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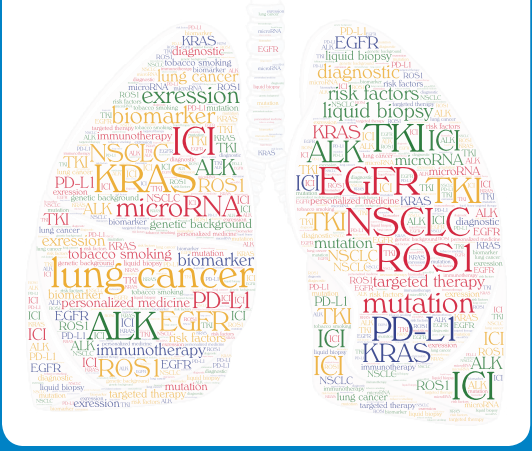

Wydawnictwo Uniwersytetu Medycznego w Łodzi

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MILENA PĄZIK[1,2*]
MAREK MIROWSKI[1]
EWA BALCERCZAK[1]

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AKTUALNE PODEJŚCIE DO DIAGNOSTYKI I TERAPII NIEDROBNOKOMÓRKOWEGO RAKA PŁUCA – KRÓTKI PRZEGLĄD

[1] Zakład Biochemii Farmaceutycznej i Diagnostyki Molekularnej, Katedra Diagnostyki Laboratoryjnej i Molekularnej, Wydział Farmaceutyczny, Uniwersytet Medyczny w Łodzi; ul. Muszyńskiego 1, 90-151 Łódź

[2] Zakład Diagnostyki Laboratoryjnej i Biochemii Klinicznej, Katedra Diagnostyki Laboratoryjnej i Molekularnej, Wydział Farmaceutyczny, Uniwersytet Medyczny w Łodzi; ul. Pomorska 251, 92-213 Łódź

* milena.pazik@umed.lodz.pl

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Łódź 2022

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WYDANIE PIERWSZE

Abstract: Non-small cell lung cancer (NSCLC), due to its incidence and mortality, is a huge worldwide health problem closely related to tobacco smoking as the main risk factor. Mostly diagnosed at an advanced stage, it results in poor overall survival. At the early stage of the disease combination of surgical resection, chemotherapy and radiation is among the most widely applied therapeutic strategies. Unfortunately, not all patients respond to the standard treatment. Therefore, strenuous efforts are made to implement new promising therapies with better efficacy and lower toxicity than the existing approach to lung cancer treatment. Immunotherapies such as tyrosine kinase inhibitors (TKI) and immune checkpoint inhibitors (ICI) are successfully used in routine clinical practice and thus molecular testing (i.e., *EGFR, KRAS, BRAF* mutations and *ALK, ROS1* rearrangements) has become an important part of the NSCLC management. Detection of the multiple genetic alterations requires application of different test methods such as FISH, immunohistochemistry, NGS and automated platforms to analyze many genes in short time. There are also some novel biomarkers that may be used in NSCLC prognosis and prediction efficacy of the corresponding drugs, although they need further testing. Another issue is application of liquid biopsy in NSCLC studies as an alternative to tissue biopsy that could be less invasive and helpful in monitoring of the disease progression. In this short review, we summarize current trends in diagnosis and personalized therapy in NSCLC as well as biomarkers useful in disease prognosis and prediction presenting their advantages and disadvantages.

Keywords: NSCLC, lung cancer, biomarker, *EGFR* gene*, ALK* gene*, ROS1* gene*, KRAS* gene, immune checkpoint inhibitors, tyrosine kinase inhibitors, liquid biopsy

Streszczenie: Niedrobnokomórkowy rak płuca (NDRP) jest najczęściej diagnozowanym nowotworem złośliwym, wiąże się z największą śmiertelnością wśród chorób nowotworowych oraz wykazuje ścisły związek z paleniem tytoniu. W większości przypadków rozpoznawany jest w bardzo późnych stadiach rozwoju choroby, co skutkuje krótkim całkowitym czasem przeżycia pacjentów. Terapia u chorych we wczesnych stadiach zaawansowania najczęściej bazuje na połączeniu resekcji operacyjnej, chemio- i radioterapii. Niestety nie wszyscy chorzy odpowiadają na standardowe leczenie, przez co współcześnie dużo uwagi poświęca się wdrażaniu nowych, obiecujących metod terapeutycznych o lepszej skuteczności i mniejszej toksyczności w porównaniu z dotychczas stosowanymi środkami. Obecnie do rutynowej kliniki praktycznej z powodzeniem została włączona immunoterapia z zastosowaniem inhibitorów kinazy tyrozynowej czy też inhibitorów punktów kontrolnych. W związku z tym testy molekularne w kierunku wykrywania mutacji w genach takich jak *EGFR, KRAS czy BRAF*, jak również rearanżacji genów *ALK* i *ROS1* stały się ważną częścią zaleceń dotyczących postępowania diagnostyczno-terapeutycznego w niedrobnokomórkowym raku płuca. W celu wykrycia istotnych klinicznie zmian genetycznych stosuje się różne metody diagnostyczne jak m.in. fluorescencyjną hybrydyzację in-situ, immunohistochemię, sekwencjonowanie nowej generacji, czy też zautomatyzowane platformy pozwalające na analizę wielu genów w krótkim czasie. Wraz z postępem nauki pojawiają się nowe, obiecujące biomarkery, które mogą mieć znaczenie prognostyczne i predykcyjne w kontekście zastosowanego, ukierunkowanego molekularnie leczenia, aczkolwiek wymagają dalszych badań klinicznych. Innym zagadnieniem jest możliwość wykorzystania do badań materiału alternatywnego dla biopsji tkankowej jakim jest płynna biopsja, wiążąca się z mniej inwazyjnym pobraniem, a przydatna w monitorowaniu progresji choroby. Rak płuca jest prawdziwym problemem zdrowotnym na całym świecie, dlatego w pracy przedstawiono przegląd obecnych trendów w diagnostyce i terapii personalizowanej z uwzględnieniem wad i zalet nowych biomarkerów prognostycznych i predykcyjnych.

Słowa kluczowe: NDRP, rak płuca, biomarker, *EGFR, ALK, ROS1, KRAS*, inhibitory punktów kontrolnych, inhibitory kinazy tyrozynowej, płynna biopsja

List of abbreviations

ALK – Anaplastic Lymphoma Receptor Tyrosine Kinase
CEA – Carcinoembryonic antigen
cfDNA – circulating cell-free DNA
CTC – circulating tumor cell
ctDNA – circulating tumor DNA
CTLA-4 – cytotoxic T-cell lymphocyte antigen 4
ddPCR – Droplet Digital PCR
EBUS-TBNA – endobronchial ultrasound-guided transbronchial needle aspiration
EDNRB – endothelin receptor type B
EGFR – Epidermal Growth Factor Receptor
EMA – European Medicines Agency
FDA – the US Food and Drug Administration
FFPE – formalin-fixed paraffin embedded
FISH – fluorescence in situ hybridization
HRAS – Harvey rat sarcoma viral oncogene homolog
ICI – immune checkpoint inhibitors
IHC – immunohistochemistry
KRAS – Kirsten rat sarcoma viral oncogene homolog
LDH – Lactate dehydrogenase
LMR – Lymphocyte to Monocyte Ratio
MET – mesenchymal-epithelial transition
NGS – Next Generation Sequencing
NLR – Neutrophil to Lymphocyte Ratio
NRAS – neuroblastoma RAS viral oncogene homolog
NSCLC – non-small cell lung cancer
NSE – neuron-specific enolase
OS – overall survival
PD-1 – Programmed death receptor 1
PD-L1 – Programmed death-ligand 1
PLR – Platelet to Lymphocyte Ratio
ROC curve – receiver operating characteristic curve
ROS1 –ROS Proto-Oncogene 1
RTK – receptor tyrosine kinase
SCLC – small cell lung cancer
SMARCA1 – SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 1
TKI – tyrosine kinase inhibitor
TMB – tumor mutation burden
TNM – tumor-node-metastasis

1. Introduction

Lung cancer is a major worldwide health problem. Currently, it is the most common cause of death due to cancer globally accounting for 1.8 million cancer-related deaths annually and the second most commonly diagnosed cancer worldwide with approximately 2.2 million new cases estimated in 2020. In men, lung cancer is the main cause of cancer morbidity and mortality within other cancers, whereas in women, it is the third one in terms of incidence and the second one in term of mortality (Sung et al., 2021). According to data collected by European Cancer Information System as required by the European Commission, in Poland 29509 new cases of lung cancer were reported in 2020, which constitutes 15% of all cancers (European Union, 2021) (Figure 1).

