Original paper

The analysis of *ALK* gene rearrangement by fluorescence *in situ* hybridization in non-small cell lung cancer patients

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Introduction: *ALK* gene rearrangement is observed in a small subset (3–7%) of non-small cell lung cancer (NSCLC) patients. The efficacy of crizotinib was shown in lung cancer patients harbouring *ALK rearrangement*. Nowadays, the analysis of *ALK* gene rearrangement is added to molecular examination of predictive factors.
Aim of the study: The frequency of *ALK* gene rearrangement as well as the type of its irregularity was analysed by fluorescence *in situ* hybridisation (FISH) in tissue samples from NSCLC patients.
Material and methods: The *ALK* gene rearrangement was analysed in 71 samples including 53 histological and 18 cytological samples. The analysis could be performed in 56 cases (78.87%), significantly more frequently in histological than in cytological materials. The encountered problem with *ALK* rearrangement diagnosis resulted from the scarcity of tumour cells in cytological samples, high background fluorescence noises and fragmentation of cell nuclei.
Results: The normal *ALK copy* number without gene rearrangement was observed in 26 (36.62%) patients *ALK* gene polysomy without gene rearrangement was observed in 25 (35.21%) samples while in 3 (4.23%) samples *ALK* gene amplification was found. *ALK* gene rearrangement was observed in 2 (2.82%) samples from males, while in the first case the rearrangement coexisted with *ALK* amplification. In the second case, signet-ring tumour cells were found during histopathological examination and this patient was successfully treated with crizotinib with partial remission lasting 16 months.
Conclusions: FISH is a useful technique for *ALK* gene rearrangement analysis which allows us to specify the type of gene irregularities. *ALK* gene examination could be performed in histological as well as cytological (cellblocks) samples, but obtaining a reliable result in cytological samples depends on the cellularity of examined materials.

Key words: *ALK* gene rearrangement, fluorescence *in situ* hybridization, non-small cell lung cancer, crizotinib.

Contemp Oncol (Pozn) 2013; 17 (6): 484–492
DOI: 10.5114/wo.2013.38758

Introduction

Driver mutations are defined as single, independently occurring somatic mutations (non-inherited) which determine the beginning of the carcinogenesis process followed by incorrect proliferation of tumour cells. Based on this description, the definition of molecularly targeted therapies should be specified as those whose efficacy depends on the presence (or on the absence) of driver mutations. Molecularly targeted therapies should block incorrect pathways of cell signalling involved in oncogenesis and in fact could be applied only in genetically selected patients [1, 2].

At present, a number of molecularly targeted therapies are available in lung cancer. Significant clinical response could be obtained in patients with *EGFR* activating mutations after treatment with EGFR tyrosine kinase inhibitors (gefitinib, erlotinib, afatinib). One of the newly defined molecular targets is the anaplastic lymphoma kinase (ALK) pathway, which is blocked by small-molecular ALK inhibitors (crizotinib, LDK378, AP26113) [3, 4]. In second line therapy of lung adenocarcinoma patients harbouring *ALK* gene rearrangement, a clinical benefit (the higher response rate and longer progression-free survival) of crizotinib compared with placebo or standard chemotherapy (docetaxel or pemetrexed) was observed (PROFILE 1001, 1005 and 1007 studies) [5, 6].

Anaplastic lymphoma kinase (a receptor tyrosine kinase anaplastic lymphoma, CD246), is a transmembrane protein – a member of the insulin-like tyrosine kinase receptor superfamily, encoded by the *ALK* gene on chromosome 2. In non-small-cell lung cancer patients, the inversion [Inv(2)(p21p23)] within the short arm of chromosome 2 is the most frequently described abnormality of the *ALK* gene, occurring in approximately 3–7% of lung adenocarcinoma patients. The inversion leads to connection of the exons of *EML4* (echinoderm microtubule-associated protein-like 4) and *ALK* gene and to creation of the chimeric protein EML4-ALK. Many variants of the *EML4-ALK* fusion gene as well as *ALK* gene rearrangement with different genes (*KIF5B* – kinesin family member 5B, *TFG*, *KLC1* – kinesin light chain

1) have been found in non-small cell lung cancer patients, e.g. KIF5B-ALK fusion is observed in approximately 0.5% of NSCLC patients.

Since KIF5B-ALK and EML4-ALK fusion proteins contain the ALK tyrosine kinase domain, it was suggested that patients harbouring the *KIF5B-ALK* fusion gene could also benefit from ALK inhibitor therapy [5–9].

