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The detection of *EGFR* mutations in patients with non-small cell lung cancer in selected molecular diagnostics centers in Poland

Wykrywalność mutacji w genie *EGFR* u chorych na niedrobnokomórkowego raka płuca w wybranych ośrodkach w Polsce zaangażowanych w diagnostykę molekularną

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Abstract

Background: The development of molecularly targeted therapies using epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) has created a need for molecular studies in patients with non-small cell lung cancer (NSCLC). TKIs (gefitinib and erlotinib) show spectacular efficacy in patients with *EGFR* activating mutations (high response rate with long progression-free survival and improved quality of life). In Poland, despite developments in the field of molecular diagnostics, there is a lack of data concerning not only the frequency of *EGFR* mutations in NSCLC patients, but also the various molecular techniques for *EGFR* mutation diagnosis that are being used in different genetic labs.

Aim of the study: The aim of this study is to describe the methods for molecular diagnostics of activating mutations in *EGFR* gene in NSCLC patients from the voivodeships of Lubelskie and Wielkopolskie (Lublin and Greater Poland).

Materials and methods: The incidence of *EGFR* mutations (deletions in exon 19 and substitution L858R in exon 21) was analyzed in 460 patients from the voivodeships of Lubelskie and Wielkopolskie (Lublin and Greater Poland).

Results: Adenocarcinoma was diagnosed in 61% of patients, NSCLC NOS (not otherwise specified) in 27%, and large cell carcinoma in 6% of patients. *EGFR* activating mutations were detected in 10.5% of patients, and were slightly more common in

Streszczenie

Wstęp: Rozwój terapii ukierunkowanych molekularnie za pomocą inhibitorów kinazy tyrozynowej EGFR (erlotynib, gefitynib), które wykazują spektakularną skuteczność u chorych z mutacjami aktywującymi genu *EGFR* (wysoki odsetek odpowiedzi na leczenie z długim czasem wolnym od progresji oraz poprawa jakości życia), stworzył konieczność prowadzenia badań molekularnych u chorych na niedrobnokomórkowego raka płuca. W Polsce, pomimo rozwoju diagnostyki molekularnej, brakuje danych nie tylko o częstości występowania mutacji w genie *EGFR*, lecz także o sposobie prowadzenia diagnostyki molekularnej w różnych ośrodkach.

Cel pracy: Opisanie metod diagnostyki molekularnej mutacji aktywujących w genie *EGFR* u osób z niedrobnokomórkowym rakiem płuca (NDRP) zamieszkujących tereny woj. lubelskiego i wielkopolskiego.

Materiał i metody: W niniejszej pracy przeanalizowano częstość występowania dwóch najczęstszych mutacji genu *EGFR* (delecji w eksonie 19 i substytucji L858R w eksonie 21) u 460 chorych z regionu Lubelszczyzny i Wielkopolski.

Wyniki: U 61% chorych zdiagnozowano raka gruczołowego, u 27% niedrobnokomórkowego raka płuca *not otherwise specified* (NDRP NOS), a u 6% – raka wielkokomórkowego. Mutacje aktywujące genu *EGFR* zdiagnozowano u 10,5% chorych, nieznacznie częściej u chorych z rozpoznaniem raka gruczołowego

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patients with adenocarcinoma (12%) than in those with NSCLC NOS (7.5%) and large cell carcinoma (7%). Mutations occurred slightly more frequently in the material from formaldehyde-fixed paraffin-embedded (FFPE) tissue obtained from NSCLC tumor surgery (12.36%) than in the material from tumor biopsy (8.8%). Materials from surgical resection were reliable for molecular examination significantly more frequently ($\chi^2 = 10.77$, $p = 0.001$) than the material from biopsy. Furthermore, the postoperative samples provided a higher DNA concentration ($p = 0.0002$) than the biopsy materials.

Conclusions: Molecular diagnosis of *EGFR* mutations during the qualification process for EGFR-TKI treatment seems justified for all patients with a diagnosis of non-squamous NSCLC, and should be conducted using the most reliable material.

Key words: NSCLC, *EGFR* mutation, cytology, histological material.

