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Personalised medicine in lung cancer

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Personalised therapy is currently a promising method of treatment for cancer patients. The dynamic development of molecular biology enabled identification of molecular subtypes of neoplasms, allowing determination of the optimal therapeutic management for the patient. Molecular diagnostics is also essential for cancer diagnosis, predicting disease development and prognosis. In the case of lung cancer, which is one of the most common malignant neoplasms, the main candidates for targeted treatment are patients with stage III and IV of the disease and with no possibility of radical local treatment. In clinical practice, the most proven therapeutic agents are inhibitors of tyrosine kinase, i.e. a receptor of the epithelial growth factor (TKI-EGFR), inhibitors of ALK, ROS1, BRAF and others, as well as immunotherapy applying monoclonal antibodies against immunological system checkpoints in cases of high level expression of programmed death receptor type 1 (PD-1) or its ligand (PD-L1), but also in cases of the high tumour mutational burden (TMB). As compared to chemotherapy, targeted therapy undoubtedly improves the treatment outcomes and, due to its lower toxicity, improves the quality of life of advanced non-small cell lung cancer patients. The aim of this paper is to characterise molecular tests which are currently applied in qualification of non-small cell lung cancer patients for targeted therapies.

Key words: lung cancer, NSCLC, FISH, NGS, targeted therapy, TKI

Introduction

Personalised therapy in oncology relies on the close relationship between molecular changes in cancer and treatment. Patients with the same diagnosis, but with different tumour molecular profiles, may undergo different course of the disease and react differently to the applied therapy. The most common action points for targeted drugs are proteins that are involved in the control of tumour cell activity, including control of various signalling pathways. These proteins show abnormal activity or function in tumour cells, leading to tumour-promoting events such as excessive cell proliferation, impaired angiogenesis, inhibition of apoptosis, and other dysfunctions of the cell cycle [1, 2]. The purpose of characterising molecular subtypes is to determine the optimal therapeutic management for the patient.

Lung cancer is one of the most common malignant neoplasms, and five-year survival is achieved only in 10–15% of patients [3]. Worldwide, over 1.5 million people a year develop non-small cell lung cancer (NSCLC), which accounts for 80–85% of all lung cancer cases. In 2018, over 2 million new cases of NSCLC were diagnosed and over 1.7 million deaths were registered [4]. There is a two-fold prevalence of cases among men compared to women (13,798 *vs.* 7,747 – in 2017 in Poland), but this difference is decreasing year by year. For 15 years, a tendency has been recorded of decreased incidence and mortality of lung cancer in men, while in 2017 for the first time, the number of women who died of lung cancer exceeded the number of patients who died of breast cancer (17.4% of deaths *vs.* 14.8% of deaths). In men, lung malignancies are still

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the dominant cause of deaths (about 30%) due to neoplastic diseases [4]. The risk of developing NSCLC is strongly correlated with smoking: 85–90% of lung cancer cases are associated with this addiction [5]. The classification of 2015 by the World Health Organization (WHO) included:

- small cell lung cancer (SCLC), which accounts for 15% of primary lung cancers,
- non-small cell lung cancer (NSCLC), which is diagnosed in 85% of cases [6].

Histopathologically, NSCLC is divided into:

- adenocarcinoma (45% of all diagnosed primary lung cancers),
- squamous cell carcinoma (about 30%),
- large cell carcinoma (10%) and
- other rare morphological types (<1%) [6].

The lung cancer is rarely diagnosed at early stages. In Poland, this diagnosis is most often made at stage IV (47–62%, depending on the voivodeship) and stage III (24–38%) [7]. In patients with advanced NSCLC, chemotherapy treatment results in a 25–30% objective response rate (ORR), while the median overall survival is 10–12 months [8]. Only about 15% of patients survive 5 years from the diagnosis [9].

In the early stages, of lung cancer the basic method of treatment is surgery (in approximately 15–20% of NSCLC patients). In stage III of the disease, tumour resection is rarely possible, and patients are treated with radiotherapy, chemotherapy or a combination of these two methods, supplemented with adjuvant immunotherapy [10]. Patients with stage III and IV of the disease, in the absence of radical local treatment, are candidates for targeted therapy [6, 11].