During crizotinib registration in the US, the genetic test based on fluorescence *in situ* hybridisation (FISH) dedicated to *ALK* gene rearrangement analysis was also approved by the Food and Drug Administration. The subsequent registration in the European Union does not specify the type of test that should be used for *ALK* gene rearrangement analysis. In addition to FISH technique, *ALK* gene rearrangement could be tested by immunohistochemistry staining or reverse transcriptase PCR technique. Selecting the appropriate technique for *ALK* gene analysis could result in qualification for crizotinib therapy and in efficacy of this drug in patients harbouring *ALK* abnormalities [10, 11].

Aim of the study

The aim of the study was to determine by FISH technique the frequency of *ALK* gene rearrangement as well as the type of its irregularity in non-small cell lung cancer patients. The usefulness of FISH technique according to the type of tissue samples and histopathological diagnosis was also evaluated. Finally, a case report of a lung adenocarcinoma patient harbouring *ALK* rearrangement successfully treated with crizotinib is presented.

Material and methods

Patient characteristics

ALK gene rearrangement was evaluated in tumour samples collected from 71 NSCLC patients in IIIB or IV stage of disease without *EGFR* activating mutation. Patient demographic and clinical characteristics are summarized in Table 1. Patients who did not smoke or those with a history of smoking < 100 cigarettes were classified as non-smokers, while individuals smoking > 100 cigarettes but who had not smoked 5 years prior to the study were considered former smokers [13]. The characteristics of tissue samples used for *ALK* analysis are summarised in Table 2.

Specimen preparation

The Vysis ALK Break Apart FISH Probe Kit (CE-IVD marked, Abbot Molecular, USA) was used to detect *ALK* gene rearrangement by fluorescence *in situ* hybridization technique. Additionally, Paraffin-Pretreatment IV and Post-Hybridization Wash Buffer Kit (Abbot Molecular, USA) was also used for the pre-staining procedure. The positive and negative control for each experiment was performed on ProbeCheck ALK Positive Control Slides and ProbeCheck ALK Negative Control Slides (Abbot Molecular, USA). At least 50 non-overlapping nuclei were evaluated for each sample.

ALK gene rearrangement was performed on tissue section collected during surgery and subsequently fixed in formalin, embedded in paraffin and stored as FFPE (formalin-fixed, paraffin-embedded) blocks. Additionally, tissue biopsies were prepared as described above and stored as cellblocks. In any case, before the start of assays, localization and content of tumour cells in the specimen were examined with H&E staining.

The paraffin sections of 3–5 μm thick were cut and mounted on positively charged glass slides. The unstained specimen and control slides were baked overnight at 60°C. Afterwards, the slides were immersed three times in xylene for 5 minutes and dehydrated twice in 100% ethanol for 1 minute at ambient temperature. In sequence, the slides were immersed for 15 minutes in Vysis Pretreatment Solution, which had been previously warmed to 80°C, and in purified water for 3 minutes. After removing excess water from slides, they were incubated for 30 minutes in

Table 1. Clinical characteristics of study group (pathomorphology division based on the recommendation proposed by IASCL/ATC/ERS International Multidisciplinary Classification of Lung Adenocarcinoma) [12]

Gender (*N*, %)	
female	24 (33.8%)
male	47 (66.2%)
Age (years, mean value ± standard deviation)	59.31 ±10.87
female	57.87 ±10.42
male	60.06 ±11.14
Pathomorphological diagnosis (*N*, %)	
adenocarcinoma	15 (21.1%)
invasive adenocarcinoma	9 (12.6%)
invasive mucinous adenocarcinoma	10 (14.1%)
invasive adenocarcinoma:	
– papillary predominant	10 (14.1%)
– acinar predominant	13 (18.3%)
– solid predominant	3 (4.2%)
– solid predominant with signet-ring cell component*	5 (7.1%)
adenocarcinoma – lepidic predominant	5 (7.1%)
large-cell carcinoma	1 (1.4%)
Smoking status	
current smoker (*N*, %)	27 (38%)
former smoker (*N*, %)	21 (29.6%)
never smoker (*N*, %)	9 (12.7%)
data not available (*N*, %)	14 (19.7%)
pack-years (median value ± standard deviation)	31.21 ±19.9

**The authors would like to point out the presence of tumour tissue with a signet-ring cell component, as it was recommended by the IASLC that this type of tumour should be included in other types of adenocarcinoma.*

Table 2. Type of sample used for *ALK* rearrangement

	***N* (%)**
Paraffin block prepared from:	53 (74.65%)
tumour tissue from distant metastasis	6 (8.45%)
tumour tissue from metastatic lymph nodes	6 (8.45%)
tumour tissue taken during surgery (including thoracoscopy and wedge resection performed during diagnostic process)	41 (57.75%)
Cellblock prepared from:	18 (25.35%)
fine needle aspiration biopsy through the chest wall (FNAB)	2 (2.82%)
endobronchial biopsy (during bronchoscopy)	7 (9.86%)
EBUS-TBNA (endobronchial ultrasound transbronchial aspiration) of primary tumour	3 (4.22%)
EBUS-TBNA metastatic lymph nodes	5 (7.04%)
sputum	1 (1.41%)

