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Analysis of *ROS1* gene rearrangement incidence among NSCLC patients with fluorescent *in situ* hybridization technique

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ABSTRACT

Introduction. The rearrangement of the gene encoding ROS protooncogene (ROS1) is observed in a very small percentage (1–2%) of patients with non-small cell lung cancer (NSCLC). The clinical characteristics of *ROS1*-positive patients are similar to those observed in the group of patients with *ALK* gene rearrangement. Detection of *ROS1* gene rearrangement is an extremely important predictive factor enabling the use of crizotinib in the 1st line of NSCLC patients with stage IIIB or IV. Due to the addition of crizotinib to the list of reimbursed drugs from January 2019, the analysis of this genetic change should be part of a molecular tests panel performed in patients with locally advanced and advanced NSCLC in the qualification for molecularly targeted treatment.

Aim of the study. Analysis of *ROS1* gene rearrangement incidence among NSCLC patients in stage IIIB or IV qualified for molecularly targeted therapies. Presentation of methodological difficulties with fluorescent *in situ* hybridization (FISH) technique which is used to detect *ROS1* genetic abnormality.

Materials and methods. The analysis of *ROS1* gene rearrangement was carried out using fluorescent *in situ* hybridization technique in tissue samples taken from 573 NSCLC patients of non-squamous cell type during routine pathomorphological diagnostics.

Results. The material obtained from the tumor was fixed in formalin and archived in paraffin. Histological material was obtained from 408 patients, and 165 — cytological (cytoblock). A reliable (diagnostic) result of the *ROS1* gene rearrangement was obtained in 439 patients (76.61%). The main difficulties for *ROS1* gene analysis were low number of cancer cells, as well as high background fluorescence interference and fragmentation of cell nuclei. *ROS1* gene rearrangement was detected in 9 patients with adenocarcinoma (1.57% among all patients), including 5 men and 4 women. In 19 patients, other abnormalities regarding the *ROS1* gene were observed, primarily the polysomy of the examined *ROS1* gene fragment (3.32%). Polysomy did not coexist with the *ROS1* rearrangement.

Conclusion. Fluorescent *in situ* hybridization is a useful tool in detecting *ROS1* gene rearrangement. The test can be performed in both histological and cytological material (cytoblock). However, the correct fixation of the material and the appropriate number of tumor cells in the tested samples is extremely important for obtaining a reliable result.

Key words: *ROS1* rearangement, fluorescence *in situ* hybridization, non-small cell lung cancer, crizotinib

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Introduction

The initiation of the carcinogenesis process is associated with the appearance of somatic (non-hereditary), single mutation in the oncogene, which results in disruption of basic physiological processes, and consequently leads to uncontrolled cell division. Based on this basic assumption, molecularly targeted therapy is treatment that blocks the abnormal signaling pathway in cancer cells. Therefore, the effectiveness of molecularly targeted therapy depends on the presence (or absence) of the driver mutation [1, 2].

At present, several molecularly targeted therapies are available for the treatment of patients with non-small cell lung cancer (NSCLC). Significant clinical response after the use of EGFR tyrosine kinase inhibitors (TKI) (such as gefitinib, erlotinib, afatinib, osimertinib, dacomitinib) is observed in NSCLC patients with a detected activation mutation in the epidermal growth factor receptor gene — *EGFR*. In Poland, gefitinib, erlotinib, afatinib and, for selected patients, osimertinib are refunded. Another type of molecularly targeted therapy is the use of anaplastic lymphoma kinase (ALK) inhibitors in patients with known *ALK* gene rearrangement. In this group of drugs, reimbursement in Poland covers crizotinib, ceritinib and alectinib, while brigatinib and lorlatinib are also registered in the European Union [3–5]. BRAF and MEK inhibitors: dabrafenib and trametinib are successfully used in NSCLC patients with mutations in the *BRAF* gene, and in the case of *NTRK* gene rearrangement — larotrectinib and entrectinib (non-reimbursed drugs in Poland) [5].

