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# The role of pAKT1 expression in diffuse large B-cell lymphoma

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## ABSTRACT

**Aim.** To evaluate the prognostic value of pAKT1 expression by tumor cells in patients with diffuse large B-cell lymphoma.

**Materials and methods.** The study included 90 patients with newly diagnosed diffuse large B-cell lymphoma (DLBCL), who were treated at the clinic of Kirov Research Institute of Hematology and Blood Transfusion from 2014 to 2017 and received standard first-line polychemotherapy according to the R-CHOP regimen. Using immunohistochemical and morphometric methods, the relative number of tumor cells expressing pAKT1 was determined. Using the two-sided Fisher's exact test, the relationship of different levels of marker expression with clinical and laboratory parameters of patients and long-term treatment results was analyzed. The impact of pAKT1 on the risk of an adverse event was assessed using the Cox regression analysis.

**Results.** Overexpression of pAKT1 is associated with unfavorable clinical characteristics of patients with DLBCL, excessive expression of the BCL2 and c-Myc oncoproteins, as well as with low rates of overall and progressive survival. Overexpression of pAKT1 is an independent prognostic factor and statistically significantly affects the risk of an adverse outcome in DLBCL.

**Conclusion.** The degree of pAKT1 expression is an informative criterion that allows to predict the course of diffuse large B-cell lymphoma. It is advisable to use the indicated marker when stratifying patients into risk groups.

**Key words:** diffuse large B-cell lymphoma, pAKT1, overexpression, survival, biomarker.

**Conflict of interest.** The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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**Conformity with the principles of ethics.** All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Kirov Research Institute of Hematology and Blood Transfusion (Protocol No. 48 of 12.10.2019).

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# Значение экспрессии pAKT1 при диффузной В-крупноклеточной лимфоме

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**РЕЗЮМЕ**

**Цель** – оценить прогностическое значение экспрессии pAKT1 опухолевыми клетками у больных диффузной В-крупноклеточной лимфомой.

**Материалы и методы.** В исследование включены 90 пациентов с впервые диагностированной диффузной В-крупноклеточной лимфомой (ДВККЛ), наблюдавшиеся в клинике института с 2014 по 2017 г. и получавшие стандартную полихимиотерапию первой линии по схеме R-CHOP. С помощью иммуногистохимического и морфометрического методов определено относительное количество экспрессирующих pAKT1 опухолевых клеток. С помощью точного двухстороннего критерия Фишера проанализирована взаимосвязь различных уровней экспрессии маркера с клинико-лабораторными показателями пациентов и отдаленными результатами лечения. Оценку влияния pAKT1 на риск наступления неблагоприятного события проводили с помощью регрессионного анализа Кокса.

**Результаты.** Гиперэкспрессия pAKT1 ассоциирована с неблагоприятными клиническими характеристиками больных диффузной В-крупноклеточной лимфомой, избыточной экспрессией онкобелков BCL2, cMyc, а также низкими показателями общей и беспрогрессивной выживаемости. Гиперэкспрессия pAKT1 является независимым фактором прогноза и статистически значимо влияет на риск возникновения неблагоприятного исхода при ДВККЛ.

**Заключение.** Степень экспрессии pAKT1 является информативным критерием, позволяющим прогнозировать течение диффузной В-крупноклеточной лимфомы. Указанный маркер целесообразно использовать при стратификации пациентов на группы риска.

**Ключевые слова:** диффузная В-крупноклеточная лимфома, pAKT1, гиперэкспрессия, выживаемость, биомаркер.

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## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is known as the most common type of lymphoid neoplasia in adults. On average, it accounts for 40% of all non-Hodgkin lymphomas, varying slightly across geographic regions. The disease is characterized by high biological and clinical heterogeneity, rapid tumor growth, and early extranodal lesions [1–3].

Standard treatment for common variants of DLBCL is first-line polychemotherapy according to the R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone). It provides good overall survival (OS) and progression-free survival (PFS). However, the disease is refractory in more than one-third of patients [4, 5].

