Original Article
EGFR mutation testing on cytological and histological samples in non-small cell lung cancer: a Polish, single institution study and systematic review of European incidence

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Abstract: The targeted treatment of advanced non-small-cell lung cancer (NSCLC) depends on confirmation of activating somatic EGFR mutation. The aim of the study was to evaluate the incidence of EGFR mutations in NSCLC detected in cytological and histological material and present literature review on European EGFR mutation incidence. 273 patients with confirmed NSCLC were entered into the study: 189 histological, paraffin-embedded materials, 12 fresh and 72 fixed cytological specimens. DNA was extracted from both types of material and the EGFR mutation in exons 18-21 was analyzed by direct sequencing. In addition the EGFR gene copy number in cases with sufficient histological material (110 patients) was evaluated by fluorescent in situ hybridization (FISH) technique. The percentage of EGFR somatic mutations was 10.62%. FISH positive results (amplification or high polysomy of EGFR gene) were identified in 33 patients (30.0%). The strongest clinicopathological correlation with the EGFR mutation was found for histological type (adenocarcinoma; p < 0.01), gender (females; p < 0.01) and FISH positive result (p < 0.05). This is the first, single institution study that estimates the EGFR mutation incidence in the Polish population. Cytological material recovered from fixed preparations and stained with hematoxylin and eosin showed DNA quality comparable to fresh tumor cells and histological samples.

Keywords: EGFR mutation, non-small-cell lung cancer, cytology, EGFR amplification

Introduction

Identification of *EGFR* gene mutations in the non-small-cell lung cancer (NSCLC) fully illustrates the impact of molecular biology in treatment decisions. The use of one of the small-molecule tyrosine kinase inhibitors (TKI), gefitinib or erlotinib, requires confirmation of somatic activating *EGFR* mutation. Both drugs belong to the group of reversible, competitive inhibitors that block the binding of ATP to the active site of the EGFR kinase [1]. The predictive value of clinicopathologic factors (Asian race, female sex, never-smoking status, adenocarcinoma histological subtype) directly correlates with the increased percentage of *EGFR* mutations.

Recent phase III clinical trials - EURTAC [2], OPTIMAL [3] , WJTOG3405 [4] and NEJ002 [5] - recruited patients with advanced NSCLC and the presence of *EGFR* activating mutations; differences were noted in the percentage of response rates (RR) as well as the duration of progression-free (PFS) and overall survival (OS). Currently, the *EGFR* mutation status still remains the strongest predictor of TKI treatment response. Recent guidelines of College of American Pathologists (CAP), International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) strongly recommend detection of *EGFR* mutations - with no particular method suggested - in all newly diagnosed advanced NSCLC patients [6]. A significant correlation between

polysomy or amplification and *EGFR* gene mutation was also reported. An increase in gene copy number has minor importance in predicting TKI response rate and is thought to be secondary phenomenon to *EGFR* mutation. Current recommendations note that further research is needed to refine the potential impact of the number of mutated copies of the *EGFR* gene as the factor modifying prospective benefits of TKI treatment. However, fluorescent in situ hybridization (FISH) is not currently accepted as an optimal method of qualification for anti-EGFR therapy.

More representative for the sample is histological material although its availability is limited. Advanced clinical stage at presentation considerably reduces surgical treatment of NSCLC. In European countries, the percentage of resectable lung cancer is up to 20%; in Poland, according to the National Lung Cancer Registry data, not more than 17% of NSCLC are resected [7] and for other patients the only source of malignant cells are histological small sample or cytological material. Availability of sufficient diagnostic material is a problem in all populations, so the latest recommendations emphasize the necessity of standardization in small sample and cytological material. Appro-priate management requires not only specification of overall pathologic diagnosis supported with immunohistochemical (IHC) staining but also retaining the biological material to further molecular analysis [6]. Personalized medicine in NSCLC takes into account individually planned diagnostic strategies; results of pathologic and molecular examination are crucial in selection of treatment option.

This study is the first presentation of a Polish, single institution results in *EGFR* mutation testing. The collected cytological material on *EGFR* detection is one of the largest series published in Europe. Moreover, gene copy number evaluated in histological samples is also reported. The *EGFR* mutation incidence in European countries is discussed and a review of cytological material application in molecular analysis is presented.

