Methodological recommendations for the diagnostics of \textit{EGFR} gene mutations and \textit{ALK} gene rearrangement in the selection of non-small-cell lung cancer patients to molecularly targeted therapies

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Abstract

Testing for \textit{EGFR} gene mutations and \textit{ALK} gene rearrangement is routinely used in advanced non-small-cell lung cancer for adequate patient selection to molecularly targeted therapies. We present Polish methodological recommendations for molecular analysis of \textit{EGFR} and \textit{ALK} genetic abnormalities. Recommendations specify clinical indications for testing, sample types and handling, as well as requirements for laboratories performing molecular diagnostics.

\textbf{Key words:} non-small-cell lung cancer, \textit{EGFR} gene mutations, \textit{ALK} gene rearrangement, genetic diagnostic tests


Streszczenie

Badania molekularne mające na celu wykrycie mutacji genu \textit{EGFR} i rearanżacji genu \textit{ALK} wykonuje sięrutynowo w zaawansowanym niedrobnokomórkowym raku płuc (NDRP) w celu właściwej kwalifikacji chorych do terapii ukierunkowanych molekularnie. Przedstawiamy polskie zalecenia metodyczne prowadzenia diagnostyki molekularnej nieprawidłowości w genach \textit{EGFR} i \textit{ALK}. Zalecenia te opisują szczegółowo wskazania kliniczne do wykonania testów, rodzaj materii oraz sposób postępowania z nią, a także wymagania stawiane laboratoriom wykonującym diagnostykę molekularną.

\textbf{Słowa kluczowe:} niedrobnokomórkowy rak płuc, mutacje genu \textit{EGFR}, rearanżacja genu \textit{ALK}, genetyczne testy diagnostyczne

Introduction

The following guidelines represent the consensus opinion of Polish experts in the field of pathomorphological and molecular diagnostics of non-small cell lung cancer (NSCLC). Along rapid development of molecularly targeted NSCLC patients pharmacotherapy, there is a high rise in the number of diagnostic genetic testing allowing proper qualification for treatment. The guidelines are to provide methodological insight into all stages of adequately conducted diagnostics. By principle, they will be the means of eliminating bad laboratory practices leading to faulty results of genetic testing or delay in the diagnostic process.

Pursuant to the official announcement of the Minister of Health regarding the list of reimbursed medicines, foodstuffs for special medical purposes and medical devices (1 March 2014), the molecularly targeted NSCLC patients treatment conducted as part of the drug scheme includes the administration of two epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI), erlotinib and gefitinib [1]. Both medicines are to be used in the first- and second-line treatment in patients with specific morphological subtypes of NSCLC, either locally advanced (unresectable) or metastatic, and the presence of activating mutations of the EGFR gene [2]. Medicines authorised for marketing in the European Union, not covered with the reimbursement system in Poland yet, are expected to be introduced into the range of molecularly targeted therapies of locally advanced and metastatic NSCLC patients in the nearest future. These comprise: irreversible EGFR HER2 and HER 4 TKI - afatinib and ALK (anaplastic lymphoma kinase) TKI - crizotinib.

1. Clinical recommendations for the diagnostics of EGFR gene and ALK gene rearrangement in NSCLC patients

A. Recommendation: Confirmed presence of EGFR gene mutations or ALK gene rearrangement in cancer cells is the key criteria for NSCLC patient qualification for molecularly targeted therapies using EGFR TKI or ALK TKI [2].

B. Recommendation: The decision to run genetic testing in an NSCLC patient should be taken by an oncologist or pulmonologist subject to individual assessment of patient condition and clinical indications for molecularly targeted treatment. EGFR gene mutations testing ought to be performed in patients diagnosed with NSCLC other than squamous cell carcinoma; whereas ALK gene rearrangement testing - in patients diagnosed with adenocarcinoma or including an adenocarcinoma component. Differentiation or histologic grade has no impact on indications for molecular diagnostics [3-8].