The International Prognostic Index (IPI) and age-adjusted International Prognostic Index (aaIPI) are used as the main tools for assessing individual risk of early disease progression. These indices are quite successfully used in clinical practice, but in some cases, they do not allow to accurately determine the

prognosis of the disease, since they mainly rely on the clinical characteristics of patients and do not take into account aspects related to biology of neoplastic cells [4, 6, 7].

It was found that molecular and biological characteristics of the tumor can influence the course of DLBCL and play an important role in the onset of unfavorable outcomes. Thus, based on the gene expression profiling (GEP), the main molecular subgroups of DLBCL (ABC and GCB) were identified. These subgroups to a large extent correlate with the disease prognosis when using standard chemotherapy regimens [2, 8]. Unfortunately, integration of GEP in clinical practice is limited due to its technological complexity and high cost. Surrogate markers in immunohistochemical analysis determined using various algorithms do not fully correlate with GEP, which reduces the accuracy of obtained prognostic information [9]. Therefore, search for other informative molecular and biological predictors of the disease course remains relevant [4].

In recent years, much attention has been paid to studying various signaling pathways involved in the pathogenesis of DLBCL. One of them is the PI3K / AKT / mTOR pathway, which has crosstalk with many other oncogenic signaling pathways and mediates a wide range of cellular functions, including proliferation, apoptosis, metabolism, and angiogenesis [10]. The main components of this signaling pathway are phosphoinositide 3-kinase (PI3K), AKT1 serine / threonine kinase, and the mTOR protein. The most important of them is the AKT1 enzyme, a key regulatory protein of this pathway.

Intact AKT1 is located in the cytoplasm of the cell. The pathway is activated by binding of a ligand to a cellular receptor and subsequent initiation of an enzymatic cascade of phosphorylation reactions. As a result, a complex of phospholipids is formed on the cell membrane, the most important of which being $PIP_3$ (phosphatidylinositol-3-4-5-triphosphate). It binds to the N-terminal domain of AKT1 and recruits it to the cytoplasmic membrane. Here, the protein kinase is activated (pAKT1) in the catalytic and hydrophobic domains through sequential interaction with PDK1 / 2 (3-phosphoinositide-dependent protein kinase-1, 2) and mTOR. Then it leaves the membrane and moves to the cytoplasm and the cell nucleus. In normal conditions, protein phosphorylation is a temporary and strictly controlled process [11].

According to foreign studies, the PI3K / AKT / mTOR signaling pathway is constitutively activated in 25–50% of DLBCL cases [12]. This contributes to a rise in the level of intracellular signaling and an increase in the survival rate and proliferation of tumor cells and is accompanied by tumor resistance to standard chemotherapy and high incidence of relapses. It was shown that this pathway can activate the nuclear factor-kappa B (NF-κB), triggering the oncogenic JAK / STAT signaling pathway. This enhances expression of apoptosis inhibitors (survivin and TIMP1) and promotes the development of immortality of tumor cells [8, 10].

There is evidence that an increased level of pACT1 indirectly affects tumor resistance to chemotherapy through negative regulation of the activity of transport proteins that control the entry and exit of substances from the cell [13]. At the same time, the prognostic value of pAKT1 expression in DLBCL has been studied insufficiently. The results of the published works are contradictory. There is no information on this topic in the domestic scientific literature.

The aim of the study was to evaluate the prognostic value of pAKT1 expression in tumor cells in DLBCL.

## MATERIALS AND METHODS

The retrospective study included 90 patients with a newly diagnosed DLBCL who were treated at the clinic of Kirov Scientific Research Institute of Hematology and Blood Transfusion from 2014 to 2017. All patients received standard first-line therapy according to the R-CHOP regimen. The average age of patients was 58 years (from 24 to 83 years). All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee. Clinical and laboratory characteristics of patients with DLBCL are presented in Table 1.

Formalin-fixed, paraffin-embedded (FFPE) lymph node and other organ and tissue samples were used, from which 3–5 μm-thick histological sections were prepared according to the generally accepted method. Verification of DLBCL diagnosis and visualization of pAKT1-positive tumor cells in the studied samples were carried out by the immunohistochemical method using antibodies to CD3, CD20, CD10, BCL6, MUM1, Ki67, BCL2, cMyc, and pAKT1 (phosphor Ser 473). The tumor subtype (GCB or non-GCB) was determined according to the classification proposed by C.P. Hans [14].