Protease Solution previously warmed to 37°C and washed in purified water for 3 minutes. Then, the slides were dehydrated in 70%, 80% and 100% ethanol for one minute each and allowed to dry. The slides were placed in a dark room, 10 µl of probe mixture was applied to a slide and immediately covered by a coverslip and sealed with rubber cement. They were placed for 3 minutes on a hotplate at 73°C and then at 37°C for overnight hybridization. At the end of the hybridization period, the rubber cement was removed from the slides and they were placed in Wash Buffer I at ambient temperature to allow the coverslips to float off the slides. Afterwards, the slides were immersed for 2 minutes in Wash Buffer II previously warmed at 74°C and air-dried in a dark room. 10 µl of DAPI counterstain was applied to the target area, covered by a coverslip, and the specimens were examined under a fluorescence microscope (Nikon Eclipse 55i, Japan).

Counterstaining procedure of *ALK* gene rearrangement in fluorescence microscope

The analysis of *ALK* gene rearrangement involves assessing the integrity of the gene. The hybridization targets of the probes are on opposite sides flanking the breakpoint of the *ALK* gene. The 3'-ALK probe that hybridizes telomerically of the breakpoint is labelled with the SpectrumOrange fluorophore. The 5'-ALK probe that hybridizes centromerically of the breakpoint is labelled with SpectrumGreen fluorophore.

The cells are considered positive (with *ALK* gene rearrangement) when adjacent orange and green signals are more than two signals' diameters apart and/or one fused signal coexists with one orange signal. A sample is considered negative if < 5 cells out of 50 (< 10%) are ALK-positive. A sample is considered positive if > 25 cells out of 50 (> 50%) are ALK-positive. A sample is considered equivocal if 5 to 25 cells (10–50%) are positive and a second reader should evaluate the sample. If the average percentage of positive cells is < 15% (< 15/100 cells), the sample is considered negative, while if the average percentage of positive cells is > 15% (> 15/100 cells), the sample is considered positive. The algorithm for assessing the FISH results for *ALK* gene rearrangement is shown in Fig. 1. Examples of cell nuclei with the *ALK* gene rearrangement are shown in Fig. 2.

The cells are considered negative (without *ALK* rearrangement) when two fusion signals or one fusion signal with one green signal without the corresponding orange signal are observed. Examples of cell nuclei without *ALK* gene rearrangements are shown in Fig. 3.

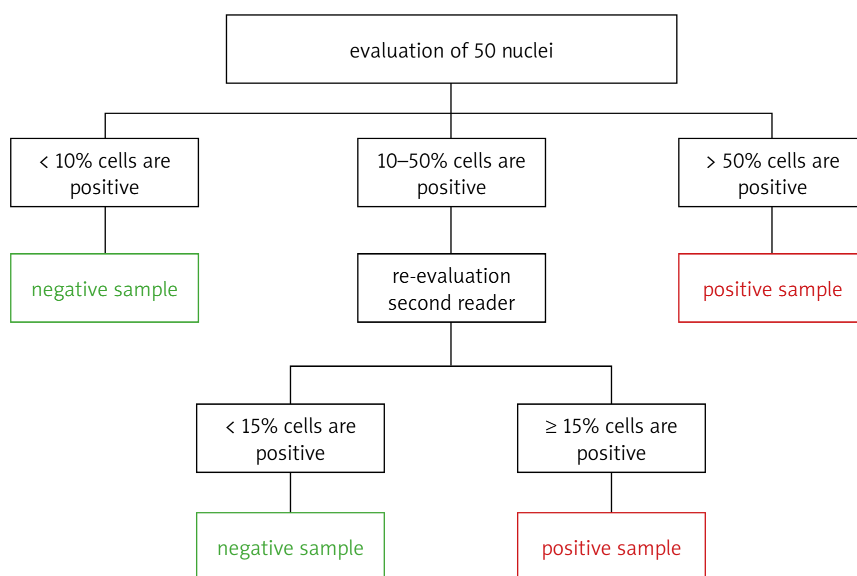

Fig. 1. The algorithm for assessing the results of FISH testing for presence of the *ALK* gene