The knowledge of molecular background of NSCLC is improving, but still in about half of the patients the molecular target remains undefined. However, numerous studies of the subpopulation of patients with various molecular changes in the neoplastic tissue allow for constant improvement of characterisation of this tumour [5, 12, 13].

Molecular testing in the qualification of NSCLC for personalised therapy

Molecular diagnostics of neoplastic tissue is essential for tumour classification, predicting disease development and prognosis, as well as choosing the optimal therapy. In the case of lung cancer, the tissue material obtained from the patient is usually small, which significantly limits the diagnostic possibilities and determines the choice of the diagnostic algorithm (Thunnissen et al., 2012) [5].

The continuous development of the molecular markers, mainly related to the application of the next generation sequencing technique (NGS) into routine laboratory practice, enables better and wider selection of NSCLC patients for targeted therapies [14–16]. Currently, multi-gene molecular profiling studies are included in the diagnostic standard in lung cancer. They allow detection of specific mutations or rearrangements of genes that are of predictive importance. Identification of these changes enables individualisation of therapy and improves treatment with an acceptable degree of toxicity. In clinical practice, the widest used drugs are inhibitors of tyrosine kinase (via a blockage of epithelial growth factor (TKI-EGFR) receptor), the inhibitors of ALK, ROS1, BRAF, NTRK, as well as immunotherapy applying monoclonal antibodies against immunological system checkpoints – mainly against the programmed cell death receptor type 1 (PD-1) or its ligand (PD-L1) (tab. I) [17].

NGS is a promising and state-of-the-art, precise and sensitive technique that allows detecting changes in the genetic material. NGS is one of the difficult diagnostic methods that require staff with high manual skills, but also with analytical and interpretative competencies. However, NGS enables analysis of many genes and, depending on the type of equipment and reagents used, many patients may be tested at the same time. The available diagnostic kits for NGS are usually designed for individual tumours and allow for a complete molecular characterisation of the examined tissue according to modern knowledge in one reaction. This significantly shortens the examination time and enables reduction of the amount of tissue necessary for its performance [13].

Molecular tests in NSCLC can be performed on formalin-fixed postoperative material (only 15–20% of primary lung cancers referred for molecular tests), in cytological material obtained during fine-needle biopsy or in the material of the so-called liquid biopsy, which is based on the use of the cell-free circulating tumour DNA (ctDNA) and RNA released from tumour cells. Recently, the increasing use of liquid biopsy to monitor therapy has been recorded [1]. Before performing molecular test in tissue, cytological material and fine needle

Table I. Genetic changes important in the molecular profiling of non-small cell lung cancer [11, 18]

No.	Gene	Change	Percentage of NSCLC
1	*KRAS*	point mutations	15–25
2	*EGFR*	amplification	20
3	*EGFR*	point mutations	10–15
4	*PTEN*	point mutations	4–8
5	*DDR2*	point mutations	4
6	*ALK*	rearrangement	3–7
7	*HER2*	point mutations	4
8	*MET*	fusion and exon 14 skipping	2–4
9	*BRAF*	point mutations	1–3
10	*PIK3CA*	point mutations	1–3
11	*AKT1*	point mutations	1
12	*MEK1*	point mutations	1
13	*NRAS*	point mutations	1
14	*RET*	rearrangement	1
15	*ROS1*	rearrangement	1

biopsy, a pathomorphological assessment of the percentage of neoplastic cells in the preparation is required [1]. In Poland, the National Health Fund (Narodowy Fundusz Zdrowia – NFZ) refunds genetic diagnostics for NSCLC patients.

Genetic changes in non-small cell lung cancer important for qualification for targeted therapy

EGFR

Mutations (pathogenic variants) in the *EGFR* (epidermal growth factor receptor – ERBB1) gene are the described as driver mutations for NSCLC and occur in approximately 10% of Caucasian patients [19]. *EGFR* belongs to the family of genes that code for receptor tyrosine kinases (RTKs). Its protein product participates in the PI3K-AKT-mTOR and RAS-RAF-MEK-ERK pathways. The most common *EGFR* mutations are found in adenomatous type cancers [3].

