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OPTIMIZATION OF MOLECULAR GENETIC DIAGNOSTICS OF PATIENTS WITH ADVANCED NON-SMALL CELL LUNG CANCER BY INTRODUCING ROS1 TESTING IN THE REPUBLIC OF KAZAKHSTAN

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ABSTRACT

Relevance: *Currently, molecular diagnosis in NSCLC in Kazakhstan includes detection of EGFR, ALK driver mutations status, and PD-L1-status, but not ROS1, what limits the access of patients with this driver mutation to vital therapy.*

The study aimed to *optimize the methods of molecular genetic diagnosis of patients with NSCLC by introducing ROS1 testing in the Republic of Kazakhstan.*

Methods: *The biopsy and surgical material of non-small cell lung cancer (NSCLC) fixed in 10% buffered formalin was studied. After the initial morphological diagnosis of adenocarcinoma, EGFR, and ALK mutation status determination, EGFR and ALK-negative tumor assays were sent for further determination of ROS1 mutation status. First, we performed immunohistochemistry (IHC) using the Ventana BenchMark Ultra platform using the ROS1 antibody (SP283) and the OptiView DAB Detection Kit imaging system. After that, samples with positive and doubtful IHC results were sent for reverse transcriptase polymerase chain reaction (RT-PCR) to confirm the ROS1 mutation status.*

Results: *A total of 99 tumor samples from patients with EGFR-negative and ALK-negative lung adenocarcinoma were studied by IHC from January 01 to September 30, 2022. The results of IHC staining were assessed as 0 (negative) – 59 samples, 1+ (negative) – 25 samples, 2+ (doubtful) – 12 samples, 3+ (positive) – 3 samples. Cases with ≥70% immunostaining were considered positive. Samples with an IHC stain score of 2+ (doubtful), 3+ (positive), and a few samples of 1+ were sent for confirmation by PCR.*

Overall, 22 samples were tested using RT-PCR, and results were considered as follows:1 (4%) – positive, 13 (59%) – negative, 8 (37%) – invalid.

Conclusion: *A large proportion of positive and equivocal results were obtained when determining ROS1 mutation status using IHC, and a large proportion of invalid results during subsequent RT-PCR testing. Choosing methods for nationwide ROS1 implementation, one should evaluate the economics of the methods to be implemented and compare them with a standard validated FISH method.*

Keywords: *ROS1 molecular genetic diagnostics, lung cancer (LC), non-small cell lung cancer (NSCLC), immunohistochemistry (IHC), reverse transcriptase polymerase chain reaction (RT-PCR), fluorescence in situ hybridization (FISH).*

Introduction: Lung cancer (LC) ranks second in incidence after breast cancer and first in cancer mortality in both sexes in the Republic of Kazakhstan (RK) over the past decades. LC incidence in men shares 20% of all cancer cases, significantly higher than in women. In 2021, 3615 new trachea, bronchi, and lung cancer cases were detected. The proportion of cases diagnosed at stages I-II was only 28%, while the proportion of advanced forms (stage IV) of trachea, bronchi, and lung cancer was 27.1 %. The LC survival rate depends on the stage of the disease at diagnosis and remains relatively low. In 2021, one-year mortality from LC in the Republic of Kazakhstan was 43.3%. In Kazakhstan, in 2021, 2,086 men and women died from LC, more than from breast, rectal, and prostate cancers combined [1]. According to Globocan estimates, in 2020 in the Republic of Kazakhstan, the primary incidence of LC was 21.8 per 100,000 population (4642 new cases), and the mortality rate was 16.7 per 100,000 population, which corresponds to 17% of all deaths from cancer [2]. The literature reports that non-small cell lung cancer (NSCLC) accounts for 80-90% of all cases of LC, while the incidence of small cell lung cancer (SCLC) has been declining over the past two decades in many countries [3]. Depending on the histological structure, NSCLC is subdivided into adenocarcinoma (most common subtype, 40-50% of NSCLC), squamous cell carcinoma (25-40% of NSCLC), large cell carcinoma (3-5% of NSCLC), adenos-

quamous (2-3% of NSCLC), and sarcomatoid (2-3% NSCLC) carcinomas [4, 5].

Fig. 1 shows the algorithm for testing patients with advanced NSCLC adopted in Kazakhstan [6].

