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ЭКСПРЕССИЯ БЕЛКА P63 В АДЕНОКАРЦИНОМАХ ЛЕГКИХ КАК ФАКТОР НЕБЛАГОПРИЯТНОГО ПРОГНОЗА

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Цель. Исследовать спектр клеточных молекулярно-биологических маркеров и выявить среди них те, которые могут быть использованы в качестве факторов прогноза клинического течения аденокарциномы легкого. ***Материалы и методы.*** В настоящей работе был использован архивный материал 129 пациентов с подтвержденным диагнозом аденокарциномы легкого. В работе применены гистологический, иммуногистохимический, молекулярно-генетический и статистический методы. ***Результаты.*** В 29 случаев (47,5%) аденокарцином легкого в разной пропорции клеток наблюдалась очаговая цитоплазматическая и/или ядерная экспрессия белка p63. При экспрессии в клетках опухоли p63 безрецидивная выживаемость (БРВ) составила, в среднем, 25,7±5,1 месяца, в то время как у пациентов без экспрессии p63 БРВ – 26,1±2,8 месяца. Этот показатель не влиял на общую выживаемость пациентов, которая в среднем составила 33,6±2,7 месяца. ***Заключение.*** Была выявлена слабо выраженная тенденция к снижению безрецидивной выживаемости пациентов с p63-позитивными АК легких. Выявление экспрессии p63 в аденокарциномах легкого может рассматриваться в качестве фактора неблагоприятного прогноза и риска более быстрого прогрессирования опухолевого процесса и требует дальнейшего изучения с увеличением статистической мощности исследования.

Ключевые слова*: экспрессия; p63; аденокарцинома легкого; рак легкого; ген TP63.*

EXPRESSION OF P63 PROTEIN IN PULMONARY ADENOCARCINOMAS AS FACTOR OF POOR PROGNOSIS

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Aim. To study the spectrum of cellular molecular-biological markers and identify those of them that can be used as prognostic factors for the clinical course of pulmonary adenocarcinoma. ***Material and Methods.*** In the given work archive material of 129 patients with confirmed diagnosis of pulmonary adenocarcinoma was used. In the work, histological, immunohistochemical, molecular-genetic and statistical methods were used. ***Results.*** In 29 cases (47.5%) of pulmonary ade-

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nocarcinoma, focal cytoplasmic and/or nuclear expression of p63 protein was observed in different proportions of cells. With expression of p63 in tumor cells, relapse-free survival was on average 25.7±5.1 months, while in patients with no expression of p63 it was 26.1±2.8 months. This parameter did not influence the overall survival of patients which was on average 33.6±2.7 months. ***Conclusion.*** A weak tendency to reduction of relapse-free survival of patients with p63-positive pulmonary carcinoma of lungs was revealed. Identification of p63 in pulmonary adenocarcinoma may be regarded as a factor of unfavorable prognosis and of risk of faster tumor progression, which requires further study to increase the statistical value of research.

Keywords: *expression; p63; pulmonary adenocarcinoma; pulmonary cancer; TP63 gene.*

Pulmonary cancer (PC) is a heterogeneous group of malignant neoplasms of lungs which may be divided to 2 main groups – small cell lung cancer (SCLC, less than 15%) and non-small cell lung cancer (NSCLC). Adenocarcinomas (AC) constitute the largest part of NSCLC and account for 30-40% of all cases of PC. According to the classification of WHO, the group of AC includes tumors of different structure and different extent of differentiation [1]. This subtype mostly affects women, non-smoking individuals, and usually localizes in the peripheral parts of lungs [2,3].

Morphological diagnosis of AC of lungs is usually based on histological preparations stained with hematoxylin and eosin. However, in some cases the diagnosis is associated with certain difficulties. Specification of the diagnosis in such difficult cases requires immunohistochemical examination with use of markers (antibodies) specific for different kinds of lung tissue, and of a wider range of markers on suspicion of the metastatic origin of the formation. Usually the diagnosis of the majority of AC cases is confirmed by a 3-marker panel which characterizes the immunophenotype of this type of tumor – cytokeratin 7 (CK7), transforming growth factor TTF-1, naps in A. Differential diagnosis of squamous cell cancer of lung suggests use of such markers as CK6 (cytokeratins that are usually intensely expressed by cells of squamous epithelium), p63 and TTF-1 [4-6]. However, immunephenotype of low-differentiated variants of AC and squamous cell cancer (SCC) of lung is often characterized by expression of markers non-inherent to well-differentiated variants. Thus, in some low-differentiated SCC of lungs, expression of TTF-1 (in 0.9% of cases) may be found, and, vice versa, p63is sometimes expressed by populations of AC cells (according to different data, in 1.3 cases of AC) [7-9]. Thus, Warth, et al. (2012) showed that in 12.1% of cases verified as AC of lung, expression of p63 was found [10]. It is important to bear in mind that the amount of p63-positive cells in AC is considerably lower than in SCC (where it is expressed by almost 100% of tumor cell nuclei) and they usually have focal, rather than diffused distribution [10].