(12%) niż z rozpoznaniem NDRP NOS (7,5%) i raka wielkokomórkowego (7%). Mutacje nieistotnie częściej rozpoznawano w materiale z bloczków parafinowych uzyskiwanym z operacji guza NDRP (12,36%) niż w materiale z biopsji zmiany nowotworowej (8,8%). Materiał pooperacyjny był natomiast istotnie częściej wiarygodnym materiałem diagnostycznym ($\chi^2 = 10,77$; $p = 0,001$) oraz dostarczał DNA o większym stężeniu ($p = 0,0002$) niż materiał pochodzący z biopsji.

Wnioski: Diagnostyka molekularna mutacji w genie *EGFR* w kwalifikacji do terapii IKT EGFR wydaje się zasadna u wszystkich chorych z rozpoznaniem niepłaskonabłonkowego NDRP, przy czym należy dążyć do przeprowadzenia jej w najbardziej wiarygodnym materiale.

Słowa kluczowe: NDRP, mutacja *EGFR*, cytologia, materiał histologiczny.

Introduction

Lung cancer is presently the most frequent cause of death among malignant neoplasms in Poland. According to the Polish National Cancer Registry, it was responsible for the deaths of 16,357 men and 5,947 women throughout the country in 2009. The numbers of deaths within the administrative regions featured in the study included 1,336 men and 500 women in the Greater Poland Voivodeship and 925 men and 226 women in the Lublin Voivodeship [1].

More than 80% of lung cancer patients are diagnosed with non-small cell lung cancer (NSCLC), most often in the locally advanced or metastatic stage, which can only be treated by means of chemotherapy and radiation therapy. In 2009, the International Association for the Study of Lung Cancer (IASLC), the American Joint Committee on Cancer (AJCC), and the European Respiratory Society (ERS) proposed significant changes to the pathomorphological classification of NSCLC in order to meet the demands of NSCLC diagnostics related to new treatment methods for this type of neoplasm. The changes were mostly related to the classification of adenocarcinoma, which surpasses other types of NSCLC in terms of its frequency of occurrence and is more common among women and non-smokers or former smokers [2-7]. Unfortunately, the frequency of occurrence of adenocarcinoma has never been precisely evaluated in Poland. Nonetheless, epidemiological data from other EU countries suggest that this type of neoplasm occurs in about 40% of NSCLC patients.

Neoplastic adenocarcinoma cells have been relatively well characterized in terms of the occurrence of molecular disorders, including, first and foremost, *EGFR* (epidermal growth factor receptor) and *KRAS* (Kirsten rat sarcoma viral oncogene homolog) gene mutations, as well as *ALK* (anaplastic lymphoma kinase) gene rearrangements. This has particular significance in relation to the possibility of conducting molecularly targeted treatment by means of EGFR tyrosine kinase inhibitors (EGFR TKI) in patients with activating mutations in the *EGFR* gene and by means of crizotinib in patients with fusion genes *EML4-ALK* or *ROS1-ALK* [8]. However, qualifying adenocarcinoma patients for molecular analysis in order to detect the presence of the aforementioned mutations is no easy task. It depends on a number of factors, in particular on the amount and quality of the available material containing the neoplastic cells that had previously been used for pathomorphological analysis [9, 10].

Therefore, the role of the pathomorphologist is not only to precisely diagnose the NSCLC subtype, but also to participate in the process of qualifying the material for molecular analysis. Whether such analysis should be conducted on the request of the oncologist, or whether it should be conducted for all patients with the specific pathomorphological diagnosis (adenocarcinoma or non-squamous carcinoma) is still a matter of discussion [9, 10]. Pathomorphological diagnosis may be aided by the evaluation of immunohistochemical reactions. The basic panel employs antibodies targeted against the thyroid transcription factor 1 (TTF1) and CK 7 (both of which are specific for adenocarcinoma), as well as antibodies against p63, CK5/6 and CK34β12 (specific for squamous carcinoma). Occasionally, particularly in cytological analysis, the exact type of NSCLC cannot be established despite the use of immunohistochemical tests. In such cases, the pathomorphologist may indicate the most probable type of neoplasm or describe it as NOS (not otherwise specified) [2-7]. Due to the probability that NOS neoplasms may include adenocarcinomas, the validity of conducting molecular diagnostics for such a diagnosis of NSCLC remains debatable. The percentage of patients diagnosed with NSCLC NOS may differ significantly depending on the medical center performing the pathomorphological diagnostics, and it has never been precisely established in Poland.