Immunoreactivity of primary antibodies was detected using secondary antibodies conjugated with peroxidase included in the reagent kit. Immunohistochemical reactions were set up according to the stan-

dard technique following the protocol for ENVISION imaging systems (DAB+, Dako, Denmark). The relative number of tumor cells with nuclear expression of pAKT1 was calculated on the AxioScope.A1 microscope (Carl Zeiss Microscopy GmbH, Germany) with a built-in photo / video camera and image analysis software. The study was carried out in five fields of view for each sample using the x10 eyepiece lens and the x100 objective lens.

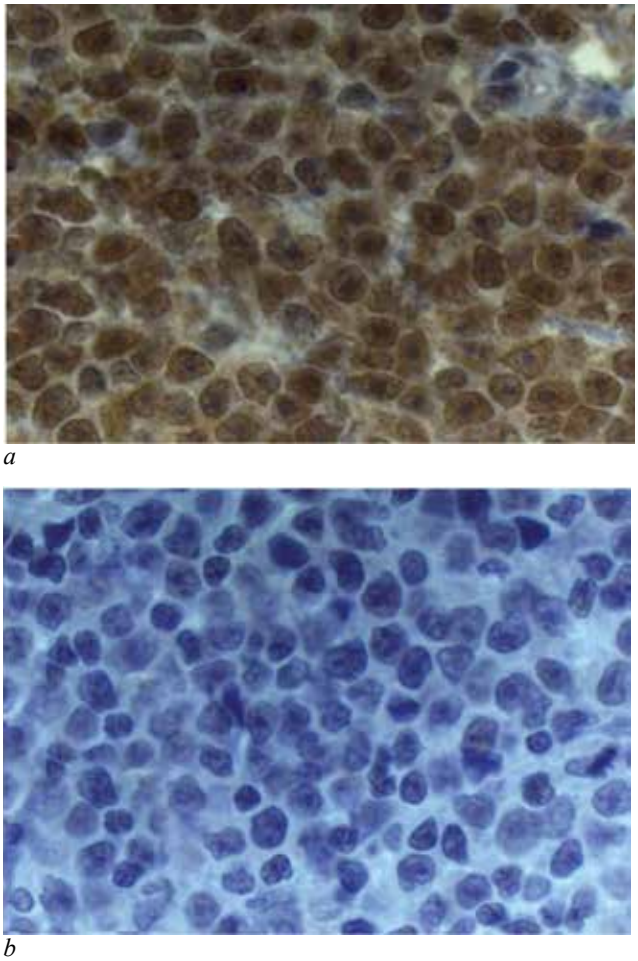
The cut-off threshold for assessing the level of pAKT1 expression was calculated using the ROC analysis. For the oncoproteins BCL2 and cMyc, the expression thresholds used in international studies and showing the most reproducible results (50 and 40% of positive tumor cells, respectively) were used [15–17]. Statistical assessment of the presence / absence of an association between the degree of pAKT1 expression and the clinical and laboratory characteristics of patients was carried out using the two-sided Fisher's exact test. OS and PFS were calculated using the Kaplan – Meier method with graphical plotting of the corresponding curves.

A comparative analysis of survival rates was performed using the log-rank test. To identify prognostic factors influencing the risk of developing an adverse event for OS and PFS, we used univariate and multivariate Cox regression analysis with the determination of a 95% confidence interval (CI) and hazard ratio (HR). The study was carried out using the IBM SPSS version 19.0 software. Differences between indicators were considered statistically significant at $p < 0.05$.

## RESULTS

During the research, all patients were divided into two groups depending on the number of pAKT1-positive tumor elements. According to the results of the ROC analysis, the optimal cut-off threshold for protein expression was set at 70% with sensitivity of 84% and specificity of 24%; the area under the curve was 0.78 ± 0.04 (95% CI 0.66–0.91). Group 1 included 36 patients (40%) with protein overexpression ( ≥ 70% of cells, Fig. 2*a*), group 2 contained 54 (60%) patients with a low degree of marker expression (< 70% of cells, Fig. 1*b*).