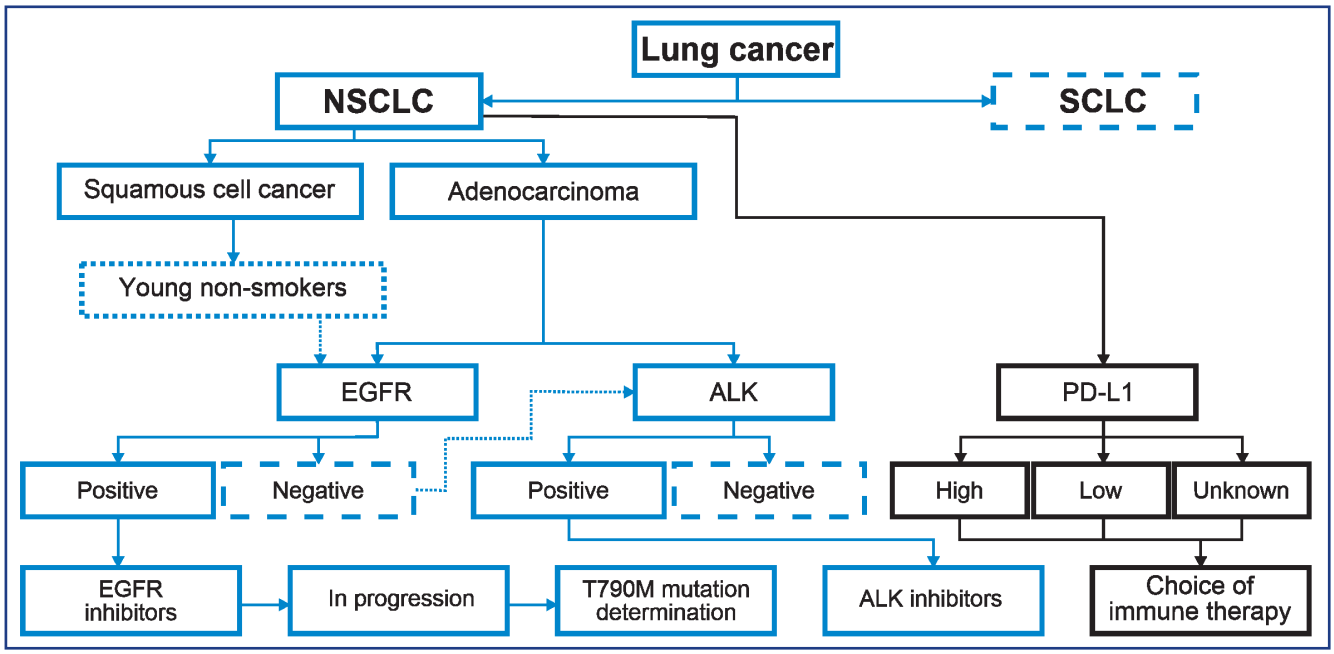

Figure 1 – Algorithm for testing patients with advanced NSCLC in Kazakhstan

Morphological diagnostics is followed by testing for biomarkers. Cancer subtype identification based on biomarker testing enables to identify patients with a high or lower probability of response to therapy to provide individualized treatment [7, 8]. Currently, the US and most European countries do mandatory testing for EGFR, ALK, and ROS1 translocation mutations, including testing for EGFR T790M mutations in disease relapse [9, 10]. Testing for ROS1 mutations was included in the updated protocol for diagnosing and treating LC in the Republic of Kazakhstan [6]. Molecular genetic tests required by the Comprehensive Cancer Control Plan for 2023-2025 in NSCLC include the detection of EGFR, ALK mutations, and PD-L1 status [11]. The project includes the determination of T790M in the progression of EGFR-positive NSCLC (liquid biopsy). Unfortunately, ROS1 mutation testing was not included in the current and previous comprehensive plans to combat oncological diseases [11, 12]. According to the Center for Morphological Studies of Kazakh Institute of Oncology and Radiology JSC (KazIOR JSC), in 2020, 1658 out of 3240 patients with newly diagnosed LC had NSCLC (adenocarcinoma), and 1161 were sampled for testing. In total, 932 patients were tested for EGFR mutation, and 167 (17.9%) were positive; 753 were tested for ALK (38 (5%) positive); 825 were tested for PD-L1 (320 (39%) positive).