ROS1 inhibitors are another group of molecularly targeted drugs that have been used in NSCLC patients. The *ROS1* gene, located on chromosome 6 (cytogenetic location: 6p22), encodes a receptor with ROS tyrosine kinase activity, belonging to the family of insulin receptors evolutionally related to the ALK receptor [6–8]. The molecular abnormalities found in NSCLC patients are the rearrangement of the *ROS1* gene. This abnormality occurs in only 1–2% of patients diagnosed with adenocarcinoma, and the clinical characteristics of patients with *ROS1* gene rearrangement are similar to patients with NSCLC with a confirmed abnormality in the *ALK* gene [6–8].

In the group of patients with *ROS1* gene rearrangement, it is possible to use the ALK, ROS1 and MET tyrosine kinase inhibitor — crizotinib. In prospective clinical trials, over 70% of NSCLC patients with *ROS1* gene rearrangement receiving crizotinib in the 1st line of treatment responded to the treatment and had a median progression-free survival time of 19.2 months [8, 9]. For these reasons, the diagnosis of *ROS1* gene rearrangement should be immediately included in the panel of molecular tests offered to patients with locally advanced and advanced NSCLC. In Poland, such a diagnostic procedure has been available to an increasing extent since January 2019, when crizotinib was reimbursed for patients with adenocarcinoma of the lung with *ROS1* gene rearrangement.

Aim of the study

The aim of this study is to evaluate the incidence of rearrangement and other molecular abnormalities of the *ROS1* gene determined by fluorescence *in situ* hybridization (FISH) in patients with locally advanced and advanced NSCLC. In addition, methodological difficulties of the FISH test used to detect *ROS1* gene abnormalities were presented.

Materials and methods

Study group characteristics

The material obtained from the tumor was fixed in formalin and archived in paraffin from 573 patients with NSCLC of a type other than squamous cell carcinoma. The *ROS1* gene rearrangement study was performed after excluding the presence of mutations in the *EGFR* gene and the rearrangement of the *ALK* gene. In 408 patients the examination was performed in histological material, and in 165 — cytological (cellblock). The demographic and clinical characteristics of the patients are summarized in Table 1.

Table 1. Demographic analysis of patients undergoing *ROS1* gene rearrangement assessment

Gender (n, %)	
Male	226 (39.44%)
Female	347 (60.56%)
Age (years, mean and standard deviation)	66.19 ± 8.44
Female	65.85 ± 8.89
Male	66.22 ± 8.13
Pathologic diagnosis of NSCLC	
Adenocarcinoma	464 (80.10%)
Other non-squamous NSCLC	109 (19.90%)
Expression of TTF1 on tumor cells	
TTF1 expression present	270 (47.12%)
TTF1 expression absent	77 (13.44%)
TTF1 expression not analyzed	226 (39.44%)
Types of analyzed material	
Histological material (small sections and surgical materials)	408 (71.20%)
Cellblock	165 (28.80%)

TTF1 — thyroid transcription factor type 1

ROS1 gene rearrangement analysis procedure using fluorescence in situ hybridization technique

The method of analyzing of *ROS1* gene rearrangement is analogous to the method of analyzing of *ALK* gene rearrangement. During the study of the rearrangement of the *ROS1* gene, its integrity is assessed, i.e. we examine the fact that a DNA strand breaks and a fragment of the *ROS1* gene moves to another place in the genome, but we do not examine the type of gene fusion that is being formed [8]. In the FISH technique, we use molecular probes — short fragments of DNA complementary to the sequences of interest in the tested DNA. In the diagnosis of *ROS1* gene rearrangement we use 2 probes: a probe with a green fluorochrome, which covers proximal DNA sequences, closer to the region sensitive to *ROS1* gene breaks, and a probe with a red or orange fluorochrome, whose sequences are complementary distally to the region sensitive to cracks in the *ROS1* gene (based on ZytoLight® Spec ROS1 Dual Color Break Apart Probe). When carrying out the FISH test, it should be taken into account that the manufacturers of molecular probes can label them in different ways, which is of great importance when interpreting the obtained results.