Following a comparative assessment of the degree of pAKT1 expression by DLBCL tumor cells with the clinical and laboratory characteristics of patients, statistically significant intergroup differences were revealed (Table 1). It was noted that the values of IPI > 2 were more often detected in the patients of group 1 compared with its values in the patients of group 2 (58% vs. 33%, $p = 0.029$). The frequency of occurrence of stage III–IV of the pathological process was significantly higher in the patients with pAKT1 overexpression than in the patients with low expression of this marker (72% vs. 48%, $p = 0.030$).



*a*

*b*

Fig. 1. Immunohistochemical staining of tumor cells with an antibody to pAKT1: high (*a*) and low (*b*) expression, x 1,000

The patients of group 1 were 1.5 times more likely to have B symptoms of the disease than those of group 2 (69% vs. 46%, $p = 0.034$). When analyzing the results of R-CHOP chemotherapy, it was found that the patients with low marker expression were characterized by higher frequency of achieving complete remissions than patients with overexpression of the marker (44% vs. 70%, respectively, $p = 0.017$). In addition, the supra-threshold values for the number of pAKT1-positive tumor cells were associated with high expression of the oncoproteins BCL2 ($p = 0.036$) and c-Myc ($p = 0.015$). No significant intergroup differences were found for other clinical and laboratory parameters.

Table 1

**Patient characteristics and results of analysis of clinical and laboratory parameters in the groups with different pAKT1 expression in patients with DLBCL**

| Parameters | Number of patients, $n = 90$, abs. (%) | pAKT1 expression | | Fisher's exact test, $p$ |
|---|---|---|---|---|
| | | high, abs., $n = 36$ | low, abs., $n = 54$ | |
| Gender:<br>males<br>females | <br>47 (52)<br>43 (48) | <br>18<br>18 | <br>25<br>29 | 0.830 |
| Age, years:<br>≥ 60<br>< 60 | <br>40 (44)<br>50 (56) | <br>17<br>19 | <br>23<br>31 | 0.672 |
| B symptoms:<br>present<br>absent | <br>50 (56)<br>40 (44) | <br>25<br>11 | <br>25<br>29 | 0.034* |
| Stage (according to Ann Arbor staging):<br>I–II<br>III–IV | <br>38 (42)<br>52 (58) | <br>10<br>26 | <br>28<br>26 | 0.030* |
| Extranodal lesions:<br>present<br>absent | <br>41 (46)<br>49 (54) | <br>18<br>18 | <br>23<br>31 | 0.523 |
| Level of lactate dehydrogenase (LDH):<br>within the normal values<br>exceeds the normal values | <br>30 (33)<br>60 (66) | <br>25<br>11 | <br>35<br>19 | 0.820 |
| Immunohistochemical subtype:<br>GCB<br>non-GCB | <br>34 (38)<br>56 (62) | <br>10<br>26 | <br>24<br>30 | 0.126 |
| IPI, risk groups:<br>IPI ≤ 2<br>IPI > 2 | <br>49 (54)<br>41 (45) | <br>15<br>21 | <br>36<br>18 | 0.029* |
| Response to first-line therapy:<br>partial response<br>response / relapse / refractory disease<br>complete response | <br>54 (60)<br>36 (40) | <br>20<br>16 | <br>16<br>38 | 0.017* |
| BCL2 expression:<br>≥ 50%<br>< 50% | <br>28 (31)<br>62 (69) | <br>16<br>20 | <br>12<br>42 | 0.036* |
| c-Myc expression:<br>≥ 40%<br>< 40% | <br>18 (20)<br>72 (80) | <br>12<br>24 | <br>6<br>48 | 0.015* |

Note: Fisher's exact test – $p$.
* statistically significant differences between the groups (here and in Table 2).