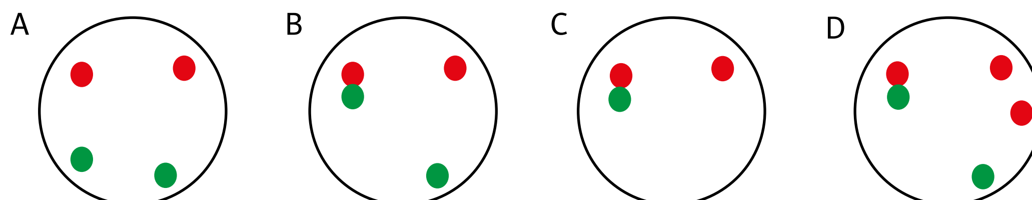

Fig. 2. Schematic diagrams of nuclei defined as positive for *ALK* rearrangement. A) two split signals with distance between the signal borders of ≥ 2 diameters of the largest of the two signals. B) one fused signal and one single red and green signal. C) one fused signal and one red without corresponding green signal providing for deletion of 5' DNA fragment. D) nuclei with one fused and more than 2 split red and green signals

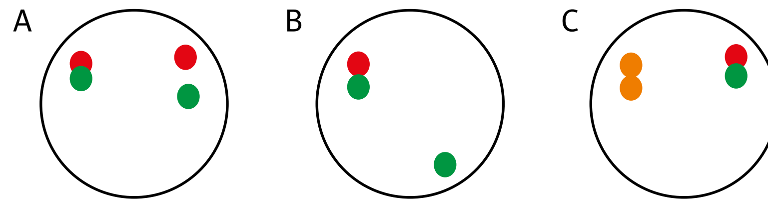

Fig. 3. Schematic diagrams of nuclei defined as negative for *ALK* rearrangement. A) two fused signals with distance between the signal borders of ≤ 2 diameters of the largest of the two signals. B) one fused signal with single green signal providing for 3' DNA fragment deletion. C) nuclei with overlapping fused signals which became yellow or orange

Moreover, an increased copy number of fused non-rearranged *ALK* signals corresponds to polysomy (≥ 4 *ALK* copies in ≥ 10% nuclei) or *ALK* amplification (≥ 10 *ALK* copies in ≥ 10% nuclei). An increased of *ALK* gene copy number observed in 10–39% of cell nuclei was considered as low-grade polysomy, while > 40% of cell nuclei was considered as high-grade polysomy. This division was adapted from the FISH test results scale proposed by Cappuzzo *et al.* for evaluation of *EGFR* gene and chromosome 7 abnormalities in NSCLC patients [14].

In the statistical analysis comparing the sample sizes the χ^2 test was used.

Results

Using FISH technique, *ALK* gene rearrangement was possible to evaluate in 56 (78.87%), samples, while 15 (21.13%) samples were classified as not interpretable. A normal *ALK* gene copy number without *ALK* gene rearrangement was observed in 26 (36.62%) patients; an increased *ALK* gene copy number (polysomy of *ALK*) without *ALK* rearrangement was found in 25 (35.21%) patients; while *ALK* gene amplification without rearrangement was observed in 3 (4.23%) patients. *ALK* gene rearrangement was observed in 2 (2.82%) patients, while in one case the rearrangement coexisted with *ALK* amplification (Table 3).

ALK gene polysomy

ALK gene polysomy was observed in 25 (35.21%) patients and it included 11 patients with high-grade *ALK* polysomy and 14 patients with low-grade *ALK* polysomy. The frequency of *ALK* polysomy was analysed according to the type of materials. In tissue samples collected during surgery (histological), low-grade *ALK* polysomy was observed in 5/53 samples, while high-grade *ALK* polysomy was found in 13/53 of such materials. Additionally, in cytological samples, low-grade *ALK* polysomy was observed in 6/18 samples, while high-grade *ALK* polysomy was found in 1/18 samples. We found high-grade *ALK* polysomy non-significantly more frequent in histological than in cytological samples (χ^2 = 3.055, p = 0.08).

The frequency of *ALK* gene polysomy according to histopathological diagnosis and smoking status in the studied population is summarised in Table 4. We did not observe significant differences in the frequency of *ALK* polysomy between analysed parameters. The average number of hybridisation signals in cell nuclei was estimated at 3.55.

ALK gene amplification

ALK gene amplification (without *ALK* rearrangement) was observed only in samples collected during surgery of primary lung tumour. We found 3 (4.23%) samples with *ALK* gene amplification, which were from the following patients: one male former smoker with histopathological diagnosis of invasive adenocarcinoma of solid predominant with mucin production; and two women, the first a former smoker with invasive adenocarcinoma of lepidic predominant type, and the second a currently smoking woman with invasive adenocarcinoma of papillary predominant type. In all three cases, *ALK* amplification was found in ≥ 16% of cell nuclei. The average number of signals from the probe complementary to the investigated region of *ALK* in patients with amplification was 4.29.