Evaluating the percentage of neoplastic cells and the quality of material presents an even greater challenge during the process of qualifying material for molecular analysis. Direct sequencing by the Sanger method, which was employed in molecular diagnostics until recently, required the presence of more than 50% of neoplastic cells within the analyzed material [9, 10]. The currently used methods, such as ARMS-PCR (amplification refractory mutation system polymerase chain reaction), enable us to obtain credible results concer-

ning the presence of mutation with 10% of neoplastic cells in the studied sample, while methods such as PNA-LNA (peptide nucleic acid-locked nucleic acid) PCR clamp remain effective with only 1% of neoplastic cells present. The ability to detect mutations in samples containing a low percentage of neoplastic cells is vital due to the great diversity of diagnostic tests being performed on NSCLC patients. The most credible object of analysis is obviously the postoperative tissue material. However, in the case of patients suffering from advanced NSCLC, the examination must often be limited to scant tissue samples from endobronchial biopsy and to cytological material obtained from transbronchial biopsy from the tumor or from metastatic lymph nodes [by means of EBUS-TBNA (endobronchial ultrasound-transbronchial needle aspiration) and EUS-TBNA (endoscopic ultrasound-transbronchial needle aspiration)], from transbronchial biopsy, biopsy from metastatic lymph nodes or distant metastases, from pleural fluid, bronchoaspirate, or from brush biopsy [11-15].

The low percentage of neoplastic cells in the examined samples is not the only issue at hand; another is the quality of the DNA which can be isolated from various materials. The process of paraffinization and deparaffinization the tissue sample makes the genetic material vulnerable to chemical and thermic factors. In the case of cytological preparations, the impact of these factors on cells may be less significant [9, 10]. The matter is complicated further by the diversity of methods employed by molecular biologists for diagnosing mutations in the *EGFR* (NSCLC) and *KRAS* genes (colorectal cancer). Although many facilities use validated molecular techniques or commercial tests and equipment with CE IVD (*in vitro* diagnostics) certification, even such tests employ various methods of isolating the DNA (in a magnetic field or by binding the DNA to siliceous deposits) and diverse ways of detecting mutations (most employ the real-time PCR method with the use of different molecular probes). It should be noted that Poland lacks proper regulation providing adequate rules for conducting molecular research.

Aim of the study

The aim of the present study is to describe the methods for molecular diagnostics of activating mutations in *EGFR* gene in NSCLC patients from the voivodeships of Greater Poland and Lublin. Detailed examination was conducted concerning the type of the material from which the DNA was isolated and the frequency of occurrence of the two most common *EGFR*-activating mutations (deletion in exon 19 and L858R substitution in exon 21) in relation to age, sex, type of material, and pathomorphological diagnosis.

Material and methods

The study encompassed 460 patients suffering from non-small cell lung cancer, diagnosed for the presence of a mutation in the *EGFR* gene between May 2011 and August 2012. The patients came from the voivodeships of Lublin (209 patients) and Greater Poland (251 patients). After obtaining their pathomorphological diagnosis, the patients were qualified for molecular tests by oncologists or pulmonologists. The group consisted of 149 women and 311 men. Their median age was 63 years (mean: 63 ±9 years). Detailed characteristics of the patients, including the pathomorphological diagnosis of NSCLC, are provided in Table I. It should be noted that the majority of the patients were diagnosed with pulmonary adenocarcinoma (281 patients: 61%), but for as many as 23% of them (n = 105) the NSCLC subtype was not diagnosed (NSCLC NOS).