Three-year OS in group 1 was 44% (the median survival was 23 months) as opposed to 87% in group 2 (the median survival was not reached), $p < 0.001$, (Fig. 2*a*). Significant differences were found in the assessment of PFS: 39% – in the patients with high expression of pAKT1, 71% – in the patients with low expression of the marker, $p = 0.005$. The median survival in group 1 was 14 months, and it was not reached in group 2 (Fig. 2, *b*).

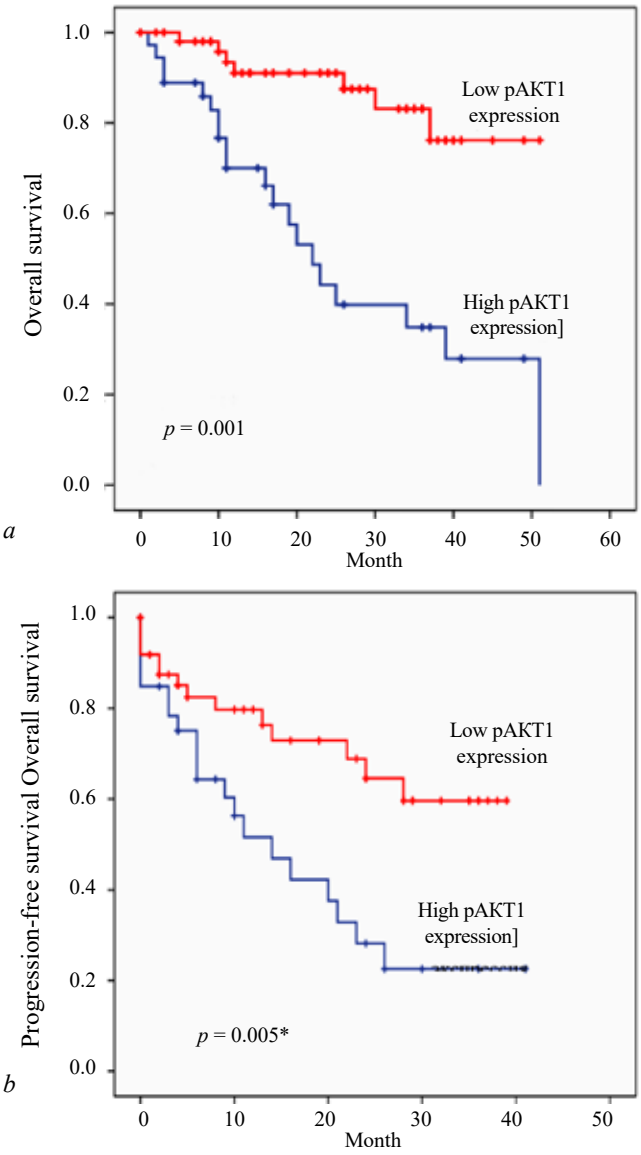



Fig. 2. Overall (*a*) and progression-free (*b* )survival of patients with diffuse large B-cell lymphoma depending on the degree of pAKT1 expression by tumor cells

Clinical and laboratory parameters of patients were initially included in the univariate Cox analysis, following which the factors adversely affecting OS in patients with DLBCL were: IPI > 2, non-GCB subtype, age ≥ 60 years, and pAKT1 expression ≥ 70% (Table 2). In the course of the multivariate regression analysis, it was found that the most significant independent predictive factors were only IPI and overexpression of pAKT1 ($p = 0.039$ and $p = 0.001$, respectively). At the same time, the content of pAKT1-positive tumor cells ≥ 70% determined a two times higher risk of an unfavorable outcome than the IPI index.

Table 2

**Univariate and multivariate Cox analysis of predictors of overall and progression-free survival in patients with diffuse B-large cell lymphoma**