Normally, expression TP63 gene is identified in basal and suprabasal cells of squamous epithelium of skin, esophagus, uterus, cervix, tonsils, urinary bladder, bronchi, mammary glands and prostate [11]. This gene plays an important role in maintenance of the population of pluripotent (stem) cells in these organs. It should be noted that point mutations in p63 gene are rarely identified, but they are rather common in different malignant tumors, including those of lungs, they induce amplification of gene and over expression of the protein coded for by this gene [12].

Aim – to study the spectrum of cellular molecular-biological markers and to identify those among them that can be used to predict the clinical course of pulmonary adenocarcinoma.

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Materials and Methods

This work used archive material of 129 patients with confirmed diagnosis of PA who were examined and given treatment in M.F. Vladimirsky Moscow Regional Research Clinical Institute from January 2014 to July 2018. Use of biological material for scientific purposes was approved by the Local Ethics Committee.

Operations and diagnostic biopsies were taken in the Thoracic Department of the Institute. The diagnosis was made after histological examination of preparations stained with hematoxylin and eosin, and if necessary – after immunohistochemical examination according to recommendations of the applicable WHO classification of lung cancer [1].

Immunohistochemical examination was conducted according to the standard protocol. The material was fixed in 10% buffered formalin, sections of 4-5 μm thickness were applied on glasses with adhesive coating, after that they were dried at 37°C during night and then for one more hour at 60°C. Then sections were subjected to deparaffinization and restoration of antigenicity in accordance with the protocol recommend-ded for each specific antibody. On the surgical material IHC reaction was conducted in the automatic mode using the auotostainer Ventana (Italy), and on the diagnostic biopsies in case of their low amount it was done on serial sections manually. The antibody panel was used: cytokeratin (CK) of wide spectrum, (AE1/AE3 clone), CK of high molecular weighs (beta E12 clone) CK7, CK5/6, p63, TTF-1, napsin A (Dako, Cell Marque, Ventana).

Mutations in EGFR gene in pulmonary cancer tissue were identified by polymerase chain reaction in real time with use of reagent kit Therascreen® EGFRRGQ PCR Kit (Qiagen, Holland) on Rotor-Gene Q apparatus (Qiagen, Holland) in accordance with the protocol of the manufacturer. DNA was isolated from paraffin blocks using Cobas DNA Sample Preparation Kit.

Categorical variables were analyzed using percentages, continuous variables were expressed in the form of mean values and standard deviations. Relapse-free survival rate was analyzed by Kaplan-Meier method; statistical significance of survival differences was checked by log rank test. Risk factors were analyzed using univariate regression. The differences were considered statistically significant at $p<0.05$. Statistical analyses were performed using soft ware SPSS version 21.0 (IBM Corporation, USA).

Of 129 patients with pulmonary adenocarcinoma (93 surgical and 43 diagnostic biopsies), 67 were men (52%) and 62 women (48%). 87 Patients were in the age group from 40 to 70 years old (67.4%), above 70 – 37 patients (28.7%), under 40 – 5 patients (3.9%).

According to TNM classification [13], patients with 1st stage PC made 10.1% (13 patents), 2nd stage – 10.1% (13 patients), 3nd stage – 17.8% (23 patients) and 4th stage – 10.8% (14 patients). In some patients TNM stage was absent. Summary data for the analyzed sample are presented in Table 1.

Results and Discussion

In 75 of 129 cases PA was diagnosed on the basis of morphological examination. All the rest cases required use of additional method – IHC which included a set of organ- and cell-specific markers corresponding to the localization of tumor in the lung. PA was determined on the basis of co-expression of CK7, TTF-1 and napsin A, and of the absence of expression of CK5/6 and p63 in tumor cells. However, in some cases there was observed expression of markers non-characteristic of the given PA subtype (Table 2). In 96.4% of cases (50 of 55) expression of CK7 in PA cells was observed, in 94.7% of cases (54 from 57) – of napsin A, in 34.8% (8 of 23) – of CK 5/6, in 47.5% (29 of 32) – of p63 (Table 2 and Figure 1).