ROS1 (ROS Proto-Oncogene 1) is a tyrosine kinase receptor. In NSCLC, ROS1 gene translocation leads to the formation of several oncogenic ROS1 fusion proteins with constitutive kinase activity, CD74-ROS1 the most common. ROS1 translocations are rarely combined with other oncogenic driver mutations, such as ALK translocations and activating EGFR mutations in NSCLC [13, 14]. The reported prevalence of ROS1-positive NSCLC is 1.6% in North America, about 2% in Europe, and 2.3% in Asia [15]. No data on the ROS1 mutation prevalence is currently available in Kazakhstan.

International guidelines recommend testing all patients with advanced adenocarcinoma of the lung for ROS1 mutation, regardless of the clinical symptoms [9, 10, 16]. ROS1 translocations can be detected using several methods: fluorescence in situ hybridization (FISH), RNA-based next-generation sequencing (NGS), or reverse transcriptase polymerase chain reaction (RT-PCR) [16].

FISH is a research-proven method to detect ROS1 gene translocations [9, 10, 16]. Immunohistochemistry (IHC) screening for ROS1 translocation in NSCLC may be preferable to FISH or molecular diagnostics under certain conditions due to a relatively low incidence of these mutations. However, interpreting ROS1 IHC is challenging since about one-third of tumors with no ROS1 mutation can present low-intensity spotted staining [16].

IHC can be used for prescreening; FISH or PCR is required to confirm the diagnosis [10, 16]. NGS is becoming an alternative molecular test for screening or confirming

the presence of gene fusion [16]. However, today, in most countries, NGS is not a routine practice due to its high cost, the need to accumulate multiple samples in one cycle, as well as the availability of sophisticated diagnostic equipment and well-trained personnel [17].

In this study, the ROS1 status of adenocarcinoma patients negative for EGFR, ALK mutations were determined using an alternative ROS1 diagnostic method – IHC followed by RT-PCR – due to the unavailability of FISH for ROS1 at KazIOR JSC during the project implementation.

The study aimed to optimize the methods of molecular genetic diagnosis of patients with NSCLC by introducing ROS1 testing in the Republic of Kazakhstan.

Materials and Methods: The biopsy and surgical samples of NSCLC fixed in 10% buffered formalin were studied. The tissue pieces were subjected to standard processing in a tissue processor and placed into paraffin (FFPE blocks). After initial morphological diagnostics of adenocarcinoma and testing for EGFR and ALK mutations, tumor assays negative for EGFR and ALK mutations were further tested for ROS1 mutations. Paraffin slices 3-4 μm thick were prepared on adhesive glass. First, we performed IHC analysis using the Ventana BenchMark Ultra platform using the ROS1 antibody (SP283) and the OptiView DAB Detection Kit imaging system. After that, IHC-positive or doubtful samples were subjected to RT-PCR to confirm ROS1 mutation status. RT-PCR utilized an AmoyDx ROS1 gene fusion detection kit according to the manufacturer's instructions.

The ROS1 oncogene encodes an orphan receptor tyrosine kinase associated with anaplastic lymphoma kinase (ALK) along with the insulin receptors. ALK and ROS1 synthesize the related tyrosine kinases, whose ATP-binding domains are 77% identical in amino acid composition [18]. Translocation leads to the fusion of the ROS1 part, including the entire tyrosine kinase domain, with 1 of 12 different partner proteins [19].

IHC detects known oncogenic mutations by mutation-specific antibodies in a tissue sample. The antibodies bind with an enzyme or fluorescent dye, which allows to identify the mutation-positive samples with a microscope [19].

To diagnose ROS1 translocations, we used VENTANA ROS1 (SP384) Rabbit Monoclonal Primary Antibody following the published recommendations [19]. IHC diagnostic assays to detect ROS1 utilized a BenchMark Ultra (Ventana) platform with a monoclonal antibody to ROS1 (SP284, Ventana) and the OptiView DAB Detection Kit. The assessments were made using a Leica DM1000 optical microscope. The cytoplasmic staining of type II non-tumor pneumocytes served as an internal positive control. The intensity of cytoplasmic staining of tumor cells was assessed at a magnification of 4 (x4): 0 (negative), 1+ (negative), 2+ (doubtful), and 3+ (positive). The case was considered positive at intensive staining of at least 70% of tumor cells.

RT-PCR was to confirm positive or equivocal ROS1 IHC results. This highly specific method detects the fusion of tumor RNA genes and does not detect alternative fusion partners [19].