| Parameter | Univariate analysis | | | | | |
|---|---|---|---|---|---|---|
| | OS | | | PFS | | |
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Gender | 1.893 | 0865–4.140 | 0.112 | 1.954 | 0.965–3.955 | 0.063 |
| Age ≥ 60 years | 2.254 | 1.013–5.015 | 0.046* | 1.511 | 0.763–2.993 | 0.236 |
| IPI > 2 | 6.319 | 2.377–16.800 | < 0.001* | 3.727 | 1.810–7.675 | < 0.001* |
| non-GCB subtype | 0.351 | 0.132–0.932 | 0.035* | 0.367 | 0.160–0.844 | 0.018* |
| Expression of pAKT1 ≥ 70% | 9.954 | 3.432–28.870 | < 0.001* | 3.527 | 1.170–7.275 | 0.001* |
| Multivariate analysis | | | | | | |
| Parameter | OS | | | PFS | | |
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Age ≥ 60 years | 1.933 | 0.813–4.595 | 0.135 | – | – | – |
| IPI > 2 | 3.061 | 1.056–8.868 | 0.039* | 2.577 | 1.191–5.574 | 0.016* |
| non-GCB subtype | 0.623 | 0.219–1.772 | 0.375 | 0.607 | 0.252–1.467 | 0.268 |
| Expression of pAKT1 ≥ 70% | 6.171 | 2.069–18.402 | 0.001* | 2.456 | 1.141–5.287 | 0.022* |

For PFS, the same factors showed statistical significance in the univariate Cox analysis, except for patient age. When assessing the effect of several predictors on disease progression, IPI > 2 and pAKT1 expression ≥ 70% were also significant independent prognostic factors with a comparable risk of an adverse event.

## DISCUSSION

DLBCL is the most common non-Hodgkin's lymphoma, which encompasses a large group of tumors that differ in their clinical, morphological, and molecular genetic characteristics. Standard therapeutic approaches provide good treatment results in only 50–60% of cases. At the stage of early diagnosis, the existing systems of stratification (IPI) do not always provide an opportunity to accurately predict the course of the disease in each individual patient, since they do not take into account the molecular and biological aspects of the pathology. This is especially relevant for low- and intermediate-risk patients. Therefore, the search for additional prognostic criteria is justified in this disease.

The results obtained in this study indicate a relationship between overexpression of pAKT1 and unfavorable prognostic clinical and laboratory characteristics: common stages of the disease, IPI values > 2, the presence of B symptoms, and high expression of the BCL2 and c-Myc oncoproteins. This association may be due to uncontrolled activation of the Mdm2 protein and impaired functioning of the p53 and p21 tumor suppressors. This leads to inhibition of apoptosis and excessive proliferation of neoplastic cells [10, 16]. In patients with supra-threshold values of pAKT1 expression, lower frequency of achieving complete remissions was noted. However, for some parameters, there is significant variation in the research results.

In a number of studies, in addition to the indicated clinical characteristics, a relationship was found between the pAKT1-positive variant of the disease and gender, age, and LDH level [8, 18, 19]. We believe that these disagreements may be associated with a lack of unified criteria for determining the threshold values for pAKT1 expression, comorbidity, and different sample sizes. In some foreign studies, an association of pAKT1 overexpression with low survival in DLBCL patients was shown [17, 18]. The results of our study confirm this.

At the same time, based on the multivariate analysis data, it was revealed that overexpression of pAKT1 is an independent predictor of low OS, which is more significant in prognostic terms than the IPI index. For PFS, the studied marker was also a significant independent prognostic factor. There is no consensus among the authors regarding this fact, the research results are contradictory. There are foreign studies in which the role of pAKT1 expression as an independent prognostic factor was not proven. It is assumed that adverse clinical manifestations associated with excessive activation of the PI3K / AKT / mTOR pathway are indirect and depend on other effectors [17]. At the same time, there are publications confirming the independent prognostic role of this marker in relation to the course of the disease [8, 18, 19].

## CONCLUSION

Overexpression of pAKT1 is associated with unfavorable clinical and laboratory parameters in patients

with DLBCL and treatment failures according to the R-CHOP regimen. This biomarker could be used as an independent predictor of the course of the disease.

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## Authors contribution

Vaneeva E.V., Rosin V.A. – design of the study, collection of data, carrying out of the practical part of the study, analysis and interpretation of data, drafting of the manuscript. Diakonov D.A., Rosin V.A. – development of the research direction, critical revision of the article for important intellectual content. Luchinin A.S. – analysis and interpretation of statistical data. Samarina S.V. – collection of clinical data of DLBCL patients. Kochetov N.L. – selection of biopsy samples.

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