Materials and methods

Patients, samples and procedures for histopathology and immunohistochemistry

Material was obtained from 273 patients (151 male and 122 female; mean age 61.5 years (25-84) with non-small-cell lung cancer diagnosed or verified and treated in the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw in the period December 2009 - March 2011. Regardless of the material type obligatory conditions for *EGFR* mutation testing were: NSCLC confirmed independently by two of three pathologists (*ASC, WTO, MW*), at least 50% of neoplastic cells per sample, availability of at least one cytological specimen, absence of overwhelming technical artefacts (excessive drying or dehydration of the cells). Samples comprised the following materials: formalin-fixed paraffin embedded (FFPE) histological sections, cytological smears fixed in alcohol and fresh cytological material collected simultaneously with fixed preparations. Fixed samples were processed routinely and finally stained with hematoxylin and eosin (HE). If necessary, additional IHC was performed to verify the microscopic diagnosis. The antibody panel, recommended for the diagnosis of adenocarcinoma by the American and European guidelines, was applied: TTF-1 (Thyroid Transcription Factor-1; 8G7G3/1 IR 056; ready to use - RTU), CK7 (Cytokeratin 7; OU-TL12/30, IR 619, RTU), CK20 (Cytokeratin 20; Ks.20.8, IR 777; RTU), p63 (4A4 M 7247; dilution 1:100), Leukocyte common antigen (LCA; 2B11 + PD7/26, IR-751; RTU). EnVision Detection System, Dako, Denmark was used to reveal antibody reactivity. The histological type of NSCLS was diagnosed according to: 2004 WHO classification, 7th edition of the American Joint Committee on Cancer and the latest classification of adenocarcinoma by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society.

Preparation of histological and cytological material for EGFR mutation assessment

Tissue samples from pulmonary resections (pneumectomy, lobectomy, segmentectomy) and small tissue samples obtained during trans-bronchial or trans-thoracic needle biopsy with diminished tumour content were macrodissected to access maximum percentage of neoplastic tumor cells in the corresponding paraffin block and then 5 to 6, "thick" 6 μm sections were deposited in PCR tubes. Material from cytological fixed smears (trans-bronchial or trans-thoracic fine-needle aspirates and bronchial brush cytology) was recovered

according to the following procedure: selected representative smears containing more than 50% tumour cells were placed in xylene to detach the coverslip (2-3 days); subsequently, cellular material was "scrapped" with sterile scalpel into PCR tube carriers. Cytological fresh samples were provided according to computed tomography guided transthoracic fine needle aspirates. One biopsy material was divided: two smears were routinely fixed and the residual material from the needle was injected directly into a PCR tube carrier. The material was stored in a refrigerator at 2-8°C until histopathological confirmation of NSCLC. After 48 hours the samples were transferred to molecular laboratory; *EGFR* mutation testing was performed concurrently from both, fresh and fixed, cytological samples.

EGFR mutation testing

A validation procedure of *EGFR* mutation testing was described previously; for DNA isolation QIAamp ® DNA Mini Kit (Qiagen) was used. Mutation screening of exons 18, 19, 20 and 21 of the *EGFR* gene was performed by Sanger direct sequencing. Each sequencing reaction was performed in both forward and reverse directions and all the electropherograms were analyzed by Mutation Surveyor (Softgenetics) software and visual inspection by highly experienced molecular biologist (AT, JAS).

EGFR gene copy number testing

Gene copy number per cell was investigated only on histological material by FISH. The LSI EGFR Spectrum Orange/CEP7 Spectrum Green probe (Vysis, Abbott Laboratories, Illinois, USA) were used in accordance with manufacturer instructions. FISH signals were evaluated under the fluorescence microscope Olympus BX41 (Olympus, Japan) equipped with single filters: DAPI, SpectrumOrange and FITC as well as triple-filter DAPI/FITC/SpectrumOrange. Images were photographed (camera F-View, Olympus, Japan) and analyzed using the Cell-F software (Olympus, Japan). FISH analysis was independently performed by pathologist and molecular biologist unaware of the clinical and molecular characteristics of patients. A scoring system was adopted from modification of classification introduced by the University of Colorado and at least 40 non-overlapping cell nuclei were examined. FISH positive NSCLC were determined if

amplification (Ratio ≥ 2, ≥ 15 copies of *EGFR* gene in ≥ 10% of the cells, the *EGFR* gene clusters) or high *EGFR* polysomy (≥ 4 copies of the *EGFR* gene in ≥ 40% of cells) were identified.

Statistical analysis

The following statistical methods were applied: descriptive statistics, statistical tests for frequency tables and multi-way tables. Qualitative variables were analyzed by the Chi2 or Fisher's exact tests; for continuous variables with normal distribution we used Student test , and for continuous variables with abnormal distribution, Mann-Whitney and Kruskal-Wallis non-parametric tests. Two-sided $p < 0.05$ were found statistically significant. Statistical analyses were performed using Statistica 7.0 (StatSoft Inc., USA). All photographs were taken using the microscope camera DP72 Olympus BX63 (Olympus, Japan). The final graphic illustrations were prepared in MS Office 2003 applications.