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Table 1

Clinico-Demographic Characteristics of Patients with Pulmonary Adenocarcinoma Included into Analysis

Parameter	Number of Patients	%
Sex		
men	67	52
women	62	48
Age		
under 40 years	5	3.9
40-70 years	87	67.4
above 70 years	37	28.7
PC degree		
1st degree	13	10.1
2nd degree	13	10.1
3d degree	23	17.8
4th degree	14	10.8

Table 2

Expression of Immunohistochemical Markers in Cells of Pulmonary Adenocarcinoma

Markers	Positive Expression	Absence of Expression
CK7	53 (96.4%)	2 (3.6%)
TTF-1	50 (90.9%)	5 (9.1%)
Napsin A	54 (94.7%)	3 (5.3%)
CK5/6	8 (34.8)	15 (65.2)
p63	29 (47.5%)	32 (52.5%)

According to the preliminary data obtained in the study, expression of only one marker in PA cells – p63 – is of prognostics significance. The difference in relapse-free survival (RFS) rate between the groups made 2.48±1.11 months, $p<0.026$. However, in case of increase in sample of patients and of the time of their observation, this tendency leveled out. But it should be noted that cytoplasmic and/or nuclear expression of p63in different proportion of PA cells was observed in 29 cases (47.5%, Figure 1). These patients presented with a more aggressive course of the disease. Thus, in patients with PA with expression of p63, RFS was on average 25.7±5.1 months, while in patients with no expression of p63 it was 26.1±2.8 months.

In conclusion, the following should be noted. P63 protein is coded for byTP63 gene that was discovered in 1998 and represents the TP53 family. It is located on 3q27-29 chromosome and consists of 15 exons. The protein coded for by this gene has a high degree of homology with p53 protein in regions of transactivation, DNA-binding and oligomerization domains, but differs from it in its C-terminal fragment. Unlike TP53 gene which codes only for one protein, although in different modifications, TP63 codes for several protein products at once. Matrix RNA (mRNA) of p63 may be synthesized from two promotors (TA and ΔN) and then be exposed to 3 alternative splicing regions (a, b and c) located in the carboxyl terminal region of the gene. In result, with participation of TP63 gene, 6 isoforms of p63 protein may be produced, both possessing (TA-forms) and non-possessing (ΔN-forms) transactivation domain