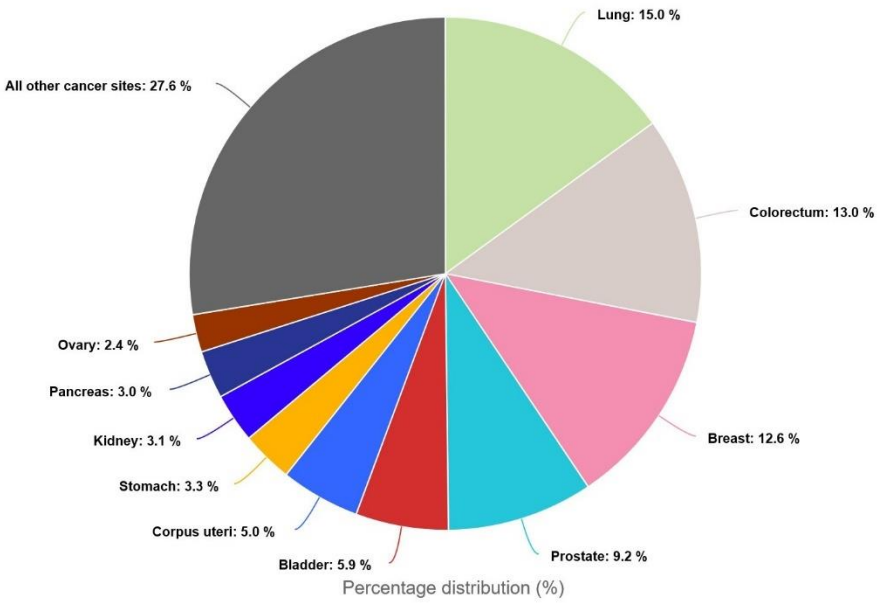

Figure 1. An estimated incidence for all cancer sites among women and men in Poland, 2020 (European Union, 2021).

Histologically lung cancer is divided into four main types, i.e., adenocarcinoma, squamous-cell carcinoma, small-cell carcinoma and large-cell carcinoma, although in practice there are, in fact, only two main types, i.e., small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for about 85% of all lung cancer cases which have totally different etiology and treatment options (Blandin Knight et al., 2017).

Lung cancer development is strongly associated with environmental and lifestyle factors, with tobacco smoking recognized as the main one causing the risk of the disease development. Others are exposure to asbestos, chrome, arsenic, radon or polycyclic aromatic hydrocarbons (Alberg & Samet, 2003; de Groot et al., 2018). Although cigarette smoking accounts for over 80% of lung cancers, approximately 10–15% of lung cancer cases occur in never- smokers (Samet et al., 2009). Additionally, NSCLC in non-smokers is different from that occurring among a group of individuals with smoking history depending on driver mutations, response to immunotherapy and overall survival (Kerrigan et al., 2021).

Lung cancer is histologically, biologically, clinically and molecularly a very heterogenous disease. Unfortunately, a majority of cases is detected at late stages and thus the overall survival rate for NCSCL is poor. Only 0% to 10% patients with stage IVA-IVB survive for five years following the diagnosis of NSCLC (Goldstraw et al., 2016). The process of lung cancer development is associated with the accumulation of many genetic and epigenetic abnormalities in cells leading to disorders of various molecular mechanisms and signaling pathways. New possibilities of early diagnosis and more precise therapy are still needed, therefore many researches concentrate on this issue. The concept of personalized medicine, which means taking a therapeutic decision based on

histology and genetic status of the patient's neoplasm is a desirable goal in modern medicine. Application of targeted therapy and immunotherapy gives hope for increase in the overall survival in NSCLC patients. However, together with implementation of effective targeted therapies, cancers develop acquired resistance to these inhibitors, which requires searching for next-generation medications. Therefore, identification of crucial genetic alterations is a vital step in the current diagnostic and therapeutic clinical practice of lung cancer.

Due to a high incidence and mortality of lung cancer patients a lot of emphasis is placed on harmonization of the diagnostic and therapeutic procedures. Zhang et al. conducted a great systematic review of existing clinical practice guidelines in NSCLC diagnostics and treatment. They have noticed different approaches to diagnosis (pathological, molecular and imaging). The most frequently mentioned genes among molecular diagnostic markers were EGFR, ALK and ROS1. Recommendations on lung cancer therapy varied depending on stage classification and assessment of the driving gene status. For example, in more than half guidelines, immunotherapy was recommended as part of the first-line treatment for patients with EGFR mutation in stage IV of NSCLC, while the rest suggested targeted therapy as a second-line treatment (Zhang et al., 2021). These differences in recommendations between guidelines encourage researches to conduct further analyses and explore novel prognostic and predictive markers of lung cancer.

The main aim of this work is to review current trends in molecular laboratory testing and development of targeted treatment in NSCLC (Figure 2).

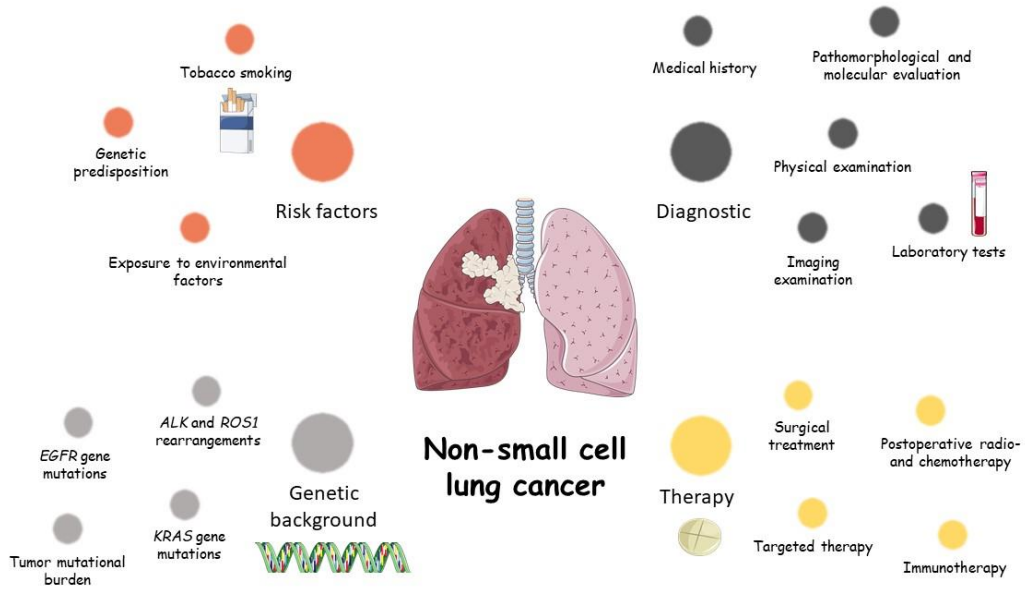

Figure 2. A summary of the key elements involved in NSCLC development, diagnosis and treatment.