Results

Histological samples were the predominant type of material (189, 69.2%), followed by fixed cytological smears (72, 26.4%) and fresh cytological cells (12, 4.4%). The percentage of samples with non-satisfactory quality of extracted DNA was 4.7% and 1.2% for histological and cytological material respectively; inadequate DNA probes were identified in 3.6% of cases. Three types of *EGFR* mutation (L858R, E746_A750 and deletion in exon 19) were found in fresh material; the results were confirmed simultaneously on fixed cytological smears derived from the same patients. There were no discrepancies between the results of *EGFR* testing from fixed and fresh cytological material; the percentage of compliance estimated 100%. The majority of NSCLC were adenocarcinomas (68.1%), squamous cell carcinomas (16.1%) and NSCLC not-otherwise specified (11%); few cases of adenosquamous carcinoma (1.5%) and large cell carcinoma (3.3%) were also diagnosed (**Figure 1A-D**). The characteristics of patients with results of *EGFR* mutation status are presented in **Table 1**.

EGFR mutations were detected in 29 patients (10.6%) but the total number of mutations was 31 as two of the patients had more than one abnormality: T790M (exon 20) and L858R
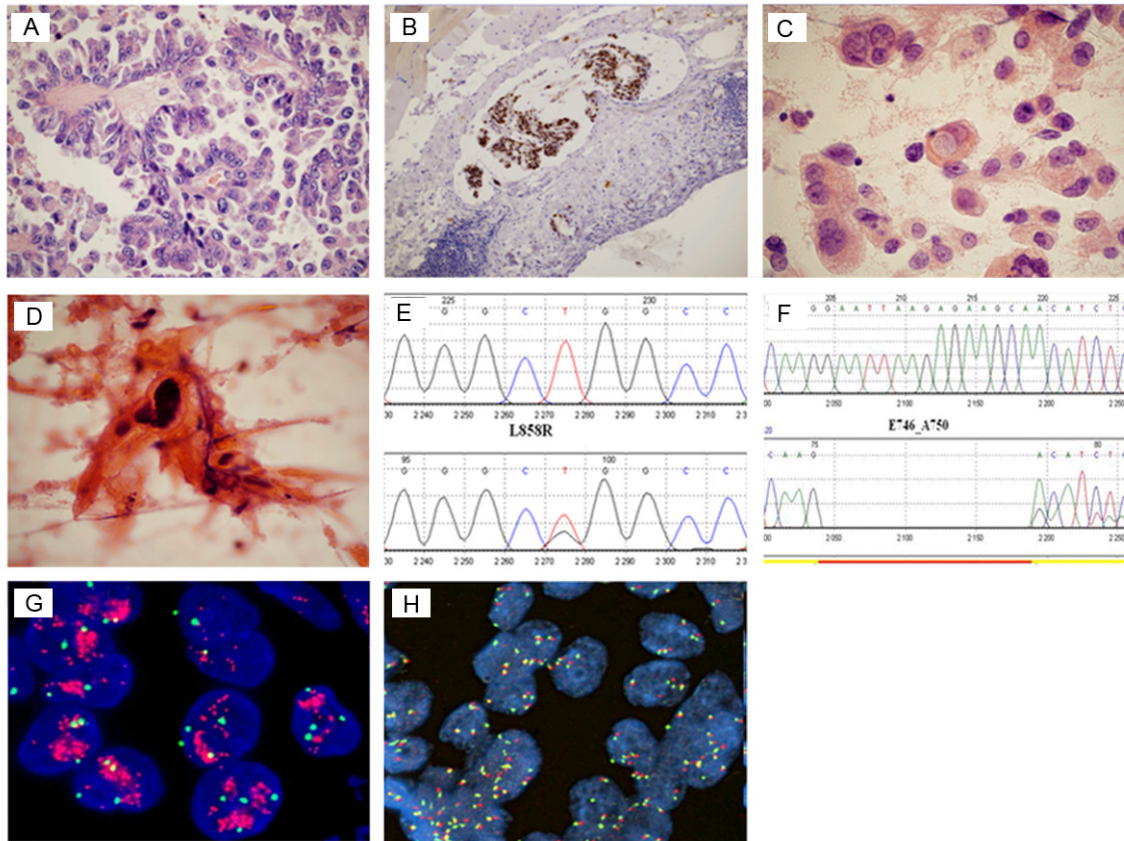
Figure 1. Images of the most frequent histological and molecular results [A: Adenocarcinoma, papillary subtype (HE, 400 x), B: Embolism of adenocarcinoma cells in the lymphatic vessel (TTF-1, 100 x), C: Adenocarcinoma with visible droplets of intracytoplasmic mucus (HE, 400 x), D: Squamous cell carcinoma (HE, 1000 x), E, F: Graphical illustration of *EGFR* gene mutations: exon 21 substitution (L858R) and exon 19 deletion (E746_A750) respectively, G, H: FISH positive results: amplification (1000 x) and high polysomy (600 x)].