Samples of the examined DNA were isolated from fresh samples (22 patients, operative material after macrodissection performed by a pathomorphologist, pleural fluid) used a DNA Blood Mini Kit (Qiagen, Canada). The DNA from paraffin neoplastic tissue or cytoblock samples (373 patients) was isolated using a QIAamp DNA FFPE Tissue kit (Qiagen, Canada). After being incubated in xylene, the cytological preparations (65 patients) were recovered mechanically. Subsequently, the DNA was isolated using the QIAamp DNA FFPE Tissue kit (Qiagen, Canada). The purity and concentra-

Tab. I. Characteristics of the study group

Sex	Pathomorphological diagnosis	Subgroup numbers
Women (n = 149) (median age: 62 years; mean ± SD: 63 ±9 years)	adenocarcinoma	99 (67%)
	large cell carcinoma	11 (7%)
	NSCLC NOS	29 (20%)
	squamous carcinoma	2 (1%)
	adenosquamous carcinoma	2 (1%)
	lung tumor – no diagnosis	6 (4%)
Men (n = 311) (median age: 63; mean ± SD: 64 ±8 years)	adenocarcinoma	182 (59%)
	large cell carcinoma	16 (5%)
	NSCLC NOS	76 (24%)
	squamous carcinoma	13 (4%)
	adenosquamous carcinoma	2 (1%)
	lung tumor – no diagnosis	22 (7%)

NSCLC NOS – non-small cell lung cancer not otherwise specified

tion of the isolated DNA was evaluated using a BioPhotometer Plus spectrophotometer (Eppendorf, Germany).

The presence of exon 19 deletions was examined using the PCR technique and the amplified PCR product fragment length analysis. In order to evaluate the L858R substitutions in exon 21, the ASP-PCR (allele-specific PCR) technique was used along with two pairs of PCR primers: a complementary primer for the nucleotide sequence in the non-mutated allele of the *EGFR* gene and a complementary primer for the nucleotide sequence in the mutated allele of the *EGFR* gene. The ASP-PCR was performed using two test tubes and PCR primer marked with Cy5 fluorochrome. The results reading was conducted on an ALF Express II sequencer by means of DNA fragment length analysis. The positive control consisted of DNA isolated from adenocarcinoma cell lines with confirmed mutation in exon 19 (cell line H1650) and in exon 21 (cell line H1975). The negative control consisted of DNA isolated from peripheral leukocytes obtained from healthy persons.

As the presence of deletions in exon 19 of the *EGFR* gene was analyzed, the PCR product was presented as a peak of 207 base pairs (in case of no mutation) or with additional shorter peak of 9, 12, 15, 18 or 24 base pairs in the occurrence of appropriate DNA fragment deletions in mutated material (deletion of only 15 base pairs was detected). Each time the presence of L858R substitutions in exon 21 was analyzed, there appeared a peak in the PCR with a complementary primer for the wild gene (heterozygosity, tissue heterogenicity). In the cases where the presence of a mutation in exon 21 of the *EGFR* gene was detected, there appeared an additional peak in the PCR with a complementary primer for the mutated DNA allele.

The sensitivity of the method was assessed through validation tests conducted by the Maria Skłodowska-Curie Institute of Oncology in Warsaw [16] and by the European Molecular Quality Network (EMQN). Mutations in the *EGFR* gene were analyzed in those cases in which the presence of at least 10% of neoplastic cells was detected.

The study was granted the approval of the bioethics committee of the Medical University of Lublin (decision no. KE-0254/131/2011).

The statistical analysis employed descriptive statistics, which allowed for the calculation of medians, means and standard deviations. The χ^2 test and the parametric Student *t*-test were also used for dependent variables.

Results

There were 48 activating mutations detected in the *EGFR* gene, including 25 deletions of 15 base pairs (ΔA746-E750) in exon 19 (52.1% of detected mutations) and 23 L858R substitutions in exon 21 (47.9% of detected mutations). The *EGFR* genes mutations were, therefore, diagnosed in 10.5% of patients. The median time between the reception of the material and the acquisition of the molecular test results was 7 days, including holidays (mean: 6.6 ±3.2 days).