Most pathogenic variants of *EGFR* are activating deletions in exon 19, which do not interrupt read-out frame (in-frame deletions) – and do not modify the three-nucleotide genetic code, hot-spot point mutations in exon 21 (e.g. L858R), and also the resistance mutation at exon 20 (T790M) [13].

If an activating *EGFR* mutation is found in the tumour tissue, treatment implemented as first line includes EGFR tyrosine kinase inhibitors (TKIs). We currently have several generations of tyrosine kinase inhibitors: the first including erlotinib, gefitinib, the second: afatinib, dacomitinib, and the third: osimertinib. In addition, it has been shown that for the less frequent *EGFR* deletions and point mutations in exons 18–21, therapy with selected TKI inhibitors can be used with good outcomes, too [13]. Rare mutations in the *EGFR* gene may coexist with common mutations of this gene. Then, it is possible to obtain a therapeutic response after the use of first-line TKI. It is an exceptional situation if an inactivating mutation (most frequently T790M) which determines TKI therapy resistance is detected to coexist with another primary activating mutation. This means that the tested neoplastic cells are insensitive to first- and second-generation TKI therapy and the patient will not benefit from such therapy, but a response to third-generation TKI treatment can be achieved [13]. However, it should be remembered that the material must be collected at the time of disease progression. In Poland currently, from 1 January 2021, the first-line treatment with osimertinib and afatinib is reimbursed in the lung cancer drug programme. In the second line treatment is reimbursed osimertinib and failure of previous treatment with other TKIs and with the presence of the T790M mutation in the *EGFR* gene. Erlotinib and gefitinib are available in the catalogue.

The methods used to detect pathogenic variants of *EGFR* include qPCR (real-time PCR, qPCR – quantitative polymerase chain reaction) or NGS. The former allows for diagnostics of a selected set of mutations (usually about 30–40 mutations in exons 18–21) and it is relatively easy among methods used in genetic diagnosis of neoplasms – usually it is performed with validated CE IVD-certified ready diagnostics sets and the result is described with fluorescence chart for particular mutations and with numerical values. The qPCR technique enables detection of mutations present even in only 1% of neoplastic cells in the examined tissue, with the use of small amounts of DNA (e.g., at a concentration of 10 ng/µl). Real-time PCR is the recommended technique for the determination of EGFR mutations. This is currently a standard method at molecular laboratories which perform genetic testing of neoplastic material. The sensitivity of the applied method should ensure reliable evaluation of the tissue material containing at least 50% of tumour cells [20].

The second technique that is used to detect variants in both the *EGFR* gene and other relevant genes, usually in combined multigene panels, is NGS. Its advantage over qPCR is the ability to detect all pathogenic and potentially pathogenic variants of all exons of gene. The standard threshold of detection of somatic mutations for NGS is defined at ≥ 5% of variant allele frequency (VAF), but it is actually possible to detect mutation at lower VAF [21].

A different diagnostic procedure is used for the T790M inactivating mutation (60% of all *EGFR* mutations). The applied techniques enable monitoring of occurrence of this mutation based on the liquid biopsy sample, i.e., on the relevantly sampled venous blood used to isolate cell-free circulating tumour DNA. In this case, the mutational status is usually assessed using qPCR but also digital PCR droplet (ddPCR) designed to study known single mutations in a very small quantity of genetic material [22]. Determination of ct*EGFR* (circulating tumour – EGFR) in liquid biopsy is also possible thanks to an automated method based on ready-to-use Idylla™ (Biocartis) cartridges, in which both the isolation process and qPCR occur in one place [23].