ALK gene rearrangement

ALK rearrangement was found in 2 samples collected from primary lung tumour. In the first case it was a 46-year-old never-smoking man with invasive adenocarcinoma of solid predominant type with a signet-ring cell component (the material from pleural invasion collected

Table 3. Results of *ALK* gene abnormalities in studied group

FISH results	Whole group (n = 71)	Male (n = 47)	Female (n = 24)
No *ALK* rearrangement, normal *ALK* gene copy number	26 (36.62%)	20 (42.55%)	6 (25%)
No *ALK* rearrangement, *ALK* gene polysomy	25 (35.21%)	14 (29.78%)	11 (45.84%)
No *ALK* rearrangement, *ALK* gene amplification	3 (4.23%)	1 (2.13%)	2 (8.33%)
ALK rearrangement, normal *ALK* gene copy number	1 (1.41%)	1 (2.13%)	0
ALK rearrangement, *ALK* gene amplification	1 (1.41%)	1 (2.13%)	0
Samples not interpretable	15 (21.12%)	10 (21.28%)	5 (20.83%)

Table 4. Occurrence of *ALK* gene polysomy according to pathological diagnosis and smoking status

Pathological diagnosis and smoking status	Low-grade polysomy (*N*)	High-grade polysomy (*N*)
Adenocarcinoma	4/15	1/15
Invasive adenocarcinoma	0/9	4/9
Invasive mucinous adenocarcinoma	1	1
Invasive adenocarcinoma:		
– papillary predominant	3/10	2/10
– acinar predominant	2/13	3/13
– solid predominant	0/3	0/3
– solid predominant with signet-ring cell component	1/5	0/5
Adenocarcinoma – lepidic predominant	0/5	3/5
Smoking status		
Current smoker	3/27	5/27
Former smoker	2/21	4/21
Never smoker	2/9	2/9
Data not available	4/14	3/14

through videothoracoscopy). *ALK* rearrangement was observed in 56% of cell nuclei (shown as coexistence of fused signals with one or two single orange signals) and the average number of signals from the probe complementary to the investigated region of *ALK* was 2.06 (Fig. 4). The patient was treated with crizotinib, which is described in the following part of the manuscript.

ALK rearrangement (observed in 20% of cell nuclei) coexisting with gene amplification was found in a 57-year-old currently smoking (31 pack-years) man with invasive adenocarcinoma of solid predominant type. The average number of signals from the probe complementary to the investigated region of *ALK* was 6. Due to the invasion into regional lymph nodes (N1, IIIA stage) and the T3 feature (tumour of 12 cm × 13 cm in diameter), the patient was qualified for surgical resection followed by adjuvant chemotherapy. Five months after surgery, local and distant metastases in the CNS were observed. The patient was qualified for radiotherapy of the CNS and did not receive chemotherapy or crizotinib therapy. He died 4 months later.

Limitations of *ALK* gene rearrangement analysis (non-diagnostic samples)

ALK gene rearrangement analysis could not be performed by FISH technique in 15 cases including 8/53 histological and 7/18 cytological samples. The difficulties in obtaining FISH results were observed significantly more frequently for cytological (χ^2 = 4.565, p = 0.03) than for histological samples. The limitation for *ALK* analysis by FISH technique resulted from:

1. The insufficient number of cells in cellblocks – too few nuclei available for enumeration (n = 4, 26.6% of non-interpretable samples). The materials were obtained by:

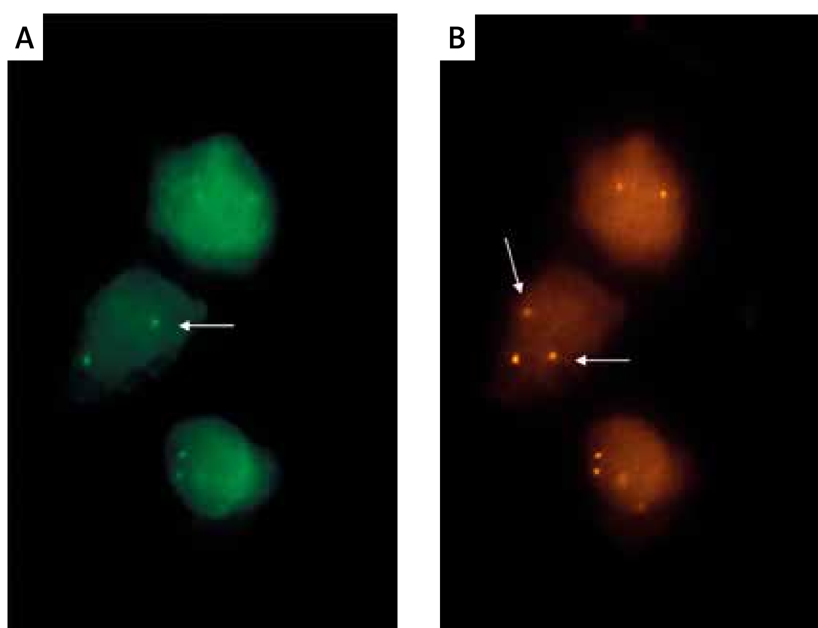

Fig. 4. Schematic nuclei with *ALK* gene rearrangement – signals of green probe (A) and split signals of red probe (B) – own material