The laboratory procedure for handling the material for studying the rearrangement of the *ROS1* gene is based on the use of ready-made kits that allow dewaxing of tissue material, fixation, digestion in a protease buffer, denaturation and hybridization with a specific molecular probe. In this procedure, one should follow the instructions provided by the manufacturer and validate the methodology used in the laboratory. The present study uses the ZytoLight® SPEC ROS1 DualColor Break Apart Probe (ZytoVision, Germany), the Vysis Paraffin Pretreatment and Post-hybridization Wash Buffer Kit (Abbott, USA), while fluorescence signals have been assessed using an Axio Scope microscope (Zeiss, Germany). It should also be remembered that, similarly to the analysis of the *ALK* gene rearrangement, not all materials can be analyzed for the *ROS1* gene rearrangement. Table 2 summarizes the materials that are delivered to laboratories and in which it is possible to perform the FISH technique.

The tumor cell nucleus is rated as positive (with the present rearrangement of the *ROS1* gene) when the gap between the orange or red and green signal is greater than the diameter of the largest signal in the pair, or when there is an isolated green signal in the presence of fusion signals (based on ZytoLight® Spec ROS1 Dual Color Break Apart Probe). Diagrams of observable signals from fluorescent probes are presented in Figure 1.

The result of the *ROS1* gene rearrangement study is considered positive when the described signal abnormalities are found in 15% of the examined tumor cell nuclei. However, to prevent bias error, it is recommended that the test be performed by two screeners [8]. A diagram of the diagnostic procedure for assessing the *ROS1* gene rearrangement is presented in Figure 2.

In order to compare means from two independent groups, the Student's T-test and Statistica v. 13.1 program were used. The assessment whether the observed distribution of a given feature depends on another variable was carried out using the Pearson χ^2 test. Survival analysis was performed using the Kaplan-Meier method using MedCalc v. 18.11.6.

Results

Analysis of the incidence of *ROS1* gene abnormalities

The *ROS1* gene rearrangement study using FISH was performed in 573 patients with non-squamous NSCLC. In 439 cases (76.61%) a reliable test result was obtained, while in 134 (23.39%) cases no diagnostic result was obtained. Among the non-diagnostic materials, there were 55 cytological materials fixed in the form of cellblocks (which constituted 33% of all cellblocks sent for examination) and 79 histological materials (which constituted 19.4% of all histological materials). Hence, the non-diagnostic result of the *ROS1* gene rearrangement study was obtained significantly more frequently in cytological than histological materials ($P = 0.00035$, $\chi^2 = 12.798$).

Table 2. Possibilities of performing the ROS1 gene rearrangement assay in various materials

Tissue material — FFPE block	Thick needle biopsy material — FFPE block	Cryobiopsy	Cytological material — cellblock	Cytological material H+E or Papanicolaou — microscopic glass slide	Liquid biopsy — peripheral blood sample
+++	+++	+ (the method must be validated by the laboratory)	++	– (only medical experiment using DNA stability in some cytological preparations stained with Papanicolau or H + E technique)	– (only medical experiment using free circulating cancer cells)