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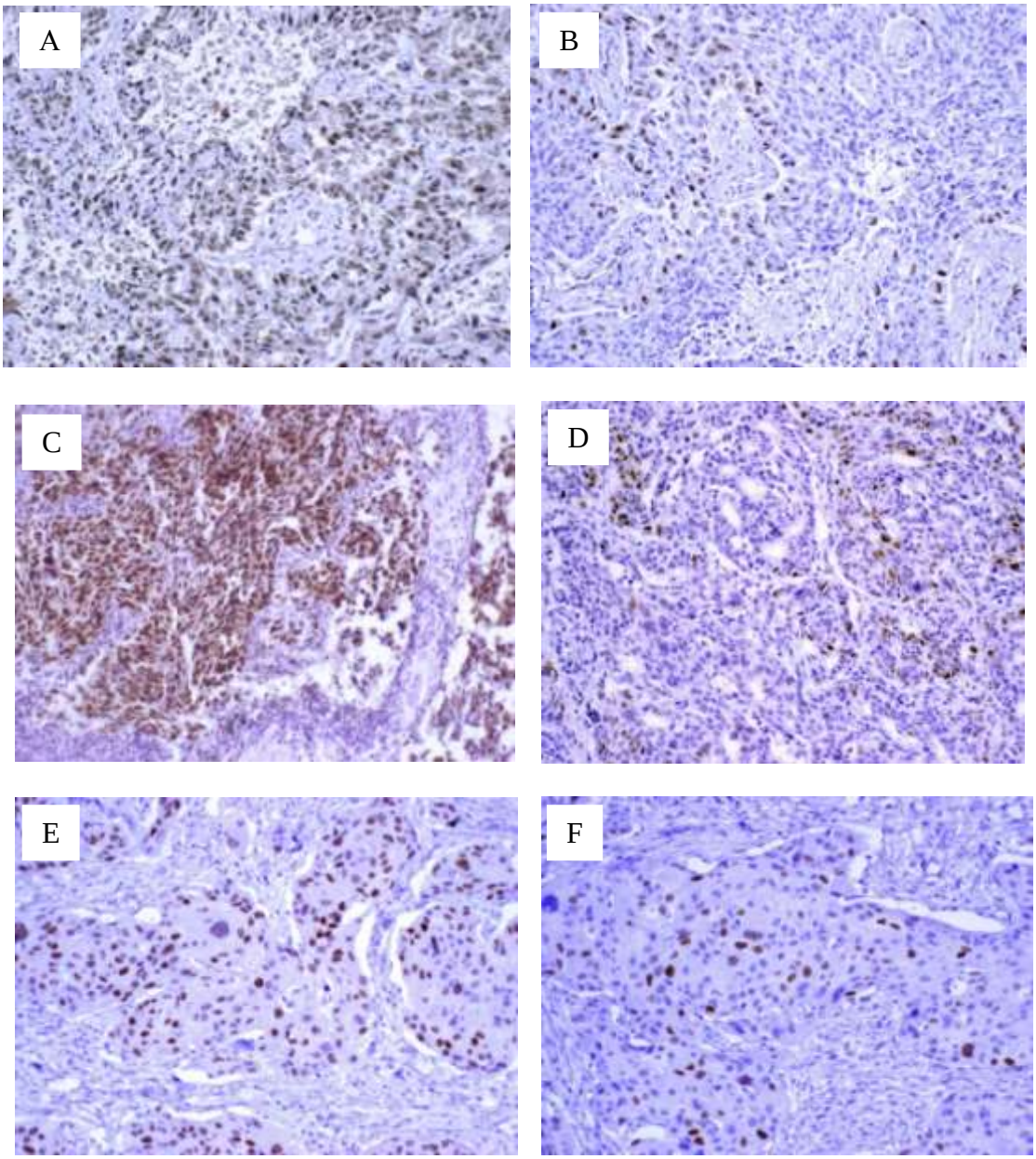

Fig. 1. Co-expression of TTF-1 and p63 cells of pulmonary adencarcinomas (immunohistochemical reaction with antibodies to TTF-1 and p63, magnification x 125 – A, B, C, D, magnificationx250 – E, F): expression of TTF-1 in most cells (A, B, E) with non-uniform and focal expression of p63 (B, D, F)

(Figure 2) [14]. TAp63 isoform activates p53 gene, thus terminating the cell cycle and starting apoptosis, and ΔNp63 isoform, on the contrary, activates the mechanism of cell proliferation and inhibits apoptosis [16,17]. TAp63 isoforms may also activate other genes containing elements of similar structure. In particular, this isoform indirectly stimulates transcription of genes of Jag1and Jag2 proteins which are ligands for Notch receptors which activation plays a key role in selection of the direction of cell differentiation. Inactivation and/or loss of TAp63 isoform in tumor cells increases their metastatic and invasive potential [15,17].

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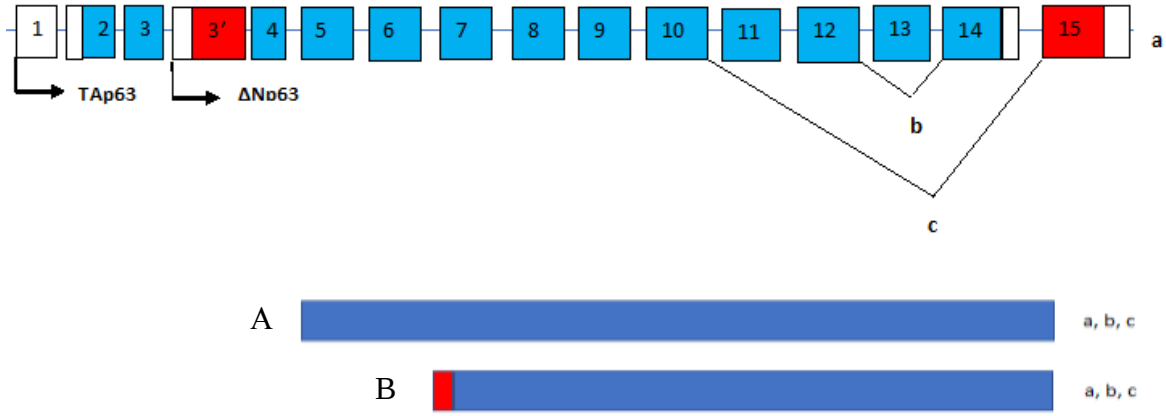