(exon 21), G719C (exon 18) and S768I (exon 20). The most common were deletions in exon 19 (17/31, 55%) (**Figure 1F**) and mutations of substitutions in exon 21 (10/31, 32%) (**Figure 1E**); 3 mutations in exon 20 (GG779F, S768I, T790M) and one in exon 18 (G719C) were also found. The contribution of *EGFR* mutation types was depicted on **Figure 2**.

In 110/189 (58.2%) of patients with available histological material *EGFR* gene copy number assessment was performed. FISH positive [amplification (**Figure 1G**) or high polysomy gene (**Figure 1H**)] NSCLC were identified in 33 patients (30.0%). The percentage of non-diagnostic samples was 9.1% (10/110). FISH positive results were found in all histological types with predominance of the adenocarcinoma (20, 18.2%) and squamous cell carcinoma (7, 6.4%).

The strongest clinicopathologic relationship with *EGFR* mutation was found for female gen-der (p < 0.01) and histologic type (adenocarcinoma *vs.* other histological types, 89.8% *vs.* 10.2%, p < 0.01). The influence of gender, FISH result and histological type on the *EGFR* mutation incidence was analyzed; the most significant increase of *EGFR* mutation was found in female group with FISH positive adenocarcinoma (**Figure 3**).

Discussion

Prospective comparisons have demonstrated significant advantage of *EGFR* TKIs over cytotoxic chemotherapy in terms of antitumor activity and quality of life in selected NSCLC patients. Ethnic differences play some role in the incidence and prognosis of lung cancer. Data analysis from two randomized phase II trials: IDEAL -1 involving 210 patients from Europe, Australia, South Africa, Japan, and IDEAL -2 including 216 patients from the United States, revealed higher overall response rate in the subgroups of

Table 1. Clinicopathologic characteristics of patients including the result of *EGFR* mutation testing

Characteristics	All patients	Patients with *EGFR* mutation	Patients with *EGFR* wild type
Subjects	273	29 (10.6%)	244 (89.4%)
Sex			
Male	151 (55.3%)	7 (24.2%)	144 (59%)
Female	122 (44.7%)	22 (75.8%)	100 (41%)
Age (years; range)	61.5 (25-84)	63.5 (40-77)	61.2 (25-84)
Male	61.9 (25-84)	62.9 (53-77)	61.9 (25-84)
Female	60.9 (37-82)	63.8 (40-77)	60.3 (37-82)
Type of material			
Histological	189 (69.2%)	18 (62.1%)	171 (70.1%)
Cytological	84 (30.8%)	11 (37.9%)	73 (29.9%)
[fixed/fresh]	[72 (26.4%)/12 (4.4%)]	[8 (27.6%)/3 (10.3%)]	[64 (26.2%)/9 (3.7%)]
Histological type			
Adenocarcinoma	186 (68.1%)	26 (89.8%)	160 (65.6%)
Squamous cell carcinoma	44 (16.1%)	2 (6.8%)	42 (17.2%)
Adenosquamous	4 (1.5%)	0	4 (1.6%)
Large cell carcinoma	9 (3.3%)	0	9 (3.7%)
NSCLC, NOS[*]	30 (11%)	1 (3.4%)	29 (11.9%)
EGFR gene copy number[#]	110	6	104
FISH positive	33 (30%)	4 (66.6%)	29 (27.8%)
FISH negative	77 (70%)	2 (33.4%)	75 (72.2%)