The study was performed on a Tanlong Real-time PCR System amplifier with the following reagents in accordance with the manufacturer's instructions: Invitrogen, DNase I, and PureLinktm FEPE (Thermo Fisher Scientific, USA) – for RNA/DNA isolation, NanoDrop 2000 (Thermo Fisher Scientific, USA) – for checking the RNA/cDNA concentration by spectrophotometry, ROS1 Gene Fusions Detection Kit (AmoyDx, China) – for amplification and detection of the results.

PCR results were interpreted according to the manufacturer's instructions for the FAM and HEX/VIC detection channels:

1) For NTC: the Ct FAM values of reaction mixture 1-4 and the Ct HEX/VIC values of reaction mixture four shall be ≥31. If not, the results were considered invalid. According to the recommendation of the manufacturer, the sample should be retested.

2) For positive control: the Ct FAM values of reaction mixtures 1-4 and the Ct HEX/VIC values of reaction mixture four should be <24. If not, the result is invalid. According to the recommendation of the manufacturer, the sample should be retested.

3) Assay for the reference gene (HEX/VIC signal) in reaction mixture 4 for each sample:

a) HEX / VIC значение Ct должно быть ≤20. The HEX/VIC value of Ct should be ≤20.

b) If the HEX/VIC value of Ct is >20, it indicates the RNA degradation or precision reaction of PCR inhibitors. According to the manufacturer's recommendation, the sample should be retested or re-extracted RNA, as there could be false negative results.

4) Analysis for each sample: Ct FAM value of reaction mixtures 1-4 for each sample was recorded:

a) If the Ct FAM value of the reaction mixture 1-4 is ≥30, the sample was identified as negative (ROS1 fusion not detected) or LOD (detection limit) of the set below.

b) If any Ct FAM value of the reaction mixture 1-4 is <30, the sample was identified as positive (ROS1 fusion gene detected).

Fig. 2 shows a simplified algorithm for testing patients with NSCLC adenocarcinoma applied in this study.