EBUS-TBNA (endobronchial ultrasound transbronchial aspiration) of lymph nodes (2 cases); FNAB (fine-needle aspiration biopsy) through the chest wall (1 case); collection of cells from sputum (1 case).
2. No signal or weak signals from hybridized probes with normal signals from the positive control slides (*n* = 8, 53.3% of non-interpretable samples). It included 6 samples obtained during surgery of primary lung tumour and in other cases the histological material obtained during surgery of distant CNS metastases and during routine diagnosis of cervical lymph nodes.
3. Tissue loss during deparaffinization procedure (*n* = 1, 6.7% of non-interpretable samples).
4. Noisy background from the probes preventing reading the signal in histological sample of primary lung tumour (*n* = 1, 6.7% of non-interpretable samples).
5. Fragmentation of the nuclei in cytological sample from endobronchial biopsy (crushed material, *n* = 1, 6.7% of non-interpretable samples).

Clinical response to crizotinib treatment of ALK-positive patient – a case report

A 46-year-old man presented at hospital with 3 months history of chest pain and increasing dyspnoea. The accumulation of pleural effusion and numerous small nodules in both lungs were visible during radiograph and CT scan of the chest. Atelectasis of some lung segments was also observed. From tissue section of pleura collected during videothoracoscopy, adenocarcinoma of solid predominant with signet-ring cells was diagnosed. Stable disease (according to the RECIST criteria) was observed after 5 cycles of cisplatin and pemetrexed first-line chemotherapy. Progression of disease manifested by increasing pleural effusion and the appearance of new nodules in both lungs was observed 6 months after the diagnosis. Molecular examination showed wild type for the *EGFR* gene and no amplification for *c-Met*. However, *in situ* fluorescence hybridization analysis revealed the presence of *ALK* gene rearrangement. Therefore, crizotinib was administered as a second-line treatment, obtaining an improvement in quality of life, relief of symptoms, partial remission in lung and disappearance of pleural effusion. Progression of disease manifested by the growth of the largest primary tumour and the appearance of new subpleural nodules was observed 16 months after starting crizotinib therapy. The patient was disqualified from palliative radiotherapy and he received 2 cycles of carboplatin and paclitaxel chemotherapy, which was insufficient to obtain stable disease. The patient remains in a good condition and is waiting for treatment involving a new generation of ALK inhibitors in a clinical trial.

Discussion

ALK gene abnormalities in NSCLC patients and their qualification for ALK inhibitor therapy

Rearrangements of the *ALK* gene were first identified in non-small cell lung cancer in 2007. Current estimates suggest that abnormalities in the *ALK* gene are well characterised and are present in approximately 2–7% of NSCLC patients. Clinical characteristics associated with *ALK* gene rearrangement are adenocarcinoma histology, especially acinar-predominant with signet-ring cell component, never/light smoking history, male gender and younger age. Moreover, *ALK* gene rearrangement rarely coexists with *EGFR* or *KRAS* mutations [15–17].

The frequency of *ALK* gene rearrangement strongly depends on the studied population and it was observed slightly more frequently in patients of eastern Asia origin. In the very first study, Soda *et al.* found 7/75 incorrect transcript of *EML4-ALK* [18]. The data presented in PROFILE 1001 and PROFILE 1005 studies showed that *EML4-ALK* fusion gene was observed in 13% of adenocarcinoma patients. The percentage of *ALK*-positive patients was increased to 22% if the studied group was limited to never smoking or patients with smoking history of < 10 pack-years. Moreover, if the patient group was restricted to *EGFR*-wild type patients, the percentage of *ALK*-positive was increased even to 33% [19]. In the PROFILE studies, *ALK* gene rearrangement was observed in 255 patients (97% were adenocarcinoma-bearing patients) with median age 52 years and approximately 70% of patients were never smokers. Currently, the median age of patients with *ALK* gene rearrangement is estimated at about 66 years [19].