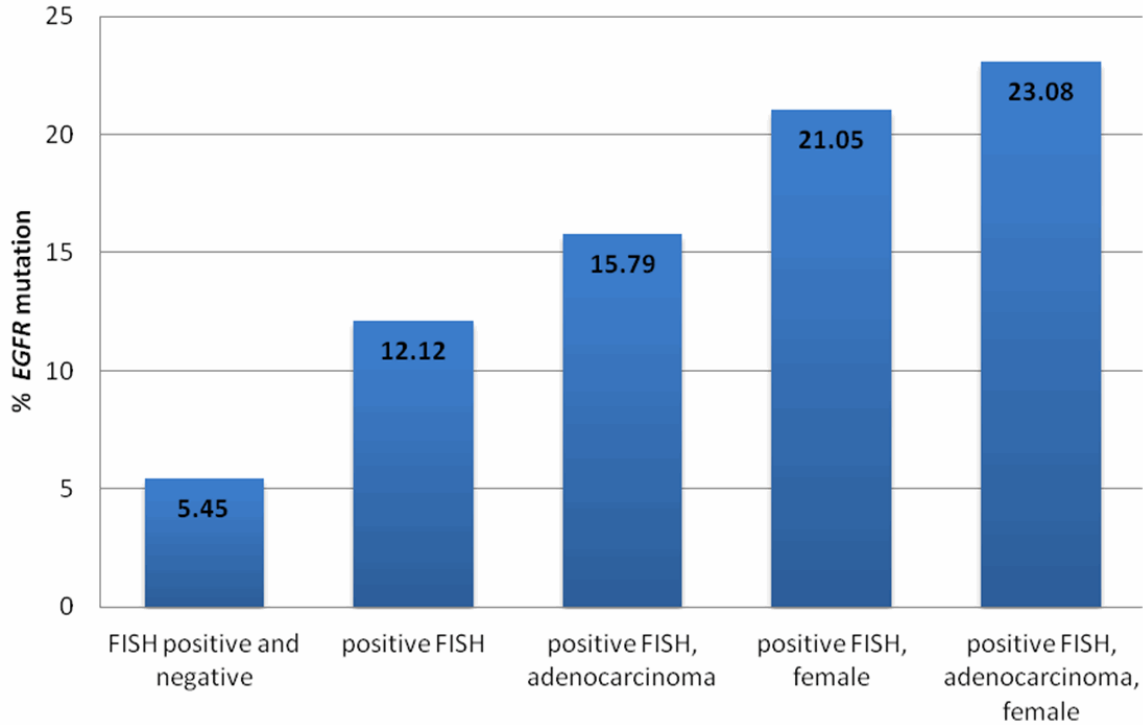[*]Not-otherwise specified. [#]Available only for histological samples.
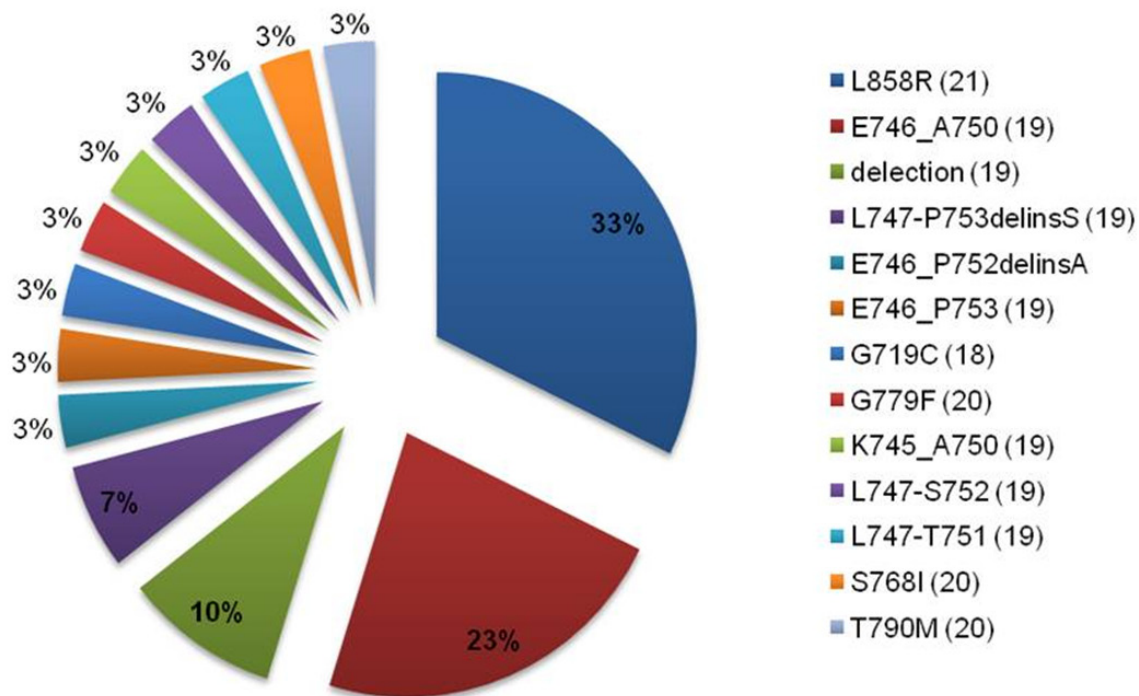
Figure 2. The contribution of *EGFR* mutations types (in brackets: exon).
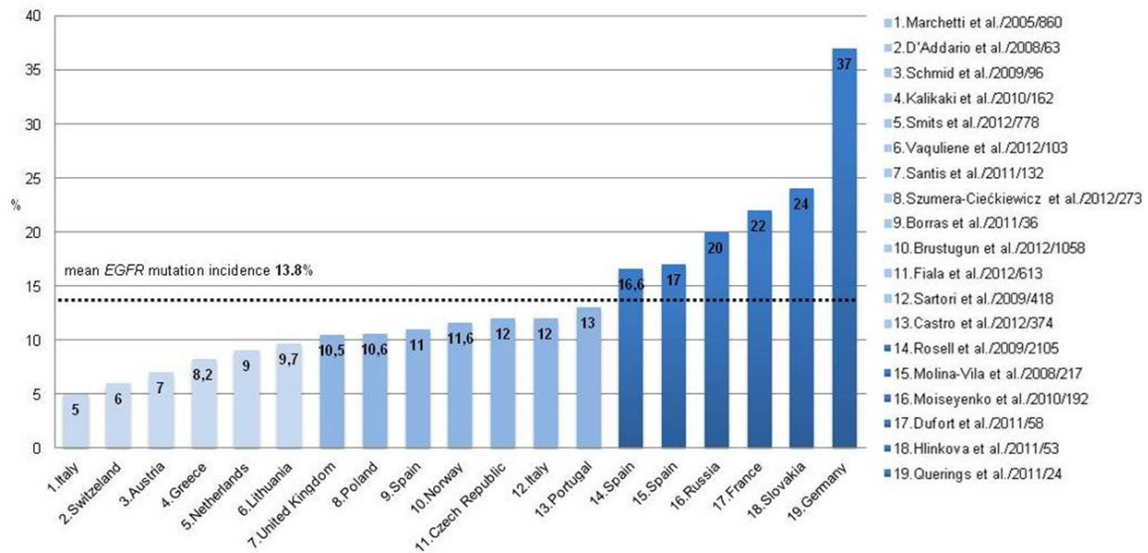
never-smokers, females and patients with adenocarcinoma. Moreover, for Japanese patients RR was 28%, while in the other groups it did not exceed 9-12% [8, 9]. Randomized phase III