2. Diagnostics and treatment

Lung cancer gives its symptoms at late stages therefore diagnostics is difficult. Patient examination should begin with taking medical history and assessment of risk factors (i.e. active and passive tobacco smoking, family history and exposure to dangerous environmental factors). Among imaging examination, the most important application has a low-dose chest computer tomography (CT) with an intravenously given contrast agent, while transbronchial needle biopsy is used to make diagnosis and assess the stage of lung cancer. Other current diagnostic methods include chest X-ray, computed tomography or sputum analysis. Pathomorphological evaluation should allow to determine features of tumor according to the TNM Staging System that includes information about the size and site of the main tumor (T), the number of nearby lymph nodes affected by cancer (N) and the presence of distant metastases (M). According to the determination of T, N and M features,

the clinical stage (I-IV) could be evaluated (Krzakowski & Jassem, 2019). In some cases, the routine microscopy analysis does not allow to precisely determine the type of tumor and then immunohistochemistry (IHC) tests are necessary. According to the WHO Classification of Lung Tumors, the most commonly used IHC adenocarcinoma markers are TTF-1 or mucin, and for squamous-cell carcinoma, p40 or p63 (Travis et al., 2015). Lung cancer, as a very heterogenous group of tumors, requires proper subclassification as well as immunohistochemical and molecular characterization, which is strongly associated with the choice of personalized treatment (targeted therapy and immunotherapy) (Osmani et al., 2018). The pathomorphological status of tumor should be assessed before commencement of treatment. Moreover, genetic tests should also be applied in lung cancer diagnostics and performed using tumor tissue as well as cytological samples (Krzakowski & Jassem, 2019). Defining molecular characteristics of lung cancer by identification of some typical mutations or altered genes expression could have an impact on further therapeutic decisions.

Therapy in NSCLC depends on cancer stage. The treatment of choice in stages I and II is radical pulmonary parenchyma resection, chemotherapy and radiation. Currently, the most frequently applied chemotherapies in the case of NSCLC comprise cisplatin, carboplatin, docetaxel, paclitaxel, pemetrexed and vinorelbine (Krzakowski & Jassem, 2019). Their main action is based on the suppression of cancer cell proliferation and growth by interfering with DNA synthesis or replication. Unfortunately, as we know, not all patients respond to this kind of treatment. Moreover, it has common side-effects such as vomiting, nausea, hair loss and general discomfort. Therefore, there are great hopes that immunotherapy will be applied in daily clinical practice.

In Poland several targeted therapy and immunotherapy agents (Figure 3) are currently available for NSCLC patients and refunded by the Ministry of Health such as:

- afatynib or dacomitinib – in the first-line treatment of III and IV stage NSCLC with confirmed activating mutation in *EGFR* gene;
- osimertinib – in the first-line treatment of III and IV stage NSCLC if activating mutation in *EGFR* gene is present or in the second-line treatment if T790M mutation in *EGFR* gene is present and earlier treatment with afatynib or dacomitinib was unsuccessful;
- crizotinib – in the first-line treatment of III and IV stage NSCLC in patients who did not undergo earlier systemic treatment and with confirmed ALK and ROS1 rearrangements;
- alectinib, ceritinib, brigatinib – in the first-line treatment of III and IV stage NSCLC in patients who did not undergo earlier systemic treatment and with confirmed ALK rearrangements or in patients with advanced ALK-positive NSCLC after previous unsuccessful treatment with other ALK-inhibitors;
- lorlatynib – in locally advanced or generic NSCLC with progression after second generation ALK-inhibitors treatment and confirmed *ALK* rearrangements;
- pembrolizumab – in the first-line treatment, advanced, previously systemic untreated NSCLC
    - as monotherapy if squamous or non-squamous cell lung cancer occurs and PD-L1 expression is > 50% and without *EGFR* mutation or *ALK* and *ROS1* rearrangements,
    - in combination with pemetrexed and a platinum-based regimen if non-squamous cell lung cancer occurs and PD-L1 expression < 50%,
    - in combination with paclitaxel and carboplatin if squamous cell lung cancer and PD-L1 expression < 50%;
- nivolumab, atezolizumab – in the second-line treatment of NSCLC, previously unsuccessfully treated with platinum-based polychemotherapy or monotherapy in advanced cancer, *EGFR* and *ALK*-negative, regardless of PD-L1 expression;
- durvalumab – in locally advanced, non-operable III stage NSCCL treatment in consolidation with radiochemotherapy.

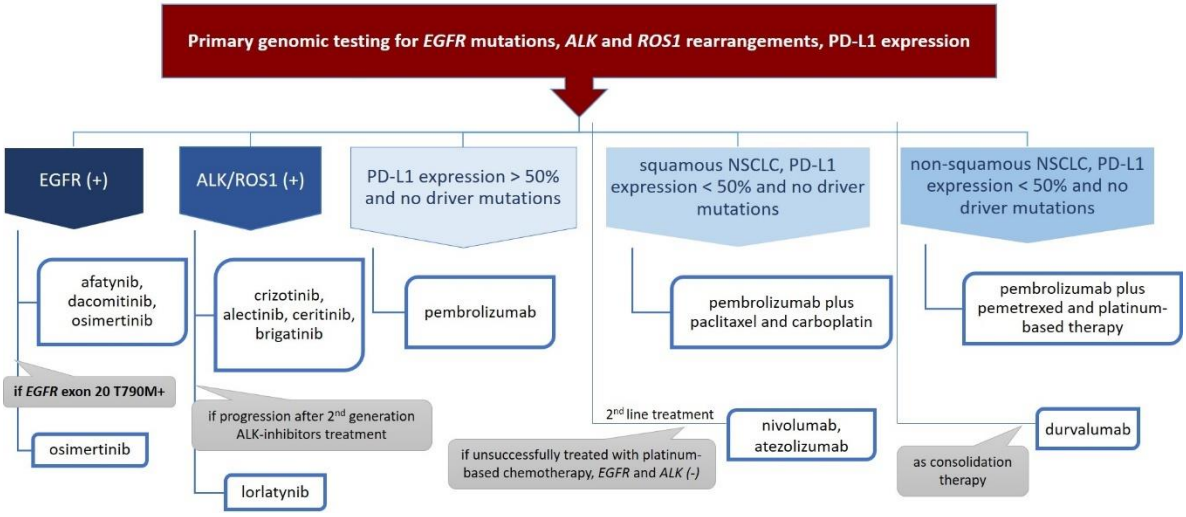

Figure 3. Current treatment options for advanced or metastatic NSCLC in Poland.

Nonetheless, before application of specific therapy, it is necessary to perform histological, cytological and molecular tests. According to the Polish drug program, qualification criteria for NSCLC patients include among others histological or cytological confirmation of specific cancer subtypes, age over 18 years, exclusion of concomitant clinically important diseases and absence of contraindications to the use of particular drugs. To assess function of the hematopoietic system, kidneys and liver, before targeted treatment is implemented, it is necessary to perform laboratory tests such as complete blood count, serum creatinine, bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. Moreover, in patients who receive PD-L1 inhibitors, levels of thyroid hormones should be evaluated. The same tests are used to monitor safety of these therapies (The Polish Ministry of Health, 2021).

Presence of ALK rearrangements could be confirmed based on immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) or next-generation sequencing (NGS), whereas ROS1 rearrangements using the FISH or NGS method and PD-L1 expression level on tumor cells determined in compliance with recommendations provided in a Summary of Product Characteristics. What is important, in order to confirm EGFR, ALK and ROS1 gene status and evaluate PD-L1 expression level validated tests have to be performed in certified medical laboratories that fulfil quality control requirements. Obviously, it also requires a considerable financial outlay.

3. Predictive and prognostic molecular markers in NSCLC

3.1. EGFR

EGFR is a part of a receptor tyrosine kinase (RTK) family in humans. EGFR signaling is activated by the binding with growth factors, which leads to the dimerization of EGFR molecules (Lemmon et al., 2014). Transphosphorylation of the receptors triggers the downstream pathways and consequently activates cell proliferation, survival signals and invasion (Lynch et al., 2004).