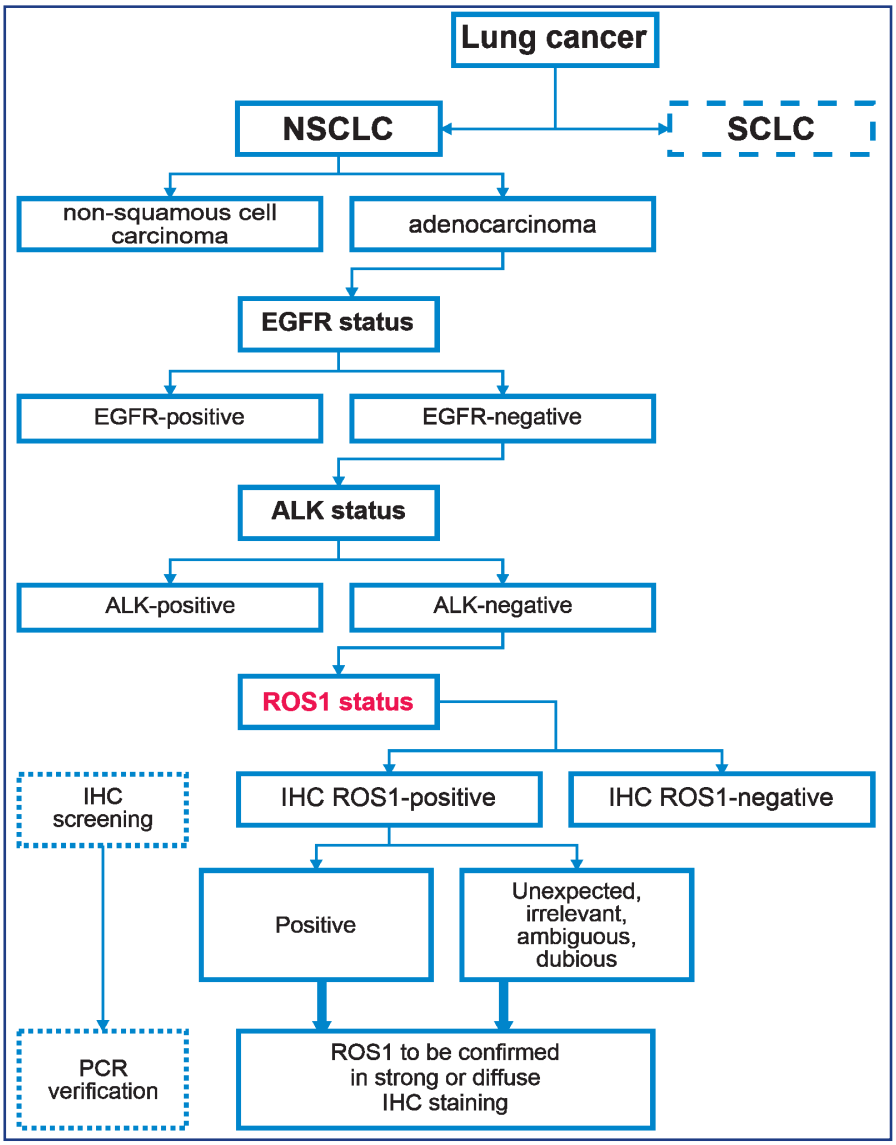

Figure 2 – Algorithm for testing patients with NSCLC adenocarcinoma

Results: A total of 99 tumor samples from patients with EGFR-negative and ALK-negative lung adenocarcinoma were studied by IHC from January 01 to September 30, 2022. The results of IHC staining were assessed as 0 (negative) – 59 samples, 1+ (negative) – 25 samples, 2+ (doubtful) – 12 samples, and 3+ (positive) – 3 samples. Cases with ≥70% immunostaining were considered positive. Samples with an IHC stain score of 2+ (doubtful), 3+ (positive), and a few samples of 1+ were sent for confirmation by PCR.

Overall, 22 samples were tested using RT-PCR at KazIOR JSC Center for Molecular Genetic Studies, and the results were considered as follows:1 (4%) – positive, 13 (59%) – negative, 8 (37%) -– invalid.

Discussion: In IHC, a large proportion of samples – 15 out of 99 samples (15%) – were found positive or equivocal. The IHC results interpretation was problematic due to many positive or equivocal results. This required additional consultations to interpret the obtained data. In some cases, heterogeneous sample staining required a FISH test to assess the tumor sites with bright and moderate staining. The aim was to identify the tumor's intrinsic heterogenicity or attribute the result to the preliminary preparation of the material.

According to RT-PCR results, 1 (4%) of 22 samples was positive, 13 (59%) – negative, and 8 (37%) were invalid. Unfortunately, a large share of invalid samples (37%) suggested a low quality of RNA isolated from FFPE blocks and inappropriate quality of the samples for PCR. The obtained results require further research for accurate conclusions.

N.I. Lindeman et al. report an IHC sensitivity of 96% (95% CI: 71-99%) and specificity of 94% (95% CI: 89-96%) compared to FISH when using D4D6 antibodies with a staining intensity of at least 2+ (as determined by studies). Due to poor IHC specificity (there is no unified assessment of IHC results, and each laboratory selects its limit for staining intensity), the authors also report

difficulties interpreting the staining intensity. However, due to IHC's high sensitivity, the tumor samples without ROS1 staining could be interpreted as negative for ROS1 translocations [16].

In a similar study, 34 111 (30.6%) samples demonstrated an immune reaction to ROS1 in IHC. However, a later FISH test revealed only 5 ROS1-positive tumors out of 34 [20]. Out of 60 samples studied by Shan L. et al., 16 (26.7%), 13 (21.7%), and 20 (33.3%) cases were found ROS1-positive by IHC, FISH, or RT-PCR, respectively [21].

In a study by T.A. Boyle et al., 33 samples were IHC-assessed for ROS1 translocations, and only six samples had a high expression of ROS1 protein. Five of them were also positive for ROS1 gene translocation in a FISH test. Twenty-seven lung cancer biopsy samples negative for ROS1 translocation in genetic testing had low or absent ROS1 protein expression. The authors proposed IHC as a practical and cost-effective ROS1 gene translocations screening method in lung cancer [22].

Although ROS1 mutations are relatively rare and found in 2-3% of lung adenocarcinomas [13-15], tumors with structural translocations involving the ROS1 gene can be successfully treated with targeted drugs. In phase I clinical trial involving 50 patients with NSCLC, a ROS1 translocation found by FISH or RT-PCR predicted a response to targeted inhibition with crizotinib of 72% and a median progression-free survival of 19.2 months [23]. Based on this study, in 2016, FDA expanded the use of crizotinib in NSCLC patients with ROS1 translocations. In a European multicenter retrospective study, 32 NSCLC patients with ROS1 translocation treated with crizotinib demonstrated an ORR of 80% and a median relapse-free survival of 9.1 months [24].