Figure 3. The influence of gender, FISH result and histologic type on *EGFR* mutation increase.

Figure 4. Frequency of EGFR gene mutations in NSCLC - European countries [listed: first author/year of publication/ number of tested cases; based on literature review].

study ISEL (gefitinib *vs.* placebo) enrolled 1692 patients with NSCLC previously treated with one or two chemotherapy regimens and has not confirmed survival improvement in patients treated with gefitinib (median survival times for gefitinib and placebo were respectively 5.6 and 5.1 months, HR: 0.89, 95% CI: 0.78-1.03). Subgroup analysis revealed a prolonged median survival time for Asian patients receiving gefitinib (9.5 vs. 5.5 months, HR: 0.66, 95% CI: 0.48-0.91) [10]. The relationship between the percentage of objective RR and ethnicity were

also observed in phase II studies on the efficacy of erlotinib monotherapy in patients with advanced NSCLC; RR was 12%, with a median OS 8.4 months [11]. Concurrently published data from Japanese trials conducted in the similarly randomized population, showed more than twice as high objective RR (30/106, 28%) with OS 13.8 months. Among patients who responded to treatment with erlotinib greater benefit was again seen in females (RR 50% vs. 17% for males, p = 0.0009) and non-smokers (RR 51% vs. 14% for active smokers, p < 0.0001) (31, 123). In the phase III trial of erlotinib *vs.* placebo, the higher RR in Asians compared to other nationalities (RR 28% vs. 10%, p = 0.02) was observed [12].

The discovery of *EGFR* activating somatic mutations and their correlation with a significantly better outcome after TKI therapy had great influence on diagnostic procedures and treatment qualification. The discrepancies in *EGFR* mutation incidence are emphasized for various regions of the world; *EGFR* activating mutations are found over 2.5 fold more frequently in Asia than in Europe (35.4% *vs.* 13.8%). For the United States and Australia, the average frequency rates are approximately 18.7% and 22% respectively. Data referring to *EGFR* mutation incidence are available from 16 of 46 European countries [based on Medline search by keywords: *EGFR* mutation and lung cancer and "name of country", to 30th June, 2013]. The lowest percentage (< 10%) is found in the following populations: Switzerland (6%) [13], Austria (7%) [14], Greece (8.2%) [15], Italy (mean 8.5%) [16, 17], Lithuania (9.2%) [18] and the Netherlands (9.71%) [19]. Two Italian studies show significant discrepancies in *EGFR* mutation frequency: Marchetti et al. [16], described 860 patients with NSCLC and 5% of *EGFR* mutation while Sartori et al. [17] observed 12% mutated patients in a group of 418 NSCLC cases. The proportion of *EGFR* mutations in European countries falls within the 10-15% range and refers to the following countries: the United Kingdom (UK) (10.5%) [20], Norway (11.6%) [21], Czech Republic (12%) [22], Portugal (13%) [23]. In Spain the percentage of *EGFR* mutations is estimated at 14-17% [24-26]. Our study is the first Polish - single institution - documentation of the frequency of *EGFR* mutations in NSCLC. It shows that 10.62% of patients harbour *EGRF*-mutated NSCLC. Similar results were achieved in the Netherlands and