The *EGFR* gene is overexpressed in approximately 40-80% of NSCLC cases. It is a key factor in the development of cancer affecting many proteins involved in carcinogenesis. The EGFR signal very often activates numerous pathways in a unique way. The signal from the EGFR receptor activates the ERK-MAPK, PI3K-AKT, SRC, PLC-1-PKC, JNK, and JAK-STAT signaling pathways (Wee & Wang, 2017). EGFR activation causes an increase in cell proliferation and a decrease in apoptosis (Figure 4). Mutations in the *EGFR* gene usually occur in specific regions called "hotspots" which appear to be tissue specific (Cohen, 2004).

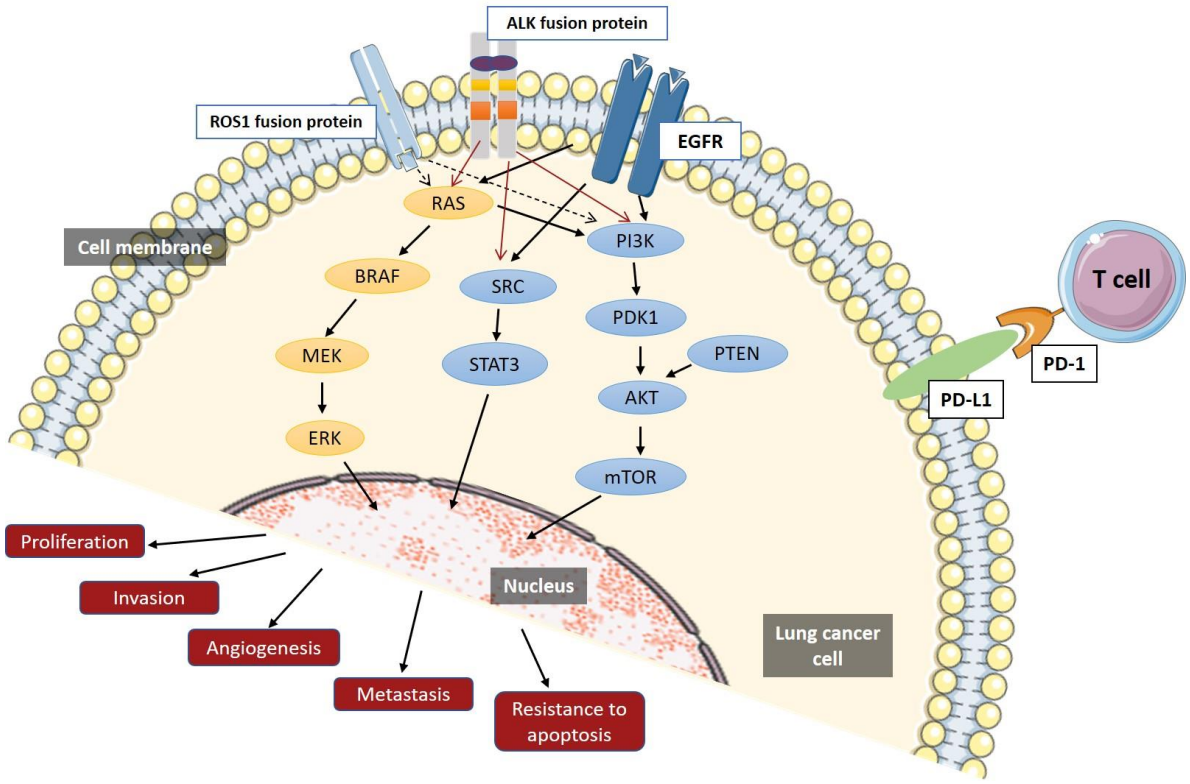

Figure 4. Major signaling pathways involved in lung cancer pathogenesis.

There are two major types of *EGFR* mutations that account for 85–95 % druggable *EGFR* alterations and are detected by all diagnostic platforms (Imyanitov et al., 2016). In-frame deletions in the exon 19 (ex19del) as well as the L858R point mutation of exon 21 constitute approximately two thirds of activating *EGFR* mutations and they are associated with sensitivity to EGFR tyrosine kinase inhibitors (Chang et al., 2016). The incidence of EGFR mutations is related to smoking and gender (more frequently in women) (Imyanitov et al., 2016).

EGFR-targeted inhibitors include monoclonal antibodies (such as cetuximab) that target the EGFR extracellular domain and tyrosine kinase inhibitors (TKIs) which are small molecules that inhibit intracellular tyrosine kinase activity of EGFR (such as erlotinib, gefitinib, afatinib and osimertinib). TKIs act by inhibiting EGFR activity through a reversible binding to the adenosine triphosphate (ATP)-binding region in the tyrosine kinase domain thus inhibiting phosphorylation and signaling (Chang et al., 2016).

Gefitinib and erlotinib were the first-generation of EGFR TKIs and showed longer progression-free survival response in cases involving *EGFR* mutations as compared to the standard chemotherapy (Fukuoka et al., 2011; Gridelli et al., 2011; Maemondo et al., 2010). Unfortunately, a secondary *EGFR* mutation in exon 20, which results in the methionine-threonine substitution at position 790 (T790M), leads to the development of acquired resistance in patients treated with these TKIs (Pao et al., 2005). The second-generation EGFR-TKIs (e.g., afatinib) act by inhibiting proliferation and triggering tumor cells apoptosis. Thus, they permanently connect to the tyrosine kinase domain of EGFR (HER1), HER2, and HER4 receptors as well as *EGFR* mutants (Ex19del, L858R and T790M) (Gelatti et al., 2019).

LUX-Lung 7 was a phase II clinical trial in which patients with an advanced adenocarcinoma and a confirmed common activating mutation in *EGFR* gene (exon 19 deletion/L858R) received afatinib or gefitinib. A second-generation EGFR TKI (afatinib) showed significantly improved progression-free survival versus gefitinib (Paz-Ares et al., 2017). The results were confirmed by other studies(de Marinis et al., 2021; Huang et al., 2021; Passaro et al., 2021). Moreover, afatinib is characterized by a better efficacy in T790M-positive cases (Huang et al., 2021). However, the second-generation TKIs

are unable to overcome T790M resistance because of their toxicity in a higher dose due to an irreversible binding to the receptors (Gelatti et al., 2019).

Osimertinib is one of the third-generation TKIs developed to overcome resistance of EGFR-mutated NSCLC patients with T790M mutation (Denis & Bennouna, 2020). Its effectiveness as high response rate with a clinically meaningful overall survival in the *EGFR* T790M cases with disease progression during a previous therapy with EGFR TKIs was widely confirmed (Ahn et al., 2019; Goss et al., 2016; Jänne et al., 2015).

3.2. ALK, ROS1

ALK is a receptor tyrosine kinase which belongs to the insulin receptor superfamily and is a significant element that binds many signaling pathways (Della Corte et al., 2018). ALK phosphorylate intracellular molecules and thus enable the signals transduction from the exterior of the cell to the nucleus. In general, ALK activity stimulates initiation of transcription of a number of genes, including *MYCN*, *JUNB*, *CEBPA*, *BCL2A1*, *MMP9*, *INK4A*, *HIF1A*, *VEGF*, *CDC42*, *FOXO*, *GSK3B*, *JNK*, *NFKB*, *NIPA* and *SHH* which affect cell growth, transformation and anti-apoptotic signaling (Figure 4). The role of *ALK* genetic aberrations in human carcinogenesis was widely recognized when it was first identified as a translocation in anaplastic large cell lymphomas (Hallberg & Palmer, 2016). Since then, various mutations of *ALK* have been identified in several human cancers, including NSCLC. To date, most of the oncogenic events of ALK have been known to promote cellular proliferation and survival signaling in cancer (Wang & Lui, 2020). There are three types of *ALK* gene mutations, i.e., rearrangement (ALK-R), amplification (ALK-A) and point mutation (Du et al., 2018). ALK rearrangements create an oncogenic ALK tyrosine kinase that activates many downstream signaling pathways resulting in increased cell proliferation and survival. *ALK* rearrangements account for 3–7% of NSCLC cases, predominantly the adenocarcinoma subtype and they occur in a mutually exclusive manner with *KRAS* and *EGFR* mutations. The discovery of *ALK* chromosomal rearrangements in NSCLC in 2007 revolutionized the treatment of ALK+ NSCLC. It is an attractive molecular target, and the development of successive generations of ALK tyrosine kinase inhibitor (TKI) has led to significant clinical improvements, especially among patients with ALK-positive NSCLC (Golding et al., 2018).