Genetic testing is not recommended if histopathological analysis of cancer specimen reveal pure squamous-cell lung cancer or small-cell lung cancer or carcinos in the event the standard hematoxylin and eosin (H+E) staining does not allow for NSCLC subtype determination, histochemical (to identify mucus in cancer cells) and/or immunohistochemical staining (IHC) is obligatory. It is recommended that expression of a minimum two markers be determined: TTF-1, which helps differentiate adenocarcinoma and p63 or p40, which expression favours squamous cell carcinoma. In the case of cytological material, every IHC should be preceded by qualification for genetic diagnostics [3−8]. Molecular testing for EGFR gene mutations is acceptable provided that it is impossible to determine the morphological subtype of NSCLC (not otherwise specified, NOS) [5-8].

C. Recommendation: Demographic criteria, such as sex, race or history of smoking do not affect the indications for EGFR gene mutations or ALK gene rearrangement testing in NSCLC patients. Considering the smoking/lack of smoking history is acceptable provided that testing is conducted on the basis of hypocellular sample or cytological material in which the presence of adenocarcinoma component was not completely excluded (NSCLC NOS) [6−8].

D. Recommendation: Specimen originating from primary NSCLC and from metastases should be treated as equally suitable for determining the presence of EGFR gene mutations and ALK gene rearrangement. There is no justification for simultaneous testing of several tissue samples of the same tumour [6−8].

2. Principles of molecular testing for EGFR gene mutations and ALK gene rearrangement

A. Recommendation: It is recommended that the oncologist or pulmonologist ordering pathomorphological testing of specimen of a patient suspected of locally advanced or metastatic NSCLC (stage IIIB or IV according to the seventh edition of TNM classification [9-10]) also instruct in writing that genetic
testing be performed, if justified by morphological diagnosis, volume, and quality of available sample. In that case, it is advised that a pathologist take decision regarding possible genetic testing for EGFR gene mutations and ALK gene rearrangement at the time of microscopic diagnosis. In default of relevant instructions, secured material should be transferred for genetic testing immediately after reception of a written order of the attending physician [6–8]. It is requisite that genetic testing be performed in lower-stage non-small cell lung cancer at disease recurrence or if unresectable, provided that qualification for molecularly targeted treatment is considered.

B. **Recommendation:** It is recommended that EGFR gene mutations testing be conducted first. ALK gene rearrangement determination in adenocarcinoma patients should be performed upon previous exclusion of the presence of somatic mutations in EGFR gene. Nowadays, there is insufficient clinical evidence to justify the analysis of other molecular markers in NSCLC patients [6–8].

C. **Recommendation:** The turnaround for EGFR gene mutations and/or ALK gene rearrangement tests should not exceed 10 working days from the time tissue samples are delivered to the genetic laboratory. Nevertheless, it is recommended that the turnaround time for genetic testing be a maximum of 5 working days [6–8].

D. **Recommendation:** It is recommended that laboratories where the turnaround time for EGFR gene mutations and ALK gene rearrangement tests exceeds 10 working days adjust their procedures and methods of analyses accordingly [6–8].

E. **Recommendation:** The time of preparing archival materials by a pathomorphological laboratory and delivering them to a genetic laboratory should not exceed 3 days from the order date. The above period may be extended if morphological reassessment is required [6–8].

3. **Requirements to be met by genetic laboratories regarding EGFR gene mutations and ALK gene rearrangement testing**

A. **Recommendation:** Operational rules regarding laboratories performing genetic testing of predictive factors for molecularly targeted therapies in neoplastic diseases shall follow the regulations set forth in the Laboratory Diagnostics Act of 27 July 2001 (as amended) and the Ministry of Health Regulation of 21 January 2009 amending the regulation on the quality standards for medical diagnostic and microbiological laboratories (Appendix 3) [11, 12]. Recommendations B to D specify laboratory operational rules included in Appendix 3 to the MH Regulation.