UK (10.5%). Three countries: France (24%) [27], Russia (20%) [28, 29] and Slovakia (24%) [29] reported higher mutations' incidence. Moiseyenko and al. [28] from Russia explained this phenomenon by the selection of histological type; the study included only adenocarcinomas, which characterized 2-3 times higher proportion of mutations than in NSCLC evaluated together. Slovakian study by Hlinková et al. [29] showed a significant difference in the percentage of detected mutations according to detection method: High resolution melting analysis confirmed *EGFR* mutation in 13/53 (24.5%) patients, while the direct sequencing in only 5/53 (9, 43%). Most studies indicate that direct sequencing has the lowest sensitivity in mutation detection and requires high content of tumour cells in a sample. The highest frequency of *EGFR*-mutated NSCLC was presented by Querings et al. [30] from Germany. Samples from 24 patients were examined using three different techniques: Sanger direct sequencing, conventional pyrosequencing and massively parallel sequencing (next-generation sequencing); the incidence of *EGFR* mutation depends on the method applied and accounted for 37.5%, 50% and 58.3%, respectively. The authors emphasized that the patients did not fulfil criteria for a representative NSCLC sample. Differences in the frequency of *EGFR* gene mutations in NSCLC, in European countries are presented in **Figure 4**.

In European studies the average number of tested patients was 400 (24-2105). It was significantly higher in Norwegian (1058) and Spanish (2105) studies [21, 26]. In 11/18 studies the number of tested patients did not exceed that of ours with mean number of 103 cases (24-217). Most studies were published in 2011 (Germany, Spain, Slovakia, France, the United Kingdom) and in 2012 (Lithuania, Portugal, Czech Republic, the Netherlands, Norway). The conclusion based on literature review is that the presented material and results are comparable to those from other European countries; both the sample size and standardization of the direct sequencing technique provide adequate basis for assessment of the frequency of mutations in the *EGFR* gene in Polish NSCLC population.

Adenocarcinoma (69%, 186) and NSCLC NOS (11%, 30) were the predominant histological type in this study although squamous cell carci-

Table 2. *EGFR* mutation testing in cytological material

Author Year/Ref.	Population	Subjects	Type of material	Method of *EGFR* mutation detection	% *EGFR* mutation
Fassina 2009/[51]	Italy	77	FC*	HRMA+	2.6
Savic 2008/[44]	Switzerland	84	SAS	DS	5.12
Schuurbiers 2010/[40]	Netherlands	35	SAS, CB	DS	7.4
Garcia-Olive 2010/[52]	Spain	51	CB	RT-PCR	8.57
Boldrini 2007/[42]	Italy	23	SAS	DS	13.04
Molina-Vila 2008/[25]	Spain	76	CB, SAS	TaqMan assay+	17.05
Nakajima 2007/[53]	Japan	43	CB	LH-MSA+	25.6
Tanaka 2007/[54]	Japan	86	FC	PNA-LNA clamp+	34
Horiike 2007/[55]	Japan	94	FFC	Scorpions ARMS+	37
Takano 2007/[56]	Japan	117	SAS/CB	HRMA	41
Shih 2006/[57]	Taiwan	62	CB	DS	47
Lozano 2011/[58]	Spain	120	SAS, FC, LBC, CB	DS	17
Bozzetti 2012/[59]	Italy	39	SAS, FC*, CB	DS	23
Pang 2012/[38]	United Kingdom	147	SAS, CB	DS	41
Smits 2012/[19]	Netherlands	34	SAS	HRMA+	9.1
van Eijk 2011/[60]	Netherlands	43	SAS	Allele-specific qPCR	2.3
Ulivi 2012/[39]	Italy	25	SAS, FC	Pyrosequencing+	12
Hasanovic 2012/[61]	USA	31**	CB	Rapid-polymerase chain reaction-based detection	38
Brustugun 2012/[21]	Norway	80	#	DS	11.3
Smouse 2009/[62]	United Kingdom	18**	CB	DS	39

*Fresh cells obtained by flushing the biopsy needles. **Adenocarcinomas only. #Abstract only (original article in Norwegian). +The results confirmed by direct sequencing. Ref. - reference; FC - fresh cells; SAS - stained archival slides; CB - cell blocks; LBC - liquid based cytology; DS - direct sequencing; HRMA - high resolution melting analysis; RT-PCR - reverse transcription-polymerase chain reaction; LH-MSA - loop-hybrid mobility shift assai; ARMS - amplification-refractory mutation system.

noma cases were also analyzed (16%, 44). The highest *EGFR* mutation rate was for adenocarcinoma group (14%, 26/186), but we also identified two cases in squamous cell carcinoma (4.5%, 2/44). Adenocarcinoma was more frequent in females (70.5% *vs.* 66.2%, p < 0.05) and *EGFR* mutations incidence was higher (17.2% *vs.* 3.97%, p < 0.01). The group with *EGFR* mutation included more than 75% females (22/29) and adenocarcinoma was diagnosed in nearly 90% (26/29). Mutations in exons 21 and 19 together accounted for 87%; in two cases simultaneous presence of both activating (L858R, G719C) and resistance (T790M, S768I) *EGFR* mutations was found. Literature review shows that the simultaneous G719C mutation (exon 18) and S768I (exon 20) is the first described case with in vivo confirmation.