So far, there is no information about the incidence of *ALK* gene rearrangement in the Polish population. In the present paper, for the first time in Poland, the percentage of *ALK* gene rearrangement amounted to 2.82%. The rearrangement was observed in two young males with invasive adenocarcinoma of solid predominant type, but in one case it was a non-smoking patient with a signet-ring cell component (one patient in five who were found to contain a signet-ring cells component). The second patient was a smoker and the rearrangement coexisted with *ALK* gene amplification. The clinical profile of our patients was similar to that described in the literature. However, *ALK* gene amplification was observed only in smokers and high-grade *ALK* polysomy was found more frequently in smoking patients (9/14). This could indicate the genetic differences in tumour tissue; moreover, *ALK* gene amplification could be involved in carcinogenesis of smoking lung cancer patients. However, a high *ALK* gene copy number, as opposed to *ALK* rearrangement, does not appear to influence the response to ALK inhibitors. The presented results of *ALK* gene rearrangement could be affected by pre-selection of patients who were qualified for crizotinib therapy based on the currently available data (patients with a high percentage of signet-ring cell component in histological specimens). Moreover, the patients were first qualified for *EGFR* testing and in case of wild-type, the analysis of *ALK* gene rearrangement was considered afterwards.

Type of materials and possibility to obtain a reliable FISH result

Recommendation for ALK rearrangement test report

In the present paper, *ALK* gene rearrangement could not be performed in 21% of cases. The best material for obtaining reliable FISH results is tissue collected during

tumour surgery, which provides a sufficient number of tumour cells and tumour tissue of a constant structure. In our study, the non-diagnosed samples were observed significantly more frequently in cytological than in histological materials. Jurado *et al.* showed the effectiveness of cytological specimens obtained by EBUS-TBNA for molecular testing of *EGFR*, *KRAS* and *ALK* rearrangement. A total of 52 of 56 (93%) patients had sufficient cytological material for complete or partial molecular testing, whereas 46 of 56 (82%) patients had sufficient material for all clinically indicated testing. *ALK* gene rearrangement was observed in 5 patients (pre-selection of patients) [20]. Similarly, Krawczyk *et al.* presented the opportunity to carry out an effective molecular diagnosis concerning *EGFR* testing in cytological materials from NSCLC patients treated in Polish cancer centres [21].

The recommended report on *ALK* testing by *in situ* hybridization must include all necessary information to give the physician a straightforward interpretation of the clinical outcome of the patient. According to the recommendation presented by Thunnissen *et al.* in 2012, the report should contain the necessary information to correctly identify the patients as well as the cancer centres ordering the *ALK* testing and the type of sample delivered for analysis (including information about storage and processing, date of receiving the sample, pathological diagnosis and the presence of necrosis) [11]. Cytological samples should be fixed in formalin and stored as cellblocks. In the case of sample scarcity, difficulties in 50 nuclei localization are expected, which could lead to re-collection of materials. Therefore, during pathological and molecular diagnosis of NSCLC patients, one should aim to obtain the most representative tumour samples (core biopsy, thoracoscopy, mediastinoscopy) properly fixed and stored as paraffin blocks. For an effective FISH procedure, a serial paraffin section should be cut and a pathomorphologist should mark the most proper place for FISH on hematoxylin- and eosin-stained slides (it should be remembered that after the FISH procedure only tumour nuclei are observed by DAPI). Moreover, paraffin-fixed tumour specimens should be cut suitably thin to avoid overlapping nuclei [10, 11].

A report on *ALK* testing should contain information about the test used for the assay, which should be certified and marked for *in vitro* diagnosis. A method to assess *ALK* rearrangement has also been described (number of evaluated nuclei, number of observers and explanation for inconclusive results in case of non-interpreted samples). It is recommended to use the abbreviation for determination of type and number of signals in nuclei, which should be precisely described on the FISH report (Table 5). This could provide additional information for the physician about other abnormalities observed in tumour nuclei (e.g. deletion of chromosome arm, polysomy or amplification of examined gene).

The FISH report should be signed by a specialist laboratory diagnostician or by the pathologist responsible for the investigation. The recommended turnaround time is < 7 working days. The inclusion of clinical interpretation of the *ALK* test result on the report is disputable.

Future perspective for *ALK* rearrangement and ALK-inhibitor therapy

The application of ALK inhibition therapy in *ALK* positive patients is a very effective strategy. Therefore, the proper identification of *ALK* gene abnormality by precise and accurate diagnostic tests is challenging (FISH, IHC, RT-PCR). Fluorescence *in situ* hybridization, which was used in all clinical trials concerning crizotinib, is defined as the "gold standard" for *ALK* gene rearrangement. FISH can detect multiple *ALK* fusion variants, but there are various challenges concerning this technique, e.g. split signals can be subtle and the inversion concerns only a small part of genetic materials. In the authors' opinion, FISH technique should not generate any problems for laboratories which successfully participate in external quality assessment programmes.

