Paweł Krawczyk[1, 2, 3], Joanna Chorostowska-Wynimko[2, 4], Rafał Dziadziuszko[5, 6], Jacek Jassem[5, 6], Maciej Krzakowski[7, 8], Renata Langfort[9, 10], Elżbieta Puacz[11], Bartosz Wasąg[3, 12], Kamila Wojas-Krawczyk[1]

[1]Immunology and Genetics Laboratory at the Department of Pneumonology, Oncology and Allergology, Medical University of Lublin, Poland
[2]Polish Respiratory Society
[3]Polish Society of Human Genetics
[4]Department of Genetics and Clinical Immunology, Institute of Tuberculosis and Lung Diseases, Warsaw, Poland
[5]Department of Oncology and Radiotherapy, Medical University of Gdańsk, Poland
[6]Polish Oncological Society
[7]Lung and Thoracic Tumours Department, Institute of Oncology, Warsaw, Poland
[8]Polish Society of Clinical Oncology
[9]Department of Pathomorphology, Institute of Tuberculosis and Lung Diseases, Warsaw, Poland
[10]Polish Society of Pathologists
[11]Department of Biology and Genetics, Medical University of Gdańsk, Poland
[12]The National Chamber of Laboratory Diagnosticians

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

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Abstract

Testing for *EGFR* gene mutations and *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 *EGFR* and *ALK* genetic abnormalities. Recommendations specify clinical indications for testing, sample types and handling, as well as requirements for laboratories performing molecular diagnostics.

Key words: non-small-cell lung cancer, *EGFR* gene mutations, *ALK* gene rearrangement, genetic diagnostic tests

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Streszczenie

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

Słowa kluczowe: niedrobnokomórkowy rak płuca, mutacje genu *EGFR*, rearanżacja genu *ALK*, genetyczne testy diagnostyczne

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Address for correspondence: Prof. Paweł Krawczyk, Pracownia Immunologii i Genetyki, Katedra i Klinika Pneumonologii, Onkologii i Alergologii Uniwersytetu Medycznego w Lublinie, ul. Jaczewskiego 8, 20–954 Lublin, tel./fax: +48 81 724 42 93, e-mail: krapa@poczta.onet.pl
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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 carcinoid. In the event the standard hematoxylin and eosin (H+E) staining does not allow for NSCLC subtype determination, histochemical (to identify mucilage in cancer cells) and/or immunochemical 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 archive 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) formali-

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. 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]

EGFR gene exon	*EGFR* gene codon	Mutation	Nucleotide substitution	Estimated incidence amongst all *EGFR* mutations (%)
18.	E709	p.E709K	c.2125G>A	1
		p.E709A	c.2126A>C	
		p.E709G	c.2126A>G	
		p.E709V	c.2126A>T	
		p.E709D	c.2127A>C,	
			c.2127A>T	
		p.E709Q	c.2125G>C	
	G719	p.G719S	c.2155G>A	2–5
		p.G719A	c.2156G>C	
		p.G719C	c.2155G>T	
		p.G719D	c.2156G>A	
19.	K739	Insertions 18-pz		1
	I740			
	P741			
	V742			
	A743			
	I744			
	E746	Deletions (bp) 9 12 15 18 24		45
	L747			
	R748			
	E749			
	A750			
	T751			
	S752			
	P753			
20.	S768	Insertions (bp) 3 9		4–10
	V769			
	D770			
	N771			
	P772			
	H773			
	V774			
	S768	p.S768I	c.2303G>T	1–2
	T790	p.T790M	c.2369C>T	2
21.	L858	p.L858R	c.2573T>G	40
		p.L858M	c.2572C>A	
	L861	p.L861Q	c.2582T>A	2–5
		p.L861R	c.2582T>G	

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

Rafal Dziadziuszko: 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

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