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. Appropriate 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 Sklodowska-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 macro-dissected to access maximum percentage of neoplastic tumour 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 DPT72 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...
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(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 gender (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
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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

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

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

*Not-otherwise specified. *Available only for histological samples.

**Figure 2.** The contribution of EGFR mutations types (in brackets: exon).
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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

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].
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 EGFR-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. Moiseenko 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

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

*Fresh cells obtained by flushing the biopsy needles. *Adenocarcinomas only. *Abstract only (original article in Norwegian).

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
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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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