The first ALK-directed TKI was crizotinib, while the next-generation of ALKIs is represented by alectinib, brigatinib, ensartinib, and lorlatinib. The data demonstrate a better long-term efficacy of second-generation inhibitors through a prolonged progression-free survival and a better safety profile than crizotinib (Peters et al., 2017).

Meanwhile, other concomitant mutations, such as *TP53* or *PIK3CA* mutations, combined with EGFR mutations or *ALK* rearrangements, have been found to affect the TKI treatment efficacy. *ALK* mutations are occasionally acquired in *ALK* fusion genes as a result of resistance to ALK inhibitors in NSCLC cases (Golding et al., 2018).

ROS1 is a receptor of the insulin receptor family with tyrosine kinase activity. *ROS1* chromosomal rearrangements were recognized as potential driver mutations in non-squamous cell lung cancer and occur in 1–2 % of NSCLC cases, most frequently in non-smokers and adenocarcinoma patients (Park et al., 2018). *ROS1* fusions result in tyrosine kinase activation and therefore are linked to sensitivity to TKIs. Patients with *ROS1*-rearranged NSCLCs present a clinical profile comparable to that observed in ALK-rearrangements (Bergethon et al., 2012). Moreover, *ROS1* and *ALK* have high homology of their amino acid sequences, which may indicate that ALK-directed TKIs (crizotinib, ceritinib, lorlatinib) also play a role in ROS1-positive tumors (Herbst et al., 2018; Zhang et al., 2016).

According to the NCCN Clinical Practice Guidelines in Oncology for Non–Small Cell Lung Cancer testing for *ALK* and *ROS1* rearrangements using FISH method should be performed prior to treatment with ALK-directed TKI (i.e. crizotinib). For this purpose, NGS could also be applied if assays were validated to *detect* ALK rearrangements (Ettinger et al., 2019).

3.3. Immune checkpoint inhibitors

Nowadays, single-agent checkpoint inhibitors as a part of immune modulation therapies are becoming a promising target in NSCLC (Diggs & Hsueh, 2017) and have revolutionized the treatment approach.

Immune checkpoints mean balance between co-stimulatory and inhibitory signals that regulate the response of the host immune system to cancer cells. Regulatory T cells constitute an element of immunity against tumors by expression of multiple immune-checkpoint receptors including PD-1/PD-L1 (programmed cell death protein 1; programmed death-ligand 1). Many tumors are highly infiltrated with TReg cells which overexpress PD1, while tumor cells increase PD-L1 expression to bind PD1 and thus avoid immunologic blockade of cancer growth (Figure 4) (Pardoll, 2012). Many types of human cancers were reported to upregulate PD-L1 expression, lung cancer too. Overexpression of PD-L1 by lung cancer cells was significantly associated with poor prognosis (Mu et al., 2011).

Therefore, inhibitors of programmed cell-death receptor (PD-1) and its related ligand (PD-L1) as anti-PD-L1 (or anti-PD-1) monoclonal antibodies inhibit PD-L1 binding to PD-1 and are widely used in treatment of malignancies. This research has led to making use of the PD-L1 expression assessed by IHC tests as a predictive factor for checkpoint inhibitor immunotherapy. Therefore, it was possible to identify patients who may benefit from PD-1/PD-L1 inhibitors treatment.

The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved nivolumab and pembrolizumab (antibodies against PD-1) as well as atezolizumab and durvalumab (antibodies against PD-L1) as a treatment in advanced NSCLC.

Immune checkpoint inhibitors may be applied both as a monotherapy and in combination with chemotherapy. KEYNOTE-042 phase 3 clinical trial showed that in patients with untreated metastatic NSCLC, if PD-L1 expression in tumor cells was > 50% and without sensitizing *EGFR* or *ALK* changes, pembrolizumab used in monotherapy improved an overall and progression-free survival (Mok et al., 2019). In the KEYNOTE-021, the study patients received pembrolizumab plus chemotherapy (pemetrexed-carboplatin) versus chemotherapy alone. As a result, the median overall survival was 34.5 in the subgroup of pembrolizumab combination vs. 21.1 months in the subgroup of chemotherapy (hazard ratio: 0.71; 95% confidence interval: 0.45–1.12) (Awad et al., 2021).

Another clinical trial – the KEYNOTE-189 showed an improved overall survival in patients with previously untreated metastatic NSCLC who received pembrolizumab plus pemetrexed-platinum versus placebo plus chemotherapy (hazard ratio: 0.56; 95% confidence interval: 0.46-0.69) (Rodríguez-Abreu et al., 2021). In other situations, when metastases in NSCLC are present, PD-L1 expression levels are higher than 1% and patients have driver oncogene molecular changes in *EGFR*, *ALK* and *ROS1* genes, they should be treated by a first-line targeted therapy for that oncogene, instead of first-line immunotherapy regimens (Ettinger et al., 2019).

In the NCCN Clinical Practice Guidelines in Oncology updated recommendations in NSCLC treatment are reported. Based on different phase III randomized trials it is strongly recommended to administer pembrolizumab as a first-line therapy option in cases with metastatic NSCLC accompanied by high PD-L1 expression (≥50%) and without genetic alterations for *EGFR*, *ALK*, *ROS1*, or *BRAF* genes (Ettinger et al., 2019).

These findings demonstrate that anti PD-1/PD-L1 treatment is a very important achievement in the therapy of metastatic NSCLC, however, the above mentioned evaluations involve patients at advanced unresectable stages of lung cancer with metastases. The role of immune checkpoint inhibitors in an earlier-stage disease is also currently investigated. There are many clinical trials to evaluate the efficacy of adjuvant checkpoint blockade in resected NSCLC, however they are still ongoing and the results will be probably known between 2024 and 2027 (Broderick, 2020).

Another checkpoint protein expressed by activated T cells is CTLA-4 (cytotoxic T-cell lymphocyte antigen 4) that binds to CD-80 and CD-86 receptors on the surface of antigen-presenting cells and thus stops activation of T cells. Therefore, blocking CTLA-4 might be useful in the inhibition of the immune system's tolerance to tumor and results in tumor reduction. At present, there are

two humanized antibodies directed at CTLA-4 – ipilimumab and tremelimumab, however, clinical trials did not confirm the efficacy of these agents as monotherapy in advanced NSCLC. Some evidence has shown the potential of applying ipilimumab in combination with nivolumab in PD-L1-positive NSCLC patients, nevertheless, so far it has not been approved by FDA (Puri & Shafique, 2020).

3.4. KRAS

RAS proteins (HRAS, KRAS, NRAS) belong to the family of small GTPases. The RAS family of genes encodes four proteins that are essential mediators of the Mitogen-activated protein kinase (MAPK) pathway and exhibit high homology and conserved amino acid sequences, i.e., the KRAS4A and KRAS4B splice variants, Harvey rat sarcoma (HRAS) and neuroblastoma RAS (NRAS) (Khan et al., 2019). Binding of ligand to receptor tyrosine kinases (RTK) or mutations in RAS leads to activation of downstream effector signaling pathways, such as BRAF, MEK1 and ERK that regulate the transcription of genes that promote cell cycle progression and survival of cancer cells. RAS also activates the phosphatidylinositol 3-kinase (PI3K) – 3-phosphoinositide-dependent protein kinase 1 (PDK1) – AKT pathway that frequently determines cellular survival (Figure4). Among the effectors of Ral is phospholipase D (PLD), an enzyme that regulates vesicle trafficking. The RAS effectors, NORE1 and RASSF1, are involved in apoptosis through MST/Hippo tumor suppressor pathway leading to apoptosis (Román et al., 2018). Missense gain-of-function mutations are the main cause for the activation of RAS protooncogenes and incessant RAS activation (Sun et al., 2013).