The main advantage of immunohistochemical (IHC) procedures is the possibility to detect tumour-specific antigens with monoclonal antibodies without cytological destruction of examined tissue. Currently, there are three primary antibodies used for ALK protein detection: 5A4, ALK1 and D5F3 [22–24]. In *ALK*-rearranged NSCLC samples, ALK protein staining is cytoplasmic and may have a granular character, and in some cases may also be relevant to membrane. One of the particular challenges of IHC is to create a precise definition of staining degree and increase the specificity of this technique. It should be noted that the *ALK*-rearranged adenocarcinoma has a much lower level of ALK protein expression than *ALK*-rearranged lymphoma. So far, there is no standardized IHC staining protocol in the literature. Moreover, *ALK* rearrangement has not been detected by IHC staining in any clinical trials concerning

Table 5. Proposed abbreviations used in the description of *ALK* gene rearrangement results

Abbreviation	Description
2F	Two fused (**F**) signals were observed – a nucleus without *ALK* rearrangement
3F; 4F; nF	Three/four/n fused signals were observed – a nucleus without *ALK* rearrangement. Depending on the amount of fused signals, it is defined as *ALK* polysomy or *ALK* amplification.
1G1F (nGnF)	One fused signal with one green (**G**) signal – a nucleus without *ALK* rearrangement. The number of fused signals and green signals could vary depending on *ALK* gene copy number.
1G1R1F (nGnRnF)	One fused signal with single green (**G**) and single red (**R**) signal – a nucleus with *ALK* rearrangement. The multiplication of each signal is also considered as *ALK* rearrangement.
1R1F (nRnF)	One fused signal with single red (**R**) signal without corresponding green (**G**) signal – a nucleus with *ALK* rearrangement. The multiplication of each signal is also considered as *ALK* rearrangement.

crizotinib; therefore there is no information about the efficacy of ALK-inhibition therapy in patients with abnormal expression of ALK protein [22–24].

RT-PCR provides a highly sensitive and rapid technique for *ALK* rearrangement detection. However, this method has several disadvantages. Due to the existence of several variants of *ALK* fusion genes (*EML4* exons of 1–13 could be fused with *ALK* exon of 20–29), multiplex validated PCR primer pairs for all known *ALK* gene fusion partners are required. Therefore, only known *ALK* alterations can be tested. RT-PCR requires genetic materials of good quality. The majority of adenocarcinoma tissue is fixed in formalin and embedded in paraffin; thus, RNA extracted from such samples is highly degraded, which could make it difficult to perform reliable RT-PCR. However, this technique can be widely used to diagnose *ALK* rearrangement in cytological (including from tissue fixed on slides) as well as in fresh frozen tissue samples [24].

Yi *et al.* evaluated FISH as the gold standard for *ALK* rearrangement examination. They found that all IHC 3+ positive cases (stained with ALK1 clone antibody) were also FISH-positive, while in cases of IHC 1+ or 2+ (24 patients) only 2 were *ALK*-positive by FISH technique. All 69 IHC-negative patients were also FISH-negative [23].

Wu *et al.* examined *ALK* rearrangement in 312 NSCLC patients. If RT-PCR technique was used as the gold standard, FISH test had a low sensitivity (58.3%), but very good specificity (99.3%), while IHC stain had better sensitivity (91.7%) than FISH, but lower specificity (79.5%) when the cut-off was IHC 2+ [24]. All the tests gave positive results if EML4-ALK expression was high, which occurs frequently in Asian patients with a specific type of *EML4-ALK* gene variants (3a and 3b). Although in Caucasian patients the first variant of the *EML4-ALK* gene is observed more frequently, unfortunately it is not associated with high expression of the specific protein.

In conclusion, it should be noted that for optimal *ALK* rearrangement testing three essential elements are required: the quality of the sample, the proper analytical procedure and the reporting of obtained results. Laboratories involved in *ALK* gene rearrangement should be validated in an external quality test conducted by European organizations (e.g. FALKE project or ALK Testing of European Society of Pathology). In Poland, the quality control of *ALK* rearrangement is carried out in two years, and the laboratories involved in the test each year pass it positively. It seems that the most important challenge for the future is to standardize the reporting of FISH results and the continuation of quality control for laboratories performing FISH tests. However, the distribution of information about *ALK* testing, and in the near future also about *ROS1* and *RET*, should be based on real access to ALK inhibitor therapy.

The authors declare no conflict of interests.

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Submitted: 4.10.2013
Accepted: 8.11.2013