clinical or investigational setting, there is little understanding whether similar principles are applicable to other types of therapy. For example, administration of immune-related drugs after chemotherapy relies on the PD-L1 status, which was observed in the tumor at diagnosis, i.e. before the start of systemic treatment. It is

established, that PD-L1 status may change over the treatment course (Hotta et al., 2019; Isomoto et al., 2020). While considering PD-L1/response correlations, which were observed in second-line trials of ICIs, it is important to recognize that they did not account for chemotherapy-induced changes of PD-L1 status.

Most of NSCLC patients develop multiple metastases and these tumor foci may demonstrate some heterogeneity with regard to molecular mechanisms of drug resistance. Repeated re-biopsies of all visible metastatic lumps are obviously not compatible with patient well-being. It is believed, that so-called liquid biopsy may provide an integral portrait of tumor evolution during the treatment course. Liquid biopsy has significant limitations, as it is often not informative in patients with low disease burden. The detection of hot-spot mutation, e.g., EGFR T790M, is easier to achieve using well-established high-sensitivity techniques, for example, droplet digital PCR (ddPCR) (Lavdovskaia et al., 2018). More complex examination, like sequencing of ALK or ROS1 genes, multigene tumor profiling or evaluation of tumor mutation burden, requires NGS analysis, which may have insufficient sensitivity (Canale et al., 2019; Horn et al., 2019; Wu et al., 2019). The analysis of plasma is acceptable for the tracking of acquired mutations in circulating tumor DNA, however is worthless for the assessment of expression markers. There are potentially relevant assays utilizing circulating tumor cells (CTCs), however it is not clear whether CTCs are representative for the entire tumor or constitute only a certain especial fraction of heterogeneous cancer cell community (Aleksakhina et al., 2019; Terlizzi et al., 2019). Some studies suggest that liquid biopsy may serve as a complementary test to tissue biopsy in order to minimize the probability of false-negative results (Aggarwal et al., 2019). The amount of circulating tumor DNA correlates with tumor response or non-response, so plasma mutation testing may be used as a marker of the efficacy of systemic therapy (Anagnostou et al., 2019; Phallen et al., 2019).

9. Conclusions and perspectives

NSCLC takes a leading position with regard to recent improvements in life expectancy as compared to other common tumor types (Howlader et al., 2020). Approximately 50–70 % Asian and 30–40 % non-Asian patients with non-squamous NSCLC carry druggable mutations, and the use of appropriate targeted drugs results in manifold increase of their overall survival (Herbst et al., 2018; Reguart and Remon, 2015; Duruisseaux et al., 2017; Ramalingam et al., 2020). The same applies for some patients, who lack actionable genetic alterations and therefore are not eligible for the treatment by kinase inhibitors, as the use of immune therapy renders long-term survival for approximately one out of five treated subjects (Nadal et al., 2019). On the other hand, it appears that the development of mutation-tailored drugs is reaching some plateau, as NSCLC exome sequencing studies did not reveal significant number of novel potentially druggable targets, and many newly developed compounds are focused on exceptionally rare genetic events (Drilon et al., 2019b; Paz-Ares et al., 2019; Imielinski et al., 2012; Kadara et al., 2017; Boeckx et al., 2020). It is also necessary to acknowledge that the progress in molecularly guided treatment is almost entirely related to non-squamous NSCLC, while the studies on squamous NSCLC failed to find its vulnerabilities (Friedlaender et al., 2019).

Within the next decade we will certainly witness further harmonization of NSCLC multigene testing. NGS appears to be capable to fulfill most of the unmet needs, however this technology has to become more cost-, time- and user-friendly in order to replace the existing diagnostic platforms.

Advances in NSCLC translational research led to significant modernization of the infrastructure for lung cancer care. The laboratory analysis of NSCLC was limited to conventional morphology for decades, so it took efforts to incorporate genetic analysis into diagnostic pipelines (Lindeman et al., 2018). Shortage in the amount of tumor material is more characteristic for lung cancer testing than for many other cancer types, therefore multiple studies were dedicated to the adjustment of relevant laboratory protocols to the biopsied and cytological material (Pennell et al., 2019; Mitiushkina et al., 2013). The need for monitoring of essential molecular characteristics of cancer disease and the development of drugs against acquired mutations in TKI-resistant tumors stimulated the concepts of re-biopsy and liquid biopsy (Aleksakhina et al., 2019; Hotta et al., 2019; Canale et al., 2019). There is also increasing demand for specialists, who combine the expertize in molecular biology, pathology and clinical aspects of cancer management.

Being in the front-line of integration of clinical research, laboratory activities and drug development, NSCLC may serve as an example of triumph of precision medicine. It is likely that the translational experience gained in the NSCLC diagnostics and treatment will be utilized in other fields of medical oncology.

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All authors declare that they have no conflict of interest.

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