The analyzed *EGFR* gene mutations occurred slightly more often in women than in men. The incidence of the mutations was not dependent on the age of the diagnosed patients. Mutations were detected insignificantly more often in the material acquired through operation (12.36%) than in the material from fine needle and endobronchial biopsies (8.8%). *EGFR* gene mutations were also insignificantly more frequent (12%) in adenocarcinoma patients than in patients with NSCLC NOS (7.5%) or large cell carcinoma (7%). It is, however, necessary to point out that in the small group of 15 squamous carcinoma patients, L858R substitution was detected in one patient only, while *EGFR* gene deletion in exon 19 was found in two out of four examined adenosquamous carcinoma patients (Table II).

The quality of the postoperative material acquired from paraffin blocks (established as the percentage and number of neoplastic cells) was significantly higher than the quality of the material acquired through biopsy ($\chi^2 = 10.77$; $p = 0.001$). The type of biopsy performed did not have significant influence on the quality of the material. However, in the case of forceps endobronchial biopsy, 40% of the acquired material exhibited a low percentage of neoplastic cells or an extremely low number of such cells. The median concentration of the DNA isolated from all the patients was 54 ng/µl (mean: 66.1 ±56.9 ng/µl). The concentration of the DNA isolated from the paraffin postoperative material (median: 60 ng/µl; mean: 73.8 ±54.1 ng/µl) was significantly higher ($p = 0.0002$) than the concentration of the DNA isolated from cytological or paraffin material acquired through biopsy (median: 38.3 ng/µl; mean: 53.1 ±53.4 ng/µl). The type of the material had no significant influence ($\chi^2 = 1.95$; $p = 0.163$) on the quality of DNA amplification in the PCR reaction. However, also in this case adequate DNA amplification was more often successfully acquired from postoperative material isolated from paraffin blocks than from biopsy material (Table III). Seven percent of the results were considered non-diagnostic.

Discussion

The introduction of EGFR tyrosine kinase inhibitors (gefitinib, erlotinib) to NSCLC patient therapy necessitated the extension of lung cancer diagnostics with molecular *EGFR* gene mutation diagnostics. In some EU countries (until recently in Poland as well) there existed certain premises to conduct such diagnostics for all patients in stage IIIB or IV as well as for those of good (PS = 0-1) and fairly good (PS = 2) performance status with diagnosed adenocarcinoma, adenosquamous carcinoma (with the predominance of adenocarcinoma), large cell carcinoma and NSCLC NOS. The relatively frequent occurrence of *EGFR* gene mutations in NSCLC patients with a pathomorphological diagnosis other than squamous carcinoma (10% of Caucasian patients) and the proven effectiveness of EGFR TKI in groups of patients with *EGFR* gene mutations justifies such a method of qualifying for molecular analysis [11-15]. Detecting a mutation in the *EGFR* gene allows for the use of EGFR TKI in monotherapy in lines of treatment I and II, which ensures a response to the treatment in nearly 70% of patients and extends the time free from disease progression

Tab. II. The influence of sex, age, type of material, and pathomorphological diagnosis on the possibility of detecting mutations in the *EGFR* gene