KRAS as a one of the protooncogenes is widely found in lung cancer and it is the second frequently mutated gene in NSCLC (Tate et al., 2019). A majority of *KRAS* gene point mutations in NSCLC occur in codon 12, 13 and 61 as transition or transversion mutations (e.g., G→A and G→C, relatively). These alterations lead to a single amino-acid substitution and thus a shift of the protein to the active GTP-bound state, activation of the RAS signaling pathway (O'Bryan, 2019). Approximately 41% of all mutation in *KRAS* in lung cancer is G12C (41%) (Román et al., 2018).

A lot of attention is paid to the role of *KRAS* mutations in the development, progress and potential therapeutic effect in NSCLC cases. *KRAS* mutations can influence therapy effectiveness by changing mRNA expression and protein function or activity (Khan et al., 2019). In their study, Sun et al. presented *KRAS* mutation tumors as related to a shorter survival in patients with advanced NSCLC and therefore as an independent predictive marker (Sun et al., 2013). Pan et al. performed a meta-analysis to evaluate a role of *KRAS* mutation in lung cancer. Based on 41 publications, they presented that *KRAS* mutation was a predictor of worse prognosis and treatment results by association with a worse overall survival at an early stage of NSCLC (Pan et al., 2016). Another meta-analysis conducted by Mascaux et al. showed that *RAS* gene mutant was a negative prognostic factor for survival in NSCLC patients. What is interesting, an analysis performed in a subgroup indicated a poorer prognosis of *RAS* alterations in adenocarcinoma, not in squamous cell carcinoma. The method of *KRAS* alterations examination was also relevant. Only when PCR was used to confirm gene changes, results were associated with a worse prognosis (Mascaux et al., 2005). However, *KRAS* status assessment as a predictive marker in response to cytotoxic chemotherapy as well as evaluation its role in predicting the clinical outcome in late stages of NSCLC was unsuccesful (Sun et al., 2013). In the NGS analysis of NSCLC samples by Scheffler et al., the authors emphasize the fact that KRAS-mutated NSCLC is a heterogenous genetic subgroup and thus there is a need of routine testing for concomitant mutations (i.e., *TP53, STK11, KEAP1, ATM, MET, ERBB2, EGFR, BRAF*) in the development of therapy approaches with specific KRAS inhibitors (Scheffler et al., 2019). Since there are many discrepancies between different studies as to whether *KRAS* mutations have (Camps et al., 2011; Jeanson et al., 2019) the prognostic and predictive role in diagnosis and treatment of NSCLC or not, targeting *KRAS* is still in an experimental phase and requires further research.

The latest achievement is that in May 2021, the Food and Drug Administration granted its accelerated consent to sotorasib, i.e., an inhibitor of the RAS GTPase family, to be applied in adult patients with *KRAS G12C*-mutated locally advanced or metastatic non-small cell lung cancer (NSCLC). Describing its function, the manufacturer states that it blocks KRAS signaling, suppresses cell growth

and promotes apoptosis only in KRAS G12C tumor cell lines. It is allowed for patients who have previously received at least one systemic therapy. FDA approval was given based on CodeBreaK 100 clinical trial (NCT03600883).

4. Novel markers

Apart from further research on these well-known genes, there is still a need for identifying novel markers useful in NSCLC prognosis and prediction. Wu et al. conducted a meta-analysis and used bioinformatics technology to assess the potential association between driver and novel gene mutations and metastasis in NSCLC. This study confirmed findings of earlier research that *EGFR* and *ALK* genes mutations were correlated with distant metastases. Moreover, it was found that some novel genes mutations were associated with NSCLC metastases (*SMARCA1, GGCX, KIF24, LRRK1, LILRA4, OR2T10, EDNRB, NR1H4, ARID4A, PRKCI, PABPC5, ACAN* and *TLN1*). What is interesting, an elevated mRNA expression level of *SMARCA1* was associated with poor prognosis in lung adenocarcinoma, while *EDNRB* in lung squamous cell carcinoma (Wu et al., 2021).

SMARCA1 gene is located on chromosome X and encodes a member of the SWI/SNF family of proteins which is expressed in different tissues, cancers as well as derived cell lines. It acts as a chromatin remodeling gene and it is involved in transcription (Ye et al., 2009). *SMARCA1* gene is a modulator of the Wnt signaling network and therefore may contribute to DNA damage, inhibition of cell growth and apoptosis (Eckey et al., 2012).

EDNRB (endothelin receptor type B) gene is located on chromosome 13 and encodes a protein belonging to the family of G protein-coupled receptors, which is an important regulatory factor in signal transduction in cells (L. Zhang et al., 2019). Many researches have shown its decreased mRNA expression in different types of cancers. For example, a study by Wei et al. reported that high *EDNRB* expression was associated with a significantly better survival in lung adenocarcinoma subtype of NSCLC (Wei et al., 2020).

A tumor mutation burden (TMB) is a novel maker, defined as the sum of somatic mutations (substitutions, short insertions and deletions), per megabase in tumor genome, that could be predictive for immunotherapy in different types of cancers.

The data show that not only high PD-L1 expression but also high TMB are good predictive factors providing benefits from immune checkpoint blockade in NSCLC patients with different oncogene alterations (Negrao et al., 2021). First-line treatment with nivolumab (an anti–programmed death 1 antibody) plus ipilimumab (an anti–cytotoxic T-lymphocyte antigen 4 antibody) in patients with advanced NSCLC and TMB over 10 mutations per megabase was associated with a longer progression-free survival as compared to chemotherapy (Hellmann et al., 2018). Moreover, Yu et al. performed a meta-analysis including 14 395 patients and noticed that combination of the PD-L1 expression and TMB level assessment could be a better biomarker for patient survival and response to first-line pembrolizumab with platinum-based chemotherapy than the PD-L1 expression or TMB only (Yu et al., 2019). Rizvi et al. hypothesized that TMB is a biomarker useful for prediction rather than prognosis since they did not find a correlation between higher TMB and survival in a group of patients not treated with ICIs. There was also no correlation between TMB and PD-L1 expression (Rizvi et al., 2018).

MicroRNAs (miRNAs) also have a potential to become biomarker of lung cancer risk even in the case of early-stage detection. They are a superfamily of non-coding RNAs that can regulate gene expression by interacting with the 3’ UTR of target mRNAs and they are detected in extracellular fluids (O'Brien et al., 2018). A lot of studies were dedicated to evaluation of miRNAs in NSCLC where a great amount of them is dysregulated. MicroRNAs in NSCLC are involved in the regulation of many cellular processes such as the epithelial to mesenchymal transition (EMT), which contributes to metastases. Many microRNAs that target growth factors and corresponding receptors can lead to increased cancer cell proliferation and evolution. Another effect of miRNAs’ dysregulation in cancer cells may lead to angiogenesis over different pathways, e.g., vascular endothelial growth factor (VEGF) or placenta growth factor (PIGF) (Petrek & Yu, 2019). In their preliminary studies, Duan et al.

identified three significant serum microRNAs, i.e., miR-492, miR-590-3p and miR-631 in early-stage NSCLC. The ROC curve analysis demonstrated the great sensitivity (86.7%) and the high specificity (71.7%), when the two parameters were considered together. In conclusion, the panel of these microRNAs might be useful in diagnosis of NSCLC at early stage and distinguish early-stage NSCLC patients from healthy people (Duan et al., 2021). In another study, a combination of miR-146a, miR-222 and miR-223 might be used as promising serum marker for detection of NSCLC in early stages (Lv et al., 2017). Among recently published results in the field of the miRNAs detection in lung cancer there are several circulating miRNAs which could play an important role in diagnosis and prognosis in NSCLC cases. These are miR-21-5p, miR-126-3p, miR-20b-5p, miR-3187-5p, miR-182, miR-200b and miR-205 (Soliman et al., 2021; Zhang et al., 2020; Zou et al., 2019).