ALK

Rearrangements (fusions) of the *ALK* (anaplastic lymphoma kinase) gene leading to its permanent activation occur in approximately 3–6% of patients with adenocarcinoma and belong to the main alteration [24, 25]. The most common one is the fusion of *ALK* with *EML4* (echinoderm microtubule-associated protein-like 4), which results from inversion of the short arm of chromosome 2, where both genes are located, and leads to the expression of the chimeric protein EML4-ALK [25]. In addition, *ALK* fuses also with *TFG* (trafficking from ER to Golgi regulator) and *KIF5B* (kinesin family member 5B). If an *ALK* rearrangement is detected in tumour tissue of patients that are qualified for therapy with ALK inhibitors of the first (crizotinib), second (alectinib, ceritinib, brigatinib) or third generation (lorlatinib). However, occurrence of another mutation in *ALK*, or activation of other pathways: EGFR or PI3 causes resistance to this therapy [25]. In NSCLC, when an *ALK* rearrangement occurs, there is an increased predisposition for the patient to develop brain metastases.

In Poland, from January 2021, crizotinib, alectinib and ceritinib in the first line of treatment are reimbursed by National Health Fund, and so are therapies with crizotinib in the second and third lines after failure of prior chemotherapy. In addition, alectinib, ceritinib and brigatinib are reimbursed after failure of therapy with another ALK inhibitor.

Immunohistochemical testing is a cheap and fast screening method for detection of expression of the EML4-ALK fusion protein in cancer cells. Under physiological conditions, ALK plays an important role in the maturation of neurons and is not expressed in normal lung tissue, so its expression in a tumour means that it has rearranged [26]. The performance of this study to qualify a patient for targeted therapy is annually evaluated externally under the European Quality Control Program. It should be emphasised that an equivocal IHC result in the form of a weak, heterogeneous ALK protein cytoplasmic test should be confirmed by testing the *ALK* gene rearrangement with (FISH) [1]. Assessment of *ALK* gene rearrangement with FISH technique applies two-colour fluorescent probes – one for the 5' end and the other for 3' end of *ALK*. If there is no rearrangement, both probes are located close to each other (the test shows them as a single, two-coloured signal), while in the case of *ALK* rearrangement, the probes for at least one copy of the gene are separated and two discrete signals (break-apart probes) or signal deletion for the gene's 5' end can be observed in the nucleus, indicating presence of the rearrangement [25]. In this test, a minimum of 50 interphasic nuclei are analysed (100 by default, but this is not always possible due to technical difficulties and a small amount of tissue material), and the cut-off limit for a positive result is the presence of rearrangements in >15% of the analysed cells, found by two examiners in independent analyses [25]. Patients with rearrangement of the *ALK* gene present in at least 15% of nuclei may be eligible for therapy with ALK inhibitors [1].

For a patient to be qualified for the drug programme, *ALK* gene rearrangement in tumour cells should be identified by IHC, FISH or NGS using a validated test.

Another *ALK* testing method is qPCR reverse transcription. This method allows identification of fusion partners and fusion variants of this gene, but requires obtaining good quality RNA from FFPE tissue (formalin-fixed paraffin-embedded tissue), which is not always possible due to degradation of the genetic material [27, 28]. Moreover, it is not possible to detect all *ALK* fusion variants with the qPCR technique. This method is not recommended in Poland in qualifying patients for therapy and therefore it is not widely used.

ALK gene rearrangements can also be tested with the NGS technique, with both *ALK* test-only kits or kits for testing several different genes in one sample. This significantly reduces the time of the analysis, and also allows to obtain more data, including not only information on the rearrangement, but also on other pathogenic variants, e.g., point mutations which are very important for the patient, determining insensitivity to therapy. Using NGS is cost-effective for a larger number of samples and not economical for single markings [13–15]. Performing this test in patient selection for targeted therapy, similarly to IHC and FISH testing, is annually evaluated outside the lab within the European quality control system.

ROS1

Rearrangements of the *ROS1* gene (ROS protooncogene-1, tyrosine kinase receptor) occur in 1–2% of NSCLC patients and determine the response to therapy with *ROS1* inhibitors (e.g., crizotinib). Similar as in the case of *ALK*, point mutations occur in the *ROS1* gene causing insensitivity to this therapy despite the occurrence of rearrangements [13]. Analogically to the *ALK* gene, diagnostics can apply FISH and NGS methods, and selection for treatment is possible based on results obtained from a laboratory that has received positive evaluation within the annual control of the European quality control system.