B. **Recommendation:** Genetic testing may be performed solely under the supervision of the laboratory diagnostician or a physician who is a staff member of the laboratory performing genetic testing and: 1) is a specialist in medical laboratory genetics and is permanently employed by the laboratory performing genetic testing or 2) has a minimum of two-year genetic laboratory work experience or 3) has been employed for a continuous period of five years by a genetic laboratory performing EGFR gene mutations and ALK gene rearrangement testing.

C. **Recommendation:** EGFR gene mutations and ALK gene rearrangement is a standard procedure to evaluate non-heritable genetic disorders in cancer cells. Thus, obtaining a separate patient consent form to perform genetic testing is unnecessary. General consent to perform diagnostic testing, including but not limited EGFR gene mutations and ALK gene rearrangement, should be obtained at admission to hospital and stored with other medical records of the patient.

D. **Recommendation:** It is recommended that genetic laboratories performing EGFR gene mutations and ALK gene rearrangement testing carry out period internal quality assessment and participated in external quality assessment (EQA) programmes [12]. Polish genetic laboratories have access to EQA programmes conducted by renown genetic diagnostics quality control centers (such as European Molecular Quality Network, European Society of Pathology) and should have quality certificates issued by at least one of them.

E. **Recommendation:** It is recommended that a Polish external quality assessment programme involving EGFR gene mutations and ALK gene rearrangement testing in NSCLC patients specimen be developed.

**Requirements regarding the methods and range of EGFR gene mutations**

A. **Recommendation:** Genetic testing for EGFR gene mutations ought to be performed only on properly secured material: 1) formal-
ne-fixed and paraffin-embedded samples (preferred material due to high stability and suitability for genetic testing even after several days from collection); 2) freshly collected tissues; 3) frozen tissues; 4) alcohol-fixed tissues. Specimen prepared with the use of other fixatives, especially ones with heavy metals content or decalcifying solutions and of acidic environment, should not be used for EGFR gene mutations assessment. It is recommended that the times of tissue fixation in a 10% buffered formalin, i.e. 6-48 hours, be strictly followed. Every case of noncompliance with routine laboratory procedures, in particular with recommended fixation times and methods of paraffin embedding should be recorded in the pathomorphological report due to their potential effect on tumour tissue DNA integrity [5−8].

B. Recommendation: Cytology specimen, in particular cytoblocks or smears on glass slides, are suitable for EGFR gene mutations analysis. Cell suspensions used for cytoblock preparation should be fixed in 10% buffered formalin or 70% ethanol solution for 6-48 hours. It is advised that each laboratory runs a complete cytology specimen development validation procedure, including fixation and genetic analysis for EGFR gene mutations [5−8, 13, 14].

C. Recommendation: It is recommended that the choice of representative material for determining EGFR gene mutations be made by a pathologist. Cancer cells and necrotic foci rate assessment, and in the case of cytology specimen — also cancer cell count — are obligatory. It is recommended that paraffin tissue blocks or cytoblocks be mass-produced according to the following scheme: 1. 3 μm-thick sections intended for cancer cell content analysis (hematoxylin and eosin staining, H+E); 2. 8–10 μm-thick sections intended for DNA isolation (in the case of hypercellular material several specimen 8-10 μm-thick are allowed); 3. and 4. 3–5 μm-thick sections are intended for ALK gene rearrangement testing (FISH and IHC methods, if a given laboratory uses said method for initial ALK gene rearrangement diagnostics); 5. 3 μm-thick sections are intended for reassessment of cancer cells content [6−8].

D. Recommendation: Diagnostic specimen-isolated DNA quantity and quality assessment is obligatory [6-8].

E. Recommendation: Genetic analysis of cytology specimen fixed on a glass slide should be performed in smears stained with hematoxylin and eosin (H+E). The use of immunohistochemical stain slides is not recommended [6−8, 13, 14]. It is recommended that cancer cells identification and location in the specimen be performed by a pathologist. Genetic laboratory procedure should involve specimen incubation in xylene solution for a minimum of 4-6 hours, optimally 12 hours, cover glass removal and DNA isolation from cells mechanically removed from the slide from the spots previously indicated by the pathologist [6−8, 13, 14].