Conclusion: In this study, IHC delivered many positive or equivocal results (15 out of 99) when determining ROS1 mutation status, and subsequent RT-PCR testing revealed a large proportion of invalid results. Choosing a ROS1 detection method for nationwide use shall consider the economic component of proposed methods and compare the results with a standard validated FISH method.

In Kazakhstan, patients with lung cancer have limited access to vital molecular diagnostics to determine ROS1 mutations. Hence, it is necessary to introduce the ROS1 test based on a recommended, approved, and reliable methodology to provide personalized, targeted therapy.

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АҢДАТПА

ҚАЗАҚСТАН РЕСПУБЛИКАСЫНДА ROS1 ТЕСТІЛЕУІН ЕНГІЗУ АРҚЫЛЫ ҰСАҚ ЖАСУШАЛЫ ЕМЕС ӨКПЕНІҢ ҚАТЕРЛІ ІСІГІ БАР НАУҚАСТАРДЫ МОЛЕКУЛАЛЫҚ-ГЕНЕТИКАЛЫҚ ДИАГНОСТИКАЛАУ ӘДІСТЕРІН ОҢТАЙЛАНДЫРУ

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Өзектілігі: *Қазіргі уақытта Қазақстанда ұсақ жасушалы емес өкпенің қатерлі ісігінің (ҰЖЕӨҚІ) молекулярлық диагностикасы EGFR, ALK-дағы драйверлік мутациялардың мәртебесін және PD-L1 мәртебесін, бірақ та ROS1 емес, анықтауды қамтиды, ол осы драйверлік мутацияға оң нәтиже берген пациенттердің өмірлік маңызды терапияны алу мүмкіндігін шектейді.*

Зерттеудің мақсаты *– Қазақстан Республикасында ROS1 тестілеуін енгізу арқылы ҰЖЕӨҚІ бар науқастардың молекулалық-генетикалық диагностикасының әдістерін оңтайландыру.*

Әдістері: *ҰЖЕӨҚІ бар науқастардың 10% буферленген формалинге салынған биопсиялық және операциялық материалы зерттелді. Аденокарциноманың бастапқы патологиялық диагностикасынан кейін, EGFR және ALK мутациясының күйін анықтағаннан кейін, EGFR және ALK теріс мәртебесі бар ісік үлгілері ROS1 мутациясының мәртебесін әрі қарай анықтау үшін жіберілді. Определение статуса мутации ROS1 мутациясының мәртебесін анықтау екі әдіспен жүргізілді: Бірінші әдіс – Ventana BenchMark Ultra платформасында ROS1 антиденесін (SP283) және OptiView DAB Detection Kit визуализация жүйесін пайдалана отырып, иммуногистохимиялық талдау (ИГХ). ИГХ нәтижелері бойынша оң және күмәнді нәтижелері бар үлгілер ROS1 мутациясының мәртебесін растау сияқты екінші әдіс үшін кері транскриптазасы бар полимеразды тізбекті реакцияға (КТ-ПТР) жіберілді.*

Нәтижелері: *01.01.2022 бастап 30.09.2022 дейін ИГХ әдісімен EGFR-теріс және ALK-теріс өкпе аденокарциномасы бар науқастардан алынған жалпы 99 ісік үлгілері зерттелді. ИГХ-бояу нәтижелері былайша бағаланды: 0 (теріс) – 59 үлгі, 1+ (теріс) – 25 үлгі, 2+ (күмәнді) – 12 үлгі, 3+ (оң) – 3 үлгі. ≥70% иммундық бояуы бар жағдайлар оң деп саналды. 2+ (күмәнді), 3+ (оң) және 1+ бірнеше үлгілері ИГХ бояуын бағалауы бар үлгілер ПТР-тестілеумен растауға жіберілді.*

КТ-ПТР бойынша барлығы 22 үлгі тексерілді, нәтижесі келесідей бағаланды: 1 (4%) – оң, 13 (59%) – теріс, 8 (37%) – сәйкес емес.