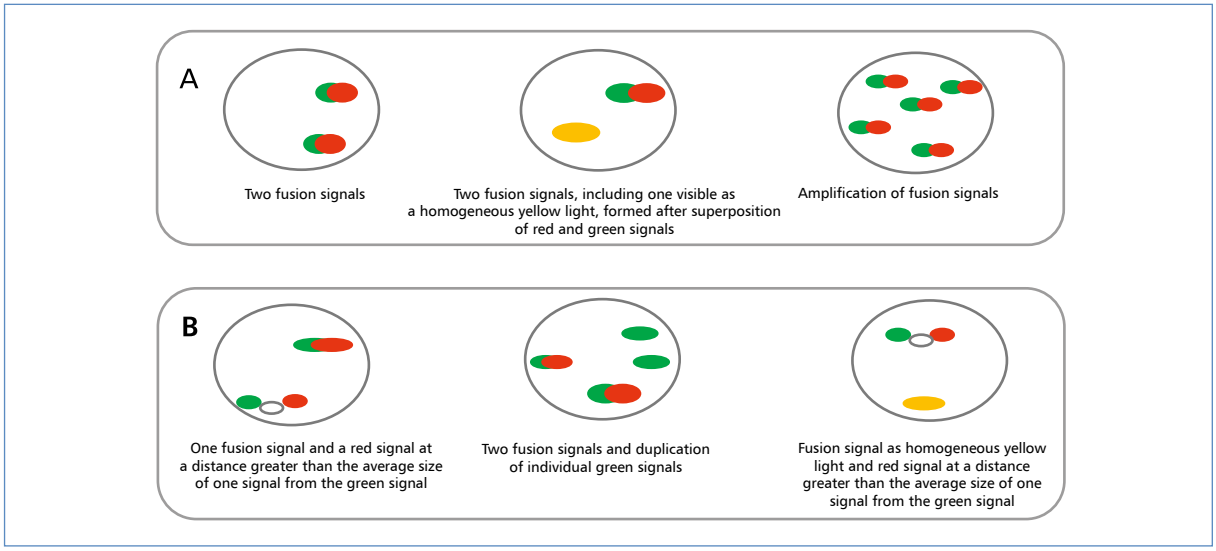

Figure 1. The representative diagrams of signals from fluorescent probes in the case of: **A** — tumor cell nuclei without rearrangement of the *ROS1* gene; **B** — nuclei of cancer cells with the current rearrangement of the *ROS1* gene

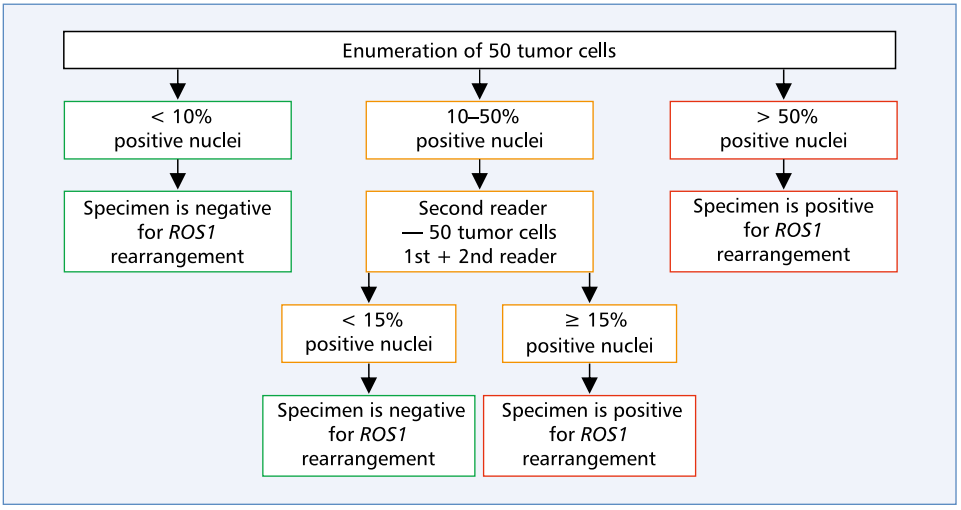

Figure 2. The scoring algorithm recommended for *ROS1* testing with FISH technique [8]

The limitations of the *ROS1* gene rearrangement analysis using FISH method resulted mainly from the insufficient number of cancer cells in the examined material and the lack of molecular probe signals due to the most likely incorrect fixation of the materials sent for testing. Pre-laboratory treatment of histological and cytological material has an extremely important impact on the possibility of obtaining a diagnostic result of FISH gene rearrangement testing.

In the examined group, rearrangement of the *ROS1* gene was detected in 9 cases, which constituted 1.57% of all examined samples. Rearrangement was detected in 5 men and 4 women ($P = 0.757$, $\chi^2 = 0.096$). Lung adenocarcinoma was diagnosed in all *ROS1*-positive patients (1.94% among patients with adenocarcinoma). In 6 *ROS1*-positive patients, expression of TTF1 protein on tumor cells was observed, and in the remaining three there was no expression of this adenocarcinoma marker ($P = 0.415$, $\chi^2 = 0.664$). In *ROS1-positive* patients, the median percentage of cancer cell nuclei with *ROS1* gene rearrangement was 18% and the median copy number of the *ROS1* gene was 2.6.