F. Recommendation: Genetic laboratory is obliged to establish the minimum rate of cancer cells content needed for reliable assessment of EGFR gene mutations during internal quality control [6−8, 12]. Microdissection of cancer cells from a given specimen to increase the cancer cell rate is allowed [6−8].

G. Recommendation: DNA isolation requires the use of reagent sets intended for in vitro diagnostics (CE-IVD symbol) [6−8, 12].

H. Recommendation: Genetic laboratory may avail of various EGFR gene mutations determination methods provided that they have been validated and specified in accordance with binding Polish regulations [Act of 18 March 2011 — establishing the Office for Registration of Medicinal Products, Medical Devices and Biocides). DNA isolation requires the use of reagent sets intended for in vitro diagnostics (CE-IVD symbol) [6−8, 12]. The use of polymerase chain reaction (PCR) -based methods, including above all real-time PCR, is recommended. The sensitivity of the EGFR gene mutations analysis method must constitute a reliable analysis of specimen containing at least 50% of cancer cells. Sanger sequencing genetic analysis may be applied to such specimen. However, it is recommended that more sensitive molecular testing methods allowing EGFR gene mutations identification in specimen with at least 10% cancer cell content be applied [6−8, 15, 16].

It is recommended that every laboratory with no technological means to perform genetic testing of low cancer cell specimen transfer the sample to other laboratory without delay and inform the ordering party accordingly [6−8].

I. Recommendation: It is required that every laboratory have at its disposal methodology allowing identification of all EGFR gene mutations of at
least 1% incidence rate (amongst the known EGFR gene mutations) [6–8, 16] (Table 1).

J. **Recommendation:** It is recommended that specimen testing for T790M mutation of the EGFR gene (related to EGFR TKI resistance) be performed with the use of high sensitivity methods allowing reliable analysis of specimen with at least 5% cancer cells content [6–8].

K. **Recommendation:** It is not recommended that IHC reactions be used for determining EGFR protein expression and EGFR gene copy number analysis by fluorescent or chromogenic in situ hybridization — for qualification for EGFR tyrosine kinase inhibitor therapy. Furthermore, KRAS gene mutations testing for qualification for EGFR TKI therapy is not recommended, wither [2, 6–8].

**Requirements regarding the methods and range of ALK gene rearrangement**

A. **Recommendation:** The presence of ALK gene rearrangement in the analysed specimen must be confirmed by fluorescent in situ hybridization (FISH) using dual-labelled break-apart probes. Immunohistochemical reaction may be used as a screening tool for qualification of specimen for ALK rearrangement testing with FISH provided that previous validation is conducted. It is recommended that sets certified for in vitro application (CE-IVD symbol) be used [6, 15–19].

B. **Recommendation:** RT-PCR is not recommended as an alternative to FISH for ALK gene rearrangement testing [6, 18–19].

C. **Recommendation:** It is recommended that the choice of representative material for determining ALK gene rearrangement be made by a pathologist in accordance with instructions set forth in Recommendation C Point 4. Furthermore, it is required that cancer cells architecture or cancer cells location be established in the cytology specimen and that its quality be determined. Specimen for ALK gene rearrangement testing should be stored in paraffin blocks (histological material or cytoblocks). Reliable determination of ALK gene rearrangement in H+E- or immunohistochemical-stained cytology is not possible [6, 18, 19].

D. **Recommendation:** It is recommended that ALK gene rearrangement testing be performed by a laboratory diagnostician or a pathologist. Simultaneous application of the positive and negative control is a prerequisite.

It is recommended that visual assessment of specimen be always performed by two independent observers experienced in interpretation and analysis of FISH and IHC findings [6, 18, 19].

E. **Recommendation:** It is recommended that a pathologist experienced in interpretation and analysis of FISH staining results participate in specimen assessment.

F. **Recommendation:** Diagnostic testing for ALK gene mutation related to acquired resistance to ALK tyrosine kinase inhibitors is not required [6, 19].