Қорытынды: *ИГХ арқылы ROS1 мутациясының мәртебесін анықтау көптеген оң және күмәнді нәтижелерге әкелді, ал кейінгі КТ-ПТР тестілеуі кезінде сәйкес емес нәтижелердің үлкен бөлігі орын алды. Әрі қарай елдік деңгейде талдау жүргізу үшін ROS1 анықтау әдісін таңдаған кезде, енгізілетін әдістердің экономикалық шығындық құрамын бағалау керек, сондай-ақ FISH стандартты валидацияланған әдістememен салыстыру қажет.*

Түйінді сөздер: *ROS1 молекулалық-генетикалық диагностикасы, өкпенің қатерлі ісігі, ұсақ жасушалы емес өкпенің қатерлі ісігі (ҰЖЕӨҚІ), иммуногистохимия (ИГХ), кері транскриптазасы бар полимеразды тізбекті реакция (КТ-ПТР), in situ флуоресценттті гибридизациясы (FISH).*

АННОТАЦИЯ

ОПТИМИЗАЦИЯ МЕТОДОВ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКОЙ ДИАГНОСТИКИ ПАЦИЕНТОВ С РАСПРОСТРАНЕННЫМ НЕМЕЛКОКЛЕТОЧНЫМ РАКОМ ЛЕГКОГО ПУТЕМ ВНЕДРЕНИЯ ТЕСТИРОВАНИЯ ROS1 В РЕСПУБЛИКЕ КАЗАХСТАН

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Актуальность: *В настоящее время молекулярная диагностика немелкоклеточного рака легкого (НМРЛ) в Казахстане включает определение статуса драйверных мутаций EGFR, ALK и статуса PD-L1, но не ROS1, что ограничивает возможности получения жизненно важной терапии пациентами с положительным результатов на эту драйверную мутацию.*

Цель исследования *– оптимизация методов молекулярно-генетической диагностики пациентов с НМРЛ путем внедрения тестирования ROS1 в Республике Казахстан.*

Методы: *Исследовался биопсийный и операционный материал пациентов с НМРЛ, фиксированный в 10%-ном забуференном формалине. После первоначальной морфологической диагностики аденокарциномы, определения статуса мутаций EGFR и ALK образцы*

опухоли с отрицательным статусом EGFR и ALK отбирались для дальнейшего выявления статуса мутации ROS1. Определение статуса мутации ROS1 проводилось двумя методами: первый метод – иммуногистохимический анализ (ИГХ) на платформе Ventana BenchMark Ultra с использованием антитела ROS1 (SP283) и системы визуализации OptiView DAB Detection Kit. По результатам ИГХ образцы с положительными и сомнительными результатами направлялись на полимеразную цепную реакцию с обратной транскриптазой (ОТ-ПЦР), чтобы подтвердить статус мутации ROS1 – второй метод.

Результаты: *С 01.01.2022 по 30.09.2022 гг. всего методом ИГХ исследовано 99 образцов опухолей пациентов с EGFR-отрицательной и ALK-отрицательной аденокарциномой легкого. Результаты ИГХ-окрашивания расценивали как: 0 (отрицательно) – 59 образцов, 1+ (отрицательно) – 25 образцов, 2+ (сомнительно) – 12 образцов, 3+ (положительно) – 3 образца. Случаи с ≥70% иммуноокрашивания считались положительными. Образцы с оценкой окрашивания ИГХ 2+ (сомнительно), 3+ (положительно) и несколько образцов 1+ были отправлены на подтверждение ПЦР-тестированием.*

Всего с помощью ОТ-ПЦР было протестировано 22 образца; результаты расценены следующим образом: 1 (4%) – положительный, 13 (59%) – отрицательный, 8 (37%) – невалидный.

Заключение: *При определении статуса мутации ROS1 с помощью ИГХ было получено большое количество положительных и сомнительных результатов, а при последующем тестировании ОТ-ПЦР – большая доля невалидных результатов. В дальнейшем при выборе методики обнаружения ROS1 для проведения анализов на страновом уровне необходимо оценить экономическую затратную составляющую внедряемых методов, а также сравнить со стандартной валидированной методикой FISH.*

Ключевые слова: *молекулярно-генетическая диагностика ROS1, рак легкого (РЛ), немелкоклеточный рак легкого (НМРЛ), иммуногистохимия (ИГХ), полимеразная цепная реакция с обратной транскриптазой (ОТ-ПЦР), флуоресцентная гибридизация in situ (FISH).*

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