An undeniable advantage of serum microRNAs is a non-invasive method of detection with an easy sample collection procedure instead of using tumor tissue and necessity of a biopsy and they seem to be promising biomarkers in tumor diagnosis. However, they also have some limitations, because miRNAs are not specific in terms of a particular cancer and so far there has been no validated method for detecting circulating miRNA in serum samples (Rijavec et al., 2019).

Some traditional blood tests used in daily practice are associated with non-specific inflammatory response during carcinogenesis. Lactate dehydrogenase (LDH) is an enzyme converting pyruvate to lactate in anaerobic conditions. Its activity increases in tissue damage, cell necrosis, hypoxia, hemolysis as well as in tumorigenesis and therefore it could be associated with poor outcome in different cancers. Based on six studies including 1136 patients, Zhang et al. performed a meta-analysis to assess if pre-treatment LDH levels may be a predictive biomarker for NSCLC patients treated with ICIs. As a result, a high pre-treatment LDH level was correlated with a significantly shorter progression free survival (PFS) and overall survival (OS) (HR = 1.53, 95% CI 1.27-1.83, $P < 0.001$, HR = 2.11, 95% CI 1.43-3.11, $P < 0.001$, respectively) (Zhang et al., 2019).

Carcinoembryonic antigen (CEA) is a glycoprotein found in many types of cells involved in different cellular processes, such as cell recognition and adhesion. It is also well-known as a biomarker associated with tumors and fetus development. There is some evidence proving that its level could be useful as a predictive marker during various NSCLC treatment. In a retrospective study conducted among 593 advanced NSCLC patients treated with first-line platinum-based chemotherapy, lower CEA and LDH levels were associated with a beneficial response to platinum-based chemotherapy in the previously untreated advanced NSCLC cohort. Moreover, the levels increased by over 20% during the therapy and a high LDH level prior to the treatment were associated with a shorter overall survival (de Jong et al., 2020). What is interesting, higher CEA levels were linked with presence of *EGFR* alterations as compared to *EGFR* wild-type patients (Facchinetti et al., 2015). In another study, reduction of neutrophil-to-lymphocyte ratio, CEA and neuron-specific enolase (NSE) levels after therapy with PD-1 inhibitors combination we associated with longer PFS and/or OS (Chen et al., 2021). Moreover, reduction of CEA and cytokeratin fragment 19 (CYFRA21-1) levels, but not NSE, after therapy with nivolumab was related to a better progression free survival (Dal Bello et al., 2019).

Some studies concentrate on detecting protein biomarkers in plasma of lung cancer cases by using different algorithms to apply screening methods in early stage of disease. Goebel et al. initially used 82 proteins and, after initial screening, reduced this panel to 33 biomarkers. Among the biomarkers that significantly upregulated in lung cancer cases, as compared to healthy non-smokers, there were IL-7, IL-10, SAA, MMP-9, IL-8, Gro, MIG, Rantes, TNFRI and Resistin, whereas sCD40L and IL-5 were down-regulated (Goebel et al., 2019). These and other similar studies show the importance of using a panel of proteins in the assessment of risk stratification.

Another interesting peripheral blood parameters that seem promising for lung cancer prognosis are hematological indexes associated with systemic inflammation. Based on a five-part differential of white blood cells, some leukocytes indexes are calculated. In a meta-analysis conducted by Li et al. in seven studies including 2106 advanced NSCLC patients with ICIs treatment, a higher pretreatment neutrophil-to-lymphocyte ratio (NLR) was significantly correlated with a shorter PFS and OS (HR = 1.39; 95% CI 1.01–1.91; $P = 0.02$, HR = 1.71; 95% CI 1.18–2.46; $P < 0.001$, respectively) (Li et al., 2020). In another meta-analysis high NLR may also not only predict worse OS

for advanced NSCLC patients who receive treatment, but also in patients with early-stage lung cancer and after undergo surgery (Yu et al., 2017). There is some evidence that suggests the pro-tumor effects of neutrophils such as secreting immunosuppressive factors, tumor initiation, progression and metastasis formation (Valero et al., 2021). On the contrary, lymphocytes are associated with host cell immunity and involved in destroying tumor cells, thus low lymphocyte levels are linked to a weaker immune response of cells (Sánchez-Gastaldo et al., 2021). Therefore these calculated ratios might be useful in predicting clinical outcomes of NSCLC patients. The main advantages of this index are its availability due to frequently performed blood tests in cancer patients undergoing oncology treatment and easy calculation based on leukocytes count.

5. Novel approach to molecular testing and treatment in NSCLC

Due to the fact that EGFR, ALK, ROS1 TKIs and ICIs are currently widely used as the part of the first-line treatment in NSCLC, it is very important to perform rapid evaluation of the gene status of cancer drivers and other targets applied in therapy.

The selection of testing pathways depends on accessible diagnostic methods (i.e., molecular tests), current recommendations on applying targeted therapy, financial support as well as turnaround time (Imyanitov et al., 2021). Another issue is the type of patient samples that may offer the highest reliability, sensitivity and diagnostic accuracy and thus they may be most appropriate for performing tests.

Cytological samples obtained during different diagnostic procedures, for example endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), could be not enough for performing molecular assays (Guibert et al., 2020). Therefore, liquid biopsy is considered a very interesting option by many researches as a less invasive and more accessible material as compared to tissue biopsy. This procedure is associated with the detection of different biomarkers in body fluids, for instance in peripheral blood, which gives a chance for detecting tumor-related mutations and other biomarkers (Rijavec et al., 2019).

Investigating *EGFR* mutation status often relied on conventional DNA sequencing in tumor samples as it was regarded as a useful method for the assessment of all known genetic alterations within the examined region, however, it requires high tumor content and is characterized by low sensitivity. Within other common techniques applied to test *EGFR* mutations are real-time PCR, reverse transcriptase PCR and NGS. These methods allow to detect multiple genetic alterations and are usually performed in tissue samples. Nonetheless, identifying genetic changes in specimens with low tumor cellularity can give false-negative results. Therefore, a promising approach is detection of *EGFR* gene mutations in circulating cell-free DNA (cfDNA). So far, only two assay kits have been approved by FDA for examination of EGFR-mutated NSCLC patients both in tissue samples and in plasma, i.e., the cobas® EGFR Mutation Test v2 (Roche, Basel, Switzerland) and TheraScreen EGFR RGQ PCR Kit (Qiagen, Hilden, Germany) (Di Capua et al., 2021). This is a real-time PCR test designed to identify the most frequent mutations in exons 18, 19, 20 and 21 of the EGFR gene, including the T790M resistance mutation (Brozos-Vázquez et al., 2021). The main aim of AURA3, phase 3 clinical trial was to assess the efficacy of osimertinib – the third generation of EGFR TKI in contrast to platinum therapy plus pemetrexed in advanced NSCLC patients with confirmed T790M mutation following first-line EGFR TKI therapy. Blood samples were collected to perform circulating tumor DNA (ctDNA) screening test for T790M variant on the above-mentioned cobas EGFR Mutation Test. They found that in T790M-positive NSCLC patients treated with osimertinib detecting *EGFR* T790M from plasma ctDNA samples was a biomarker useful in predicting which patients could benefit from TKI treatment. However, in patients with T790M-negative result in ctDNA, collecting biopsy sample is recommended to confirm or exclude EGFR gene alteration (Mok et al., 2017). Based on these results, the currently applied Polish guidelines indicate that in patients with progression during EGFR TKIs treatment it is important to re-sample material in order to evaluate molecularly possible T790M mutation in *EGFR* gene as strongly associated with developed resistance to TKI. It is recommended

to perform tests in cfDNA first, and, only if these results are negative, re-biopsy or needle biopsy should be performed (Krzakowski & Jassem, 2019).

Nowadays, a lot of clinical researches focus on an alternative biomarkers analysis in circulating tumor cell (CTC) and ctDNA in blood plasma. CTCs are released from solid tumor tissue or its metastases, while ctDNA is a fraction of cfDNA deriving from apoptotic or necrotic tumor cells (Yang et al., 2021).