In 138 (24.08%) patients, cancer cell nuclei with *ROS1* gene rearrangement were observed, however, with a result that did not meet the criteria for inclusion for a molecularly targeted treatment (< 15% of cancer cell nuclei with *ROS1* gene rearrangement). In 19 patients (3.32% of analyzed cases) *ROS1* gene polysomy was observed (≥ 4 copies of the *ROS1* gene in the cell nucleus), however, in no case did this abnormality coexist with *ROS1* gene rearrangement. In the whole study group, the median copy number of the *ROS1* gene was

2.4. The number of copies of the *ROS1* gene did not significantly depend on sex, age, pathomorphological diagnosis, as well as the presence of TTF1 and CK7 expression on cancer cells.

Life expectancy of patients with known ROS1 gene status

In the studied group, 6 patients with *ROS1* gene rearrangement received molecularly targeted treatment with crizotinib (the remaining three patients in this group had an adverse course of the disease which prevented systemic treatment). In patients without *ROS1* gene rearrangement, 54 patients with PD-L1 expression on over 50% of cancer cells (9.57%) received first-line treatment with pembrolizumab, and 412 patients received chemotherapy (73.05%), among whom 2nd line immunotherapy received 16 patients (this number will increase significantly during observation of patients, since we began observing patients from January 2019). 98 patients did not receive any systemic treatment due to poor fitness and the presence of concomitant diseases (17.38%).

The median of overall survival (mOS) did not depend on sex, age, pathological diagnosis, presence of rearrangement of the *ROS1* gene and the number of copies of the *ROS1* gene in cancer cell nuclei. mOS in patients with non-squamous NSCLC type with TTF1 expression on cancer cells was 13 months, and in patients without this marker only 7 months (HR = 0.5634, P = 0.01). mOS in patients receiving first-line chemotherapy followed by second-line immunotherapy was 29 months (95% CI: 20.0–29.0), in patients receiving only chemotherapy — 14 months (95% CI: 10.0–30.0) and in patients without systemic treatment (due to poor performance) — 2 months. mOS in patients with *ROS1* gene rearrangement treated with crizotinib and in patients with PD-L1 expression on more than 50% of cancer cells receiving 1st line immunotherapy with pembrolizumab was not achieved. These differences were statistically significant (P < 0.0001). In the group of patients treated with crizotinib, at the time of statistical analysis, five patients were still alive (from 2 to 13 months of treatment), and one patient died 7 months after the implementation of molecularly targeted treatment.

Discussion

Rearrangement of the *ROS1* gene was first detected in a patient with lung cancer in 2007 [10]. Currently, this change is relatively well known — it is estimated that this rearrangement occurs in 1–2% of NSCLC patients. Patients with *ROS1* gene rearrangement are usually a group of young patients with adenocarcinoma (around 40–50 years old), however, there is a noticeable increase in the incidence of *ROS1* rearrangement also in patients over 70 years of age. 70% of patients with *ROS1* gene rearrangement have never smoked and 30% still smoke or smoked in the past [9, 11]. No significant differences were observed in the occurrence of rearrangement depending on the race of patients with NSCLC — in a study conducted by the IASLC (International Association for the Study of Lung Cancer) the rearrangement of the ROS1 gene was found in 2.3% of Asian patients, in 2% of patients of the race Caucasian and 1.6% of patients living in North America. However, local differences are described in the incidence of *ROS1* rearrangement — in a study conducted in northern India, this abnormality was found in 2.8% of NSCLC patients [12]. To date, it is difficult to determine the frequency of this genetic abnormality in the Polish population of NSCLC patients. In the presented study, *ROS1* gene rearrangement was detected in 1.57% of NSCLC patients with non-squamous cell type and in 1.94% of patients with adenocarcinoma, which confirms the worldwide incidence of this genetic abnormality.