**Reporting EGFR gene mutations and ALK gene rearrangement genetic testing**

A. **Recommendation:** The EGFR gene mutations and ALK gene rearrangement report is required to include test findings presented according to the Human Genome Variation Society (HGVS) terminology [20] and its clearly-worded interpretation comprehensible for an oncologist or a pulmonologist and a pathologist.

B. **Recommendation:** It is recommended that the EGFR gene mutations testing report include, in particular: 1) data allowing definite patient identification; 2) identification data of the centre ordering genetic testing and the surname of the ordering oncologist or pulmonologist; 3) identification data of the pathomorphological laboratory where the pathomorphological analysis was performed and the surname of the pathologist conducting the analysis; 4) the reference no. of specimen referred for genetic testing accompanied by detailed pathomorphological diagnosis and specimen cancer cells content, and immunohistochemical findings, if any; 5) description and sensitivity of the method used for EGFR gene mutations assessment; 6) list of mutations tested; 7) descriptive evaluation of the quality of isolated DNA; 8) results of genetic testing along clinical interpretation; 9) specimen reception date and specimen analysis date; 10) signature of the laboratory diagnostician performing the assay and the person authorising the results: a laboratory diagnostician specialising in medical laboratory genetics, a pathologist or clinical genetics specialist. It is not recommended that genetic testing report contain detailed suggestions regarding the selection of a specific molecularly targeted medicine [6–8, 12].
Table 1. Known mutations in the EGFR gene (NM_005228.3) with at least 1% incidence amongst all EGFR gene mutations [16]

<table>
<thead>
<tr>
<th>EGFR gene exon</th>
<th>EGFR gene codon</th>
<th>Mutation</th>
<th>Nucleotide substitution</th>
<th>Estimated incidence amongst all EGFR mutations (%)</th>
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<td>p.E709G</td>
<td>c.2126A&gt;G</td>
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<td>c.2126A&gt;T</td>
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<td>c.2127A&gt;C, c.2127A&gt;T</td>
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<td>p.E709Q</td>
<td>c.2125G&gt;C</td>
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<td>p.G719A</td>
<td>c.2156G&gt;C</td>
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<td>c.2155G&gt;T</td>
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<td>c.2156G&gt;A</td>
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<td>p.L861R</td>
<td>c.2582T&gt;G</td>
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bp — base pair
C. **Recommendation: ALK gene rearrangement**

Genetic testing report should include data stipulated in Points 1–4 and 6–7 of Recommendations B Point 6, and, obligatorily, a description of the method used for ALK gene rearrangement testing, information regarding the number of cell nuclei assessed (FISH) and the level of EML4-ALK protein expression (IHC) [6–8].

**Conflict of interest**

**Paweł Krawczyk:** Boehringer Ingelheim, AstraZeneca, Eli Lilly — advisory boards; Abbott, Roche, Eli Lilly, AstraZeneca, Boehringer Ingelheim, BMS, MSD — lecture

**Joanna Chorostowska-Wynimko:** Boehringer Ingelheim, Novartis — advisory boards; Roche, Boehringer Ingelheim, AstraZeneca — lecture

**Rafał Dziadzioaszko:** Boehringer Ingelheim, Pfizer — advisory boards; Boehringer Ingelheim, Pfizer, Eli Lilly, AstraZeneca — lecture

**Jacek Jassem:** Roche — research grant; Boehringer Ingelheim, Eli Lilly, AstraZeneca — advisory boards; Roche — lecture

**Maciej Krzakowski:** Boehringer Ingelheim, Eli Lilly — expert panel meeting

**Renata Langfort:** Roche, Eli Lilly, AstraZeneca, Boehringer Ingelheim — lecture

**Elżbieta Puacz:** no conflict of interest

**Bartosz Wasąg:** Roche — lecture

**Kamila Wojas-Krawczyk:** no conflict of interest

**References**


17. Act of 18 March 2011 — establishing the Office for Registration of Medicinal Products, Medical Devices and Biocides (Dz. U. 2011 No. 82 Item 451).