PD-1 and PD-L1

PD-L1 (programmed death-ligand 1) is a cell surface ligand and its overexpression in neoplastic cells is conditioned by loss of the *PTEN* gene and induction of the PI3K-AKT pathway. In turn, PD-1 (programmed cell death protein 1) is a receptor on the surface of CD81+ T cells, and its expression increases during tumour cell infiltration [29]. This reduces the lymphocytes' ability to produce cytokines and proliferate, which disrupts the immune system. If an IHC test using DAKO PD-L1 IHC 22C3 antibodies concentration or Ventana PD-L1 SP263 antibodies confirms presence of PD-L1 in 50% or more cancer cells, patients are qualified for treatment with anti-PD-1 antibodies in monotherapy (pembrolizumab), restoring lymphocytes' cytotoxic activity. On the other hand, when PD-L1 expression is below 50%, patients benefit from treatment with immunotherapy in combination with chemotherapy (currently reimbursed in the Polish drug program from January 2021).

It should be remembered that expression of PD-L1 in neoplastic cells is not essential for the immunotherapy treatment to be beneficial for the patient. This is the case of nivolumab, atezolizumab – in Poland reimbursed under the drug program in the second-line treatment, after failure of chemotherapy. It is not required either for application of durvalumab – as consolidation treatment after radical radiochemotherapy. However, immunotherapy is only effective in a small percentage of patients, possibly due to the highly complex immune microenvironment of the tumour.

Globally, clinical application of immunotherapy in NSCLC originated in 2015 – based on the CheckMate 017 study [30]. Clinical trials show divergent results regarding the role of PD-L1 expression as a predictive factor for immunotherapy result. This is probably due to differences in the evaluation of expression and methods of testing with immunohistochemical methods. The change in expression may be also affected by prior treatment (e.g., chemotherapy). Moreover, tumours are

characterised by heterogeneity of PD-L1 expression within the tumour as well as different expression between the primary tumour and lymph nodes [31–33].

Other genes

Next-generation sequencing makes it possible to analyse the entire sequence of many genes in a single assay, but the amount of data obtained from such a study also carries the risk of misinterpretation and difficulties in interpreting their meaning in relation to clinical data. Therefore, according to the latest recommendations, in patients with NSCLC, the examination of tumour tissue for **diagnostic purposes** should include a specific panel of genes whose pathogenic variants are predictive or prognostic [13]. According to the recommendations by the European Society for Medical Oncology (ESMO) and the Scale for Clinical Actionability of molecular Targets (ESCAT), such genes in patients with advanced non-squamous NSCLC include:

- *MET* – which encodes the receptor for hepatocyte growth factor receptor (HGFR). The most common mutation is either exon 14 deletion (exon 14 skipping) associated with poor prognosis (approximately 3% of patients), or gene amplification (also in approximately 3% of patients) inducing resistance to EGFR inhibitors – usually a result of cell clonal selection in patients after this therapy. Application of capmatinib was an attempt to overcome this resistance. Crizotinib has been shown to be effective in patients with high amplification of the *MET* gene.
- *BRAF* – the most common V600E mutation, occurring in approximately 2% of patients. Drugs approved for first-line treatment in cases of this mutation are dabrafenib and trametinib.
- *NTRK* – 0.23–3% of patients have *NTRK/1/2/3* gene fusions, which determine formation of oncoproteins. In 0.1–1%, NSCLC does not coexist with other genetic disorders. Drugs approved for treatment in cases of these mutations are entrectinib and larotrectinib.
- *RET* – fusions occur in 0.6–0.9% of NSCLC patients, and in 1–2% of adenocarcinoma patients. Rearrangement of this gene does not usually coexist with other genetic changes in NSCLC cells. On 10 December 2020, the European Medicines Agency (EMA) approved selpercatinib in monotherapy for RET-positive non-small cell lung cancer after prior immunotherapy and/or platinum-based chemotherapy.
- *KRAS* – 12% of patients have these mutations, 97% of which are in exons 2 and 3 (mainly in G12, G13 and Q61).
- *HER2 (ERBB2)* – gene amplification and hot-spot mutations are observed in 2–5% of patients. A therapeutic effect of afatinib and dacomitinib was recorded in patients with these mutations.
- *BRCA1/2* – point mutations were observed in 1.2% of patients.
- *PIK3CA* – mainly hot-spot mutations, but also amplifications present in 1.2–7% of patients, often coexisting with mutations of other genes.
- *NRG1* – gene fusions occur in 1.7% of patients.