Several methods aimed at CTCs detecting are still being developed and the only assay currently approved by FDA for enumeration of CTC is the CellSearch (Veridex LLC) that uses antibodies against tumor-associated markers to gain CTC. Another method is ISET which means isolation of epithelial tumor cells by size using a filtration technique.

Among methods applied to detect ctDNA are BEAMing technology and droplet digital PCR (ddPCR) (Rijavec et al., 2019). BEAM method is a combination of digital polymerase chain reactions (PCR) with magnetic beads and flow cytometry (Denis et al., 2017). ddPCR is a highly sensitive method based on microfluidics technology that enables independent quantification of nucleic acids and uses the ultimate dilutions of the PCR mix volume following the Poisson distribution without creating standard curves (Jiang et al., 2019). NGS as a method of high efficiency sequencing enables detection a variety of genetic alterations, from single base mutations to small and large genomic amplifications or deletions to aberrations as translocations. This technique could detect both well-known and novel, previously undescribed genetic changes in the sequenced genes (Rolfo et al., 2018).

There is a number of studies dedicated to evaluation of the relevance of liquid biopsy by means CTC and ctDNA detecting in NSCLC as an alternative method to tissue biopsy. However, they are still unvalidated and give contradictory results. Syrigos et al. carried out an extensive literature review on prospective research evaluating the prognostic significance of CTC count in NSCLC patients. A majority of obtained results, especially in a cohort larger than 100 patients, have showed that increased CTC count at baseline and/or on disease progression was independently associated with reduced PFS and OS. Therefore, these studies give hope for potential CTCs as a non-invasive prognostic biomarker (Syrigos et al., 2018). Jiao et al. compared blood and tissue genomic profiles among 185 advanced lung adenocarcinoma patients and showed that 80% of the samples had identical sequencing results in tissue DNA and plasma cfDNA in eight driver genes (*EGFR*, *ALK*, *ROS1*, *RET*, *MET*, *NTRK*, *BRAF* and *HER2*) (Jiao et al., 2021). Another systematic review focused on whether a targeted next NGS of liquid biopsy in advanced NSCLC could be an alternative to tissue biopsy mutational testing. For this purpose, data from 38 studies were studied, including a ctDNA analysis in 1141 NSCLC patients. To give an example, the calculated positive percent agreement between NGS in tissue and liquid biopsy was 67.8% (428/631) for *EGFR* and 64.2% (122/190) for *KRAS.* This shows that targeted NGS in liquid biopsy has lower efficacy in detecting mutations than in tissue biopsy. However, different studies applied variable NGS panels or diverse cut-offs. Moreover, sometimes tissue and plasma samples were collected at different time points (Esagian et al., 2020). Due to many limitations such as lack of standardization, lack of sensitivity, detecting low ctDNA levels, liquid biopsy probably could not replace tissue biopsy that is still the gold standard. Nevertheless, in the future, targeted NGS testing on ctDNA might be a useful tool in diagnosis, prognosis and monitoring NSCLC.

Immunotherapy with the use of anti-PD-1 drugs or anti-PD-L1 is a part of first-line treatment in patients with disseminated NSCLC, if PD-L1 cells expression level is 50% or more. Therefore, qualifying for immunotherapy with ICI requires evaluation of PD-L1 protein expression (Krzakowski & Jassem, 2019). Immunohistochemistry (IHC) is the most frequently used method available for testing PD-L1 expression in tumor cells and with using formalin-fixed paraffin embedded (FFPE) histology samples. However, in a great number of cases histological specimens could not be obtained or, after making diagnosis, are insufficient to perform a further biomarker analysis (Tejerina et al., 2021). Thus, many studies have focused on an alternative using cytological specimens to PD-L1 test as a promising material for diagnosing and evaluating patients who will benefit from ICIs drugs. According to the Guidelines of the College of American Pathologists, both cell blocks and other cytologic preparations might be used for testing molecular biomarkers in lung

cancer (Lindeman et al., 2018). Some studies tried to evaluate an analysis of the PD-L1 expression in CTCs of NSCLC patients (Guibert et al., 2018; Ilié et al., 2018; Wang et al., 2019). Results obtained by Guibert et al. showed that CTCs were more frequently PD-L1 positive than tissue (83% vs. 41%), however, they did not find any correlation between tissue and CTC PD-L1 expression ($r = 0.04$, $p = 0.77$) (Guibert et al., 2018). Ilie et al. evaluated that PD-L1 expression in CTCs correlated with PD-L1 expression in tumor tissue with a 93% concordance (Ilié et al., 2018). Assessment of CTC provides grounds to believe that it may be a good alternative for adjusted treatment. Nevertheless, it needs to be standardized and validated in more studies.

Among the newest drugs recently approved by FDA, though not yet applied in Poland, are amivantamab-vmjw and tepotinib. Tepotinib, as another TKI to use in patients with metastatic NSCLC and confirmed *MET* exon 14 skipping mutations was approved based on the VISION clinical trial (NCT02864992). *MET* alterations result in dysregulation of the RAS–RAF and PI3K signaling pathways and thus favor proliferation, invasion and metastasis of the tumor cells. TKI targeting *MET* showed a good response rate (46% with 95% confidence interval [CI]) in patients with confirmed *MET* exon 14 mutations detected in tissue samples as well as in cfDNA (Paik et al., 2020). Amivantamab-vmjw is an antibody directed against bispecific EGF-receptor and MET-receptor dedicated to the treatment of locally advanced or metastatic NSCLC patients with detected *EGFR* exon 20 insertion mutations. Its main function is to inhibit two different driver pathways involved in NSCLC development. The efficacy of amivantamab was assessed in the CHRYSALIS clinical trial (NCT02609776) and showed an overall 40% response rate (95% CI) (Park et al., 2021).

As novel driver mutations in common genes are revealed and corresponding drugs are designed, examined and approved for use in NSCLC patients, there exists a great need for applying companion diagnostic devices that allow to facilitate and speed up the diagnostic process as well as to select patients who are likely to experience clinical benefits. Until now, FDA has approved some qualitative immunohistochemical, real-time PCR, FISH and NGS assays to use in NSCLC. They are dedicated to assess PD-L1 protein expression, the most common *EGFR* mutations (i.e., exon 19 deletions, L858R and T790M) and also rearrangements involving the *ALK* and *ROS1* genes. These tests are indicated to aid in selecting NSCLC patients for treatment with the corresponding targeted therapies in accordance with the approved therapeutic product labeling (Food and Drug Administration, 2021).

6. Conclusions

Currently, tissue biopsy is still the gold standard in tumor molecular profiling, however, it is an invasive method. Despite strong arguments suggesting the importance of molecular markers testing in patients with lung cancer, there are many obstacles for applying them in clinical practice. Due to their small portion or poor quality, tissue samples obtained in biopsy are often insufficient for biomarker testing and provide just a small part of the tumor that is not representative for the tumor heterogeneity. Moreover, it is difficult to collect consecutive samples in the course of therapy to monitor possible genetic alterations that might lead to treatment resistance. Another problem is performing a single test for each biomarker to detect one type of mutation instead of testing in panels with NGS assays that give a lot of information about multiple genetic alteration in shorter time (Pennell et al., 2019). A considerable barrier are also financial costs of testing, as well as a limited access to laboratories using such an advanced molecular technology.

Another issue is application of liquid biopsy, especially as ctDNA, in clinical practice. There are indications that ctDNA extraction from plasma might be useful in preliminary molecular diagnosis and monitoring progression during personalized therapy (Rolfo et al., 2018).

So far, targeted therapies based on *EGFR* mutations, *ALK* rearrangements and PD-L1 expression have been implemented successfully into clinical practice, which emphasizes a need for assessment of tumor molecular status in each newly diagnosed NSCLC patient.

The role of biomarker testing in targeted therapies and immunotherapy is obvious, however, further studies are essential to improve the identification of potential prognostic and predictive

biomarkers, increase sensitivity of different laboratory techniques used to evaluate genetic alterations and validate the findings on large NSCLC cohorts.

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