Despite the sporadic occurrence of this rearrangement of the *ROS1* gene, the benefits of its diagnosis and the introduction of molecularly targeted therapy in *ROS1*-positive patients can be significant. In the PROFILE1001 clinical trial, 53 patients with locally advanced and advanced NSCLC with detected *ROS1* gene rearrangement were treated with crizotinib. The response rate to treatment was 72%, and the median overall survival was 51.4 months [13]. In another study, the efficacy of crizotinib in 1st line of treatment (n = 30) was compared to chemotherapy based on platinum and pemetrexed (n = 47) in NSCLC patients with *ROS1* gene rearrangement. The median follow-up was 28.1 months. The objective response rate in the crizotinib group was higher than in the group receiving chemotherapy (86.7% *vs.* 44.7%, respectively; P < 0.001). In addition, a significant increase in progression-free survival time (18.4 months) was observed in patients treated with crizotinib compared to patients receiving chemotherapy (8.6 months; P < 0.001). The median overall survival was not reached for patients receiving crizotinib, but it was 28.4 months for patients receiving chemotherapy (cross-over effect) [14].

From January 2019, crizotinib was reimbursed in Poland as a molecularly targeted therapy for patients with stage IIIB or IV NSCLC with *ROS1* gene rearrangement. The problem that clinicians planning therapy with crizotinib in ROS1-positive patients may encounter is the development of resistance to this drug during treatment. Gainor et al. observed that as many as 53% of patients undergoing crizotinib treatment develop resistance, which is most likely associated with the appearance of new mutations in the *ROS1* gene [15].

The problem of crizotinib resistance may be solved by research into the 2nd generation of ROS1 inhibitors. An example of the usefulness of this group of drugs may be the proven efficacy of lorlatinib and repotrectinib observed in *ROS1*-positive patients progressing after the use of crizotinib [16].

In Poland, the fluorescence in situ hybridization method using specific molecular probes is used to diagnose the *ROS1* gene rearrangement. It is an effective and proven diagnostic method, characterized by high sensitivity and specificity, and the kits for this diagnostic method have CE-IVD (*in vitro* diagnostic) certificates. The false-positive results described in the literature may result from the detection of an inactive fusion in the *ROS1* gene resulting from post-transcriptional processing, but this is a casuistic situation. As a result of the rearrangement taking place, the *ROS1* gene may fuse with other genes, e.g. *TPD52L1*, present near the location of the *ROS1* gene. The existence of such a partner gene fusion may be a diagnostic problem in the FISH method [8, 17]. However, to date, it is the technique most widely used in the diagnosis of rearrangement of the *ROS1* gene, and the only limitation of this method is the possibility of damage to the genetic material of cancer cells during improper fixation and protection of tissue material, and too low number of cancer cells in the assessed materials. Research is ongoing on the possibility of detecting the presence of an abnormal fusion protein containing *ROS1* on the surface of cancer cells by immunohistochemistry (IHC) [8]. The IHC method has obtained the CE-IVD certificate in recent months. In some laboratories, it is already routinely used for screening for *ROS1* gene abnormalities. However, it should be remembered that all positive IHC results for the presence of a *ROS1*-containing fusion protein must still be confirmed by FISH. Another technique that can be used in analyzing *ROS1* gene abnormalities is next-generation sequencing (NGS) [8].

In summary, analysis of the *ROS1* gene rearrangement among patients with locally advanced or advanced non-small cell lung cancer should be the standard in the diagnosis of predictive factors. Patients with *ROS1* gene rearrangement, thanks to new generations of drugs, have a chance to significantly extend life expectancy and improve its quality. The technique of fluorescence in situ hybridization is the basic diagnostic method, but it should be remembered that pre-laboratory treatment of histological and cytological material has an extremely important impact on the possibility of obtaining a diagnostic and reliable result of gene rearrangement testing using this method.

Conflicts of interest

The authors declare to have no conflict of interest.

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