According to ESCAT, in patients with advanced squamous-cell NSCLC, fusions of the *NTRK* gene (present in 0.23–3% of patients), mutations of *PIK3CA* (16% of patients) and *BRCA1/2* (1.2% of patients) are diagnostically and clinically significant [13].

Tumour mutational burden (TMB)

Recently, the quantitative biomarker of TMB (tumour mutational burden) is gaining increased interest as a predictive factor in immunotherapy. The TMB test is performed with NGS technique, and the TMB value is determined by the number of mutations per million base pairs in DNA isolated from the tumour [34]. TMB result is reported as: high (TMB-high), intermediate (TMB-intermediate), low (TMB-low) or undetermined (TMB-undetermined), depending on the number of mutations detected in the tumour [35]. NSCLC patients with high TMB have been shown to benefit clinically with immunotherapy targeted at immune checkpoints (immune checkpoint inhibitors – ICIs) [16]. This effect is associated with increased expression of neoantigens induced by the presence of a mutation that mobilises the immune system to recognise and destroy cancer cells [36]. High TMB correlates with increased progression-free survival (PFS) and increased response rate in patients after immunotherapy [37].

Perspectives

In selected patients, targeted therapy undoubtedly improves treatment results and control of advanced non-small cell lung cancer, as compared to chemotherapy. The quality of life of patients treated in this way also improves because the toxicity of this therapy is lower. Application of first-generation TKI targeted at EGFR also improves PFS as compared to chemotherapy [38]. However, over time, patients inevitably develop drug resistance. The most common resistance mechanisms include appearance of the T790M mutation of *EGFR*, *RAS* gene mutation, and *MET* amplification. Resistance to first- and second-generation inhibitors has been shown to occur on average after 10–14 months [39]. In recent years, the third generation of EGFR-TKI has been developed – a drug that is active both in the first line in the presence of the *EGFR* mutation, and in the second line of treatment after other TKI inhibitors, in the presence of the T790M resistance mutation in the *EGFR* gene. Studies have shown that there is drug resistance to third generation EGFR-TKI in the form of mutations of *EGFR*, *PIK3CA*, *KRAS*, *BRAF* and *MET*. *MET* inhibitor can increase sensitivity to first-generation EGFR-TKI [40]. In general, regardless of the *EGFR* and *KRAS* mutations, approximately 5% of NSCLC patients have a rearrangement (fusion) of the *ALK* gene. A greater risk of brain metastases is observed in such cases [41]. It was also found

that the second-generation ALK inhibitor has a high intracranial efficacy compared to the first-generation ALK inhibitor [42], but with time new mutations appear, conditioning resistance to the treatment.

Other immune checkpoints besides PD-1/PD-L1 are also being sought to increase the number of patients who can benefit from this form of treatment. Another direction involves application of the combined therapy, e.g., chemotherapy with checkpoint inhibitors (such a combination of drugs is already registered and available in Poland within the drug programme – from 1 January 2021).

There are currently many clinical trials underway concerning targeted therapies in NSCLC, both alone and in combination, as well as their sequential administration. Further, there are also trials on application of molecularly targeted drugs and immunotherapy in neoadjuvant and adjuvant treatment, as well as maintenance therapy. Chemotherapy is no longer the best systemic treatment available for all NSCLC patients. Therapeutic decisions should be based on examination of the molecular characteristics of the tumour.

Expected benefits for patients, such as prolongation of overall survival and obtaining the longest possible remission in the future will probably result from finding the optimal ways of combining targeted therapy, immunotherapy and chemotherapy.

Conflict of interest: none declared

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