The presence of *EGFR* mutations is essential for qualification to targeted therapy, but alternative predictors are still being sought. The results from clinical trials BR. 21 (erlotinib vs.

placebo) [31] and ISEL [32] (gefitinib *vs.* placebo) suggest that patients with increased *EGFR* gene copy number may have longer survival (BR. 21: HR 0.43, 95% CI 0.23 - 0.78, p < 0.004; ISEL: HR 0.61, 95% CI 0.36 - 1.04, p = 0.067). In contrast, results the SATURN [33] and INTEREST [34] studies did not confirm an increase of survival rates in patients with *EGFR* gene amplification. The only study in which TKI was administrated based on FISH result was a phase II study ONCOBELL [35]. In 25 of 37 patients (69.4%) amplification or high polysomy of the EGFR (FISH positive) were confirmed; in that group RR was significantly higher (68% *vs.* 9.1%, p < 0.001) and PFS longer (7.6 *vs.* 2.7 months, p = 0.02). The CAP/IASLC/AMP recommendations state that the *EGFR* mutation status is the most reliable predictive marker for anti-EGFR treatment in NSCLC, while the number of copies of the gene should be assessed in the framework of the research. In our 110 study cases of NSCLC the *EGFR* gene copy number analysis was performed; positive FISH results

were identified in 30% of patients. The strongest relation was determined between an increased EGFR copy number, adenocarcinoma and gender (female) (**Figure 3**).

Based on the results of the available studies no disparity exists between *EGFR* mutation incidence and the type of tested material. Masago et al [36] compared the percentage of *EGFR* mutations assessed in small biopsy samples and surgical materials derived from the same patients. In 18/19 patients the results were similar; in one case only exon 19 sequencing was performed as the tumour cells content was diminished. The authors underline the high compliance of molecular analysis and recommend testing of all available materials. Bozzetti et al [37] confronted the differences between testing the material from fresh cytological and histological NSCLC specimens. Cytological samples were sufficient for the analysis of the *EGFR* gene mutation in 93% of cases; 100% compliance was achieved between *EGFR* mutation frequency detected in cytological and histological material. Both studies were conducted prospectively on small groups of patients; difficult access of comparative cytological and histological material limits research in this field. Crucial point for *EGFR* detection is not the type of material or method used for collection but the content of cancer cells and DNA quality [17, 38, 39]. Cytological material for assessment of *EGFR* gene mutations can be recovered from: paraffin blocks (cell - blocks), archival smears or fresh tumor cells, rarely from frozen material. The use of archival smears has numerous advantages; molecular testing may be performed without the necessity to repeat diagnostic procedures (bronchofiberscopy, mediastinoscopy) and the costs are lower. It has been shown that the method of staining smears (Papanicolaou, Romanovsky, HE) has no effect on the quality of DNA and PCR reactions [40, 41]. Studies on the usefulness of fixed cytological preparations in the *EGFR* mutation testing confirmed that sufficient DNA quality can be obtained from 92.9% to 100% of archival smears [41-44]; moreover, the results are comparable to formalin fixed paraffin embedded tissues [45-50]. In the present study the *EGFR* gene mutation was examined on cytological fixed and fresh material; the compliance of results was 100%. Medline database review [by keywords: cytology and small sample and lung cancer and *EGFR* mutation, 30th June,

2013] revealed many publications but when we cut off the studies with less than 20 tested samples only several articles are left (**Table 2**). The mean number of *EGFR* mutation tests was 58 (range 23-84); in most of them the direct sequencing was used as a gold standard. This study represents one of the largest European series of *EGFR* mutation testing performed on cytological specimens.

In conclusion, we presented the results of Polish, single institution study on the incidence of *EGFR* mutations in NSCLC. Histological and cytological samples were examined; the percentage of *EGFR* mutation was 10.6% and gene copy number was increased in 30% of cases. Against literature background, our study group is representative in terms of numbers tested and the methodology used. Stained archival, cytological samples are observed to be a valuable source of neoplastic cells in molecular testing; the incidence of *EGFR* mutations was comparable in histological and cytological preparations. Accurate selection of material by the experienced pathologist still remains the mandatory step in qualification to *EGFR* mutation testing.

Disclosure of conflict of interest

None.

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