		EGFR gene wild type	*EGFR* gene mutation	*p* χ^2	Deletion of *EGFR* gene in exon 19	L858R substitution of *EGFR* gene in exon 21
All patients	*n* = 460	412 (89.5%)	48 (10.5%)		25 (5.5%)	23 (5%)
Sex	women (*n* = 149; 32%)	128 (86%)	21 (14%)	0.1065 2.605	8 (5%)	13 (9%)
	men (*n* = 311; 68%)	284 (91%)	27 (9%)		17 (5.5%)	10 (3.5%)
Age	median	63 years	63 years		63 years	62 years
	mean ± SD	63 ±8 years	63 ±8 years		64 ±8 years	63 ±9 years
	≥ 63 years (*n* = 223; 48%)	197 (88%)	26 (12%)	0.4961 0.463	15 (7%)	11 (5%)
	< 63 years (*n* = 237; 52%)	215 (91%)	22 (9%)		10 (4%)	12 (5%)
Type of material (*n* = 412)	postoperative material (paraffin blocks) (*n* = 186; 45%)	163 (88%)	23 (12%)	0.2469 1.341	14 (7.5%)	9 (4.5%)
	biopsy material (paraffin blocks, cyto-blocks, cytological preparations) (*n* = 226; 55%)	207 (92%)	19 (8%)		8 (3.5%)	11 (4.5%)
Type of material (*n* = 460)	material from primary tumor surgery (paraffin block) (*n* = 126; 27%)	110 (87%)	16 (13%)		10 (8%)	6 (5%)
	material from metastatic tumor surgery (paraffin block) (*n* = 33; 7%)	28 (85%)	5 (15%)		3 (9%)	2 (6%)
	postoperative material from metastatic lymph node (paraffin block) (*n* = 27; 6%)	25 (93%)	2 (7%)		1 (3.5%)	1 (3.5%)
	fresh frozen material (*n* = 22; 5%)	21 (95%)	1 (5%)		1 (5%)	0 (0%)
	endobronchial biopsy (paraffin block) (*n* = 40; 9%)	37 (92.5%)	3 (7.5%)		1 (2.5%)	2 (5%)
	transthoracic biopsy (paraffin block) (*n* = 37; 8%)	34 (92%)	3 (8%)		1 (3%)	2 (5%)
	transthoracic biopsy (cytological preparation) (*n* = 17; 4%)	16 (94%)	1 (6%)		0 (0%)	1 (6%)
	EBUS-TBNA (cytological preparation or cytoblock) (*n* = 22; 5%)	20 (91%)	2 (9%)		2 (9%)	0 (0%)
	lesion biopsy (paraffin block) (*n* = 48; 10%)	41 (85%)	7 (15%)		3 (6%)	4 (8%)
	other cytological material (cytological preparation) (*n* = 40; 9%)	38 (95%)	2 (5%)		0 (0%)	2 (5%)
	no information about place of material collection (paraffin block) (*n* = 48; 10%)	42 (88%)	6 (12%)		3 (6%)	3 (6%)
Pathomorphological diagnosis	adenocarcinoma (*n* = 281; 61%)	246 (88%)	35 (12%)		17 (6%)	18 (6%)
	large cell carcinoma (*n* = 27; 6%)	25 (93%)	2 (7%)		1 (3.5%)	1 (3.5%)
	NSCLC NOS (*n* = 105; 23%)	97 (92%)	8 (8%)		4 (4%)	4 (4%)
	squamous carcinoma (*n* = 15; 3%)	14 (93%)	1 (7%)		0 (0%)	1 (7%)
	adenosquamous carcinoma (*n* = 4; 1%)	2 (50%)	2 (50%)		2 (50%)	0 (0%)
	lung tumor – no diagnosis (*n* = 28; 6%)	28 (100%)	0 (0%)		0 (0%)	0 (0%)
	adenocarcinoma (*n* = 281; 61%)	246 (88%)	35 (12%)	0.2452 1.351	17 (6%)	18 (6%)
	NSCLC NOS (*n* = 105; 23%)	97 (92%)	8 (8%)		4 (4%)	4 (4%)
	adenocarcinoma (*n* = 281; 61%)	246 (88%)	35 (12%)	0.645 0.212	17 (6%)	18 (6%)
	large cell carcinoma (*n* = 27; 6%)	25 (93%)	2 (7%)		1 (4%)	1 (4%)
	adenocarcinoma (*n* = 281; 61%)	246 (88%)	35 (12%)	0.2926 1.108	17 (6%)	18 (6%)
	other NSCLC pathomorphological diagnosis (*n* = 151; 33%)	138 (91%)	13 (9%)		7 (5%)	6 (4%)

EBUS-TBNA – endobronchial ultrasound-transbronchial needle aspiration, NSCLC NOS – non-small cell lung cancer not otherwise specified

Tab. III. The influence of the type of material used for molecular analysis of mutations in the *EGFR* gene on the percentage of neoplastic cells, their number, DNA concentration and the quality of DNA amplification