Fig. 2. Scheme of TP63 gene and of its proteins:
A. Scheme of exons and respective domains of TP63 gene; B. Isoforms of p63 proteins [14,11]

It was shown in some recent research that p63 gene promotes transformation of stem cells for differentiation to squamous cell [14]. It was demonstrated on experimental models that expression of this gene in embryogenesis is necessary for normal morphogenesis of the epidermis including maturation of teeth, hair, mammary glands, prostate, sweat and lacrimal glands and of their organs and systems [15]. Transformation of non-differentiated stem cells to tissue stem basal cells leads to formation of normal epithelium. In an adult organism, p63 plays an important role in maintenance of the population of stem cell in the squamous and stratified epithelium. In norm, basal, ciliated, goblet, alveolar cells also intensely express p63. With this, cells that express p63 are also diffused in the terminal bronchioles. Expression of p63is as well seen in myoepithelial cells localized in the sub mucosal layer of bronchial glands [12,16,17].

In the given research, expression of p63 was found in 47.5% of cases of PA, while different authors give different information concerning the rate of expression of this marker. Thus, according to P.P. Massion, et al. (2003), expression of p63 was observed in 18% of cases (17 of 93) [18], according to G. Pelosi, et al. (2002) – в 16% (15 of 95 cases), according F. Bir, et al. (2014) – in 25% [12], and according to B.Y. Wang, et al. (2002) – in 60% of cases [19]. These differences may very likely be attributed to different approaches to estimation.

In our research a mild tendency to decline of relapse-free survival of patients with p63-positive PA was found. Similar data were obtained in some international research works. Thus, according to M.L. Iacono, et al. (2011), expression of N-terminal end isoform of p63 in tumor cells was reliably associated with survival of patients with NSCLC, and the multivariate analysis showed 8.09-fold increase (at p=0.001) in the relative risk for unfavorable outcome. The authors also note that detection of such type of protein in the normal lung tissue furthers its malignant transformation [20]. The research of T. Narahashi, et al. (2006) showed correlation between expression of p63 in the cytoplasm of tumor cells and poor prognosis for patients with PA, and the survival of the group with cytoplasmic expression of this protein and the group where the expression was absent was 2.6 against 4.64 [21]. E. Ko, et al. (2013) showed p63 gene and RASSF1A gene to be independent factors for recurrences of tumor growth after surgical removal of pulmonary cancer in the 1-2 stage without involvement of lymph node [22].

The work of M-Ch. Aubry, et al. (2015) showed correlation between expression of p63 in PA and amplification of TP63 gene.

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Besides, the authors revealed a complex restructure in B3GALNT1 gene located on the 3nd (3q26.1a) chromosome [23]. However, the clinical significance of this restructure of the gene is yet unclear, and further investigations are required in this direction. Amplification of p63 gene and its expression were also observed in dysplasia and *in-situ* carcinoma of lung. This permitted the authors to suggest an important role of p63 gene and of its expression in the cells of pulmonary epithelium in the mechanism of carcinogenesis.

Anomalies in EGFR gene were detected in 25 patients (31%): y 15 patients – del19ex, in 10 patients – L858R. To note, the main group of patients with identified mutations were women (19 cases).

No dependence was found between expression of p63 in cells of PA and the identified disorders in EGFR gene. Disorders in p63 gene were identified both in the group positive for the studied protein in which there were 5 patients with different mutations in gene (3 del19ex and 2L858R), and in the group of patients without p63 expression (Table 3).

Table 3

Identified Mutations of EGFR in Patients with Expression and Absence of Expression of p63

	Positive p63 Expression	**Absence of p63 Expression**
Mutation in EGFR gene	5	4
Absence of mutation in EGFR gene	10	12

Conclusion

A mild tendency was found to decline of relapse-free survival of patients with p63-positive pulmonary adenocarcinomas. Expression of p63 in pulmonary adenocarcinomas may be regarded as a factor of poor prognosis and of risk for faster progress of tumor process and requires further investigation with increase in the study power.

We believe that in-depth analysis of medical history of patients with pulmonary adenocarcinomas, increase in the sample volume with analysis of stages of the disease at the moment of making diagnosis, as well as study of expression of different p63 isoforms in them, will permit to make a more reliable judgment about prognostic significance of this factor for the course of the disease.

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