Type of material	Sufficient (over 20% of neoplastic cells, extremely low number of cells) (*n*; %)	Scant (10-20% of neoplastic cells) (*n*; %)	Median (mean ± SD) of isolated DNA concentration (ng/µl)	Good DNA amplification (*n*; %)	Weak DNA amplification (*n*; %)
material from primary tumor surgery (paraffin block)	108; 86%	18; 14%	65.3 (73.8 ±42.9)	100; 79%	26; 21%
material from metastatic tumor surgery (paraffin block)	29; 88%	4; 12%	72 (86.4 ±78.2)	28; 85%	5; 15%
postoperative material from metastatic lymph node (paraffin block)	24; 88%	3; 12%	45.4 (66.7 ±67.2)	25; 92%	2; 8%
fresh frozen material	22; 100%	0; 0%	78.4 (100.8 ±90.4)	22; 100%	0; 0%
endobronchial biopsy (paraffin block)	24; 60%	16; 40%	38.4 (48.9 ±40.8)	25; 63%	15; 37%
transthoracic biopsy (paraffin block)	26; 70%	11; 30%	26 (46.8 ±45.5)	27; 73%	10; 27%
transthoracic biopsy (cytological preparation)	11; 67%	6; 33%	21.2 (34.9 ±37.1)	10; 56%	7; 44%
EBUS-TBNA (cytological preparation or cytoblock)	15; 68%	7; 32%	38.5 (48.4 ±31.7)	19; 86%	3; 14%
lesion biopsy (paraffin block)	42; 88%	6; 12%	47.55 (65.5 ±75.5)	42; 88%	6; 12%
other cytological material (cytological preparation)	30; 75%	10; 25%	41.45 (55.9 ±51.4)	32; 80%	8; 20%

EBUS-TBNA – endobronchial ultrasound-transbronchial needle aspiration

to about 10 months, providing a significant improvement of the quality of life and a different profile of undesired effects than in the case of chemotherapy [17].

Until recently, the scope and method of conducting molecular diagnostics of *EGFR* gene mutations had not been sufficiently researched in Poland. Approximate calculations, based on epidemiological data from other EU countries, suggest that in Poland NSCLC constitutes more than 80% of all lung cancer cases. Among these NSCLC patients, 60% suffer from non-squamous carcinoma. On the basis of the data from the National Cancer Registry, it is possible to estimate that in 2009 about 8,200 people developed non-squamous carcinoma, including 5,900 men and 2,300 women. In the Greater Poland Voivodeship, these figures were as follows: about 680 patients, including 500 men and 180 women; and in the Lublin Voivodeship: 430 patients, including 320 men and 110 women [1]. When transposing the epidemiological data from 2009 to our data concerning the number of non-squamous lung carcinoma patients undergoing molecular tests between the first half of 2011 and the second half of 2012, it is possible to estimate very approximately that our diagnostics covered about 40% of patients from both analyzed geographical regions of Poland. Molecular diagnostics was conducted more often on women (50% of patients) than on men (37% of patients). The molecular diagnostics encompassed about 45% of non-squamous NSCLC patients in the Lublin Voivodeship, while in Greater Poland this percentage amounted to 35%. In the Lublin Voivodeship, 60% of women and 45% of men underwent the diagnostic procedure, in Greater Poland over 40% of women and over 30% of men.

It is important to remember that the data are highly approximate in nature and can only be treated as such. The calculation error is influenced not only by the obvious inaccuracy resulting from comparing the data from 2009 and 2011-2012, but also by the way in which molecular diagnostics is performed in Poland. Molecular analyses from various clinical centers within the area of a single voivodeship may be performed in different genetic laboratories in Poland on the basis of contracts between hospitals, pathomorphology and molecular biology centers. Moreover, many patients undergoing surgery because of early stage NSCLC do not qualify for molecular tests and, possibly, they should not be included in such calculations. Since July 2012, the method of qualifying a patient for a molecular test has changed due to the implementation of new prescription drug programs, according to which only adenocarcinoma patients can be treated using EGFR TKI. Therefore, molecular tests are no longer performed on patients with diagnosed NSCLC NOS and large cell carcinoma, even though our data show that 7.5% of such patients have an activating mutation in the *EGFR* gene. What is more, 23% of the patients in our material were diagnosed with NSCLC NOS, 6% with large cell carcinoma. Thus, in view of the current legislation, 1/3 of patients have no access to molecular diagnostics and EGFR TKI treatment.

European institutions: IASLC, European Society for Medical Oncology (ESMO), European Thoracic Oncology Plat-

form (ETOP), Austrian Society of Pathology, Spanish Society of Medical Oncology (SEOM), Spanish Society of Pathology (SEAP) recommend molecular diagnostics of the *EGFR* gene for all adenocarcinoma patients. The results of the molecular test should be received no later than within 5-7 working days. However, patients suffering from other forms of NSCLC (including squamous carcinoma), except for neuroendocrine and carcinoid tumors, should be examined for the presence of *EGFR* gene mutations after receiving a referral from an oncologist (especially non-smoking patients). There are no guidelines concerning the place from which the material for tagging is taken. The majority of data describing the incidence of *EGFR* gene mutations refer to the analyses of mutations in primary NSCLC tumors. However, taking into consideration the heterogeneity of NSCLC tumors, it seems advisable to analyze both primary and metastatic neoplastic changes [11-15].

European recommendations emphasize that the material for a molecular test should contain 5-20% neoplastic cells (depending on the molecular biology method used for mutation analysis) and the number of these cells should not be lower than 200-400. The recommended material for this type of diagnostics is, therefore, a sample taken from a tumor during surgery and stored in the form of paraffin blocks. However, molecular diagnostics can also be performed on material obtained through biopsy; the isolation of proper DNA from cytological preparations is also successful in the majority of cases. Moreover, the low percentage of neoplastic cells in biopsy preparations excludes the use of direct sequencing, which has to be substituted by ARMS-PCR, amplified DNA fragment length analysis or DHPLC (denaturing high-performance liquid chromatography). In case of doubt as to the presence of a mutation, two different methods are employed [11-15]. Controversy arises when it comes to the use of microdissection of neoplastic cells in order to increase their percentage. Laser radiation used for microdissection may cause serious damage to the DNA of neoplastic cells, resulting in its fragmentation and reducing the accuracy of the results of *EGFR* gene mutation tests.

Our results confirm that postoperative paraffin material is adequate for molecular tests in the highest degree. More than 85% of such material contained a high percentage of neoplastic cells. The DNA isolated from these cells allowed for the proper amplification of selected fragments of the *EGFR* gene. The isolation of neoplastic DNA from biopsy material was more difficult and proved to be successful for only 60-88% of patients. The lowest percentage of patients for whom the acquired material was fully reliable was found in the groups of patients diagnosed by means of EBUS-TBNA (68%), transthoracic biopsy (67%) and endobronchial biopsy performed during bronchoscopy (60%). The amount and quality of the DNA acquired from cytological preparations and paraffin blocks of biopsy materials were similar.

Summing up, it should be noted that, despite the approximate nature of the data, less than half of all non-squamous NSCLC patients in the Lublin and Greater Poland voivodeships underwent *EGFR* gene molecular diagnostics. These patients, according to the recommendations binding until July 2012, should have been covered by the EGFR TKI therapy qualifying process. The restriction of molecular tests to pulmonary adenocarcinoma patients only, included in the currently binding prescription drug programs, prevents the use of EGFR TKI in almost 30% of patients with *EGFR* gene mutations diagnosed with non-squamous lung cancer in the analyzed regions. The results that we obtained could be taken into consideration during the creation of new prescription drug programs. They also point to the necessity of creating detailed qualifying procedures for *EGFR* gene mutation tagging. In order to maximize the efficiency of *EGFR* gene molecular diagnostics, it is necessary to use those methods of acquiring material for pathomorphological analyses which provide a high percentage and number of neoplastic cells and abandon those procedures which should rather be used to qualify early stage NSCLC patients for surgery. The use of low sensitivity molecular biology methods (direct sequencing) makes it impossible to perform *EGFR* gene molecular diagnostics in more than 30% of patients because of the low percentage of neoplastic cells. Therefore, these patients require the use of sensitive PCR techniques, such as ARMS-PCR, ASP-PCR, amplified DNA fragment length analysis or PNA-LNA PCR clamp.

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