DOI 10.1007/s10517-014-2710-y
Cell Technologies in Biology and Medicine, No. 3, November, 2014

Relationship of Argyrophilic Proteins of Nucleolal Organizer Regions in Ki-67$^+$ Cells with Clinical and Morphological Parameters in Lung Adenocarcinoma

A. F. Lazarev, D. S. Kobyakov, A. M. Avdalyan, E. L. Lushnikova*, and L. M. Nepomnyashchikh*

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 3, pp. 169-173, July, 2014
Original article submitted May 14, 2014

Ninety-four lung adenocarcinoma samples obtained during surgeries were examined using a combination of immunohistochemical staining for Ki-67 antigen and silver nitrate staining for argyrophilic nucleolar organizer (Ag-NOR) proteins. In lung adenocarcinoma, we studied the correlation between the area of Ag-NOR proteins in Ki-67$^+$ cells and clinical and morphological TNM parameters: maximum tumor diameter (T) and stage of the disease and tumor differentiation degree (N). Survival of patients with small area of Ag-NOR proteins in Ki-67$^+$ cells was higher that in patients with great area of these proteins. The area of Ag-NOR proteins in Ki-67$^+$ cells is an independent prognostic factor in lung adenocarcinoma. The area of Ag-NOR proteins in Ki-67$^+$ cells correlates with clinical and morphological TNM parameters and survival of patients with lung adenocarcinoma.

Key Words: *lung adenocarcinoma; argirophilic proteins of nucleolar organizer regions in Ki-67$^+$ cells; survival*

Lung adenocarcinoma accounts for about $^1/_3$ of all histological types of lung neoplasms. An actual task is selection of morphological criteria related to the key clinical and morphological parameters and survival of lung adenocarcinoma patients, because they will help to predict the course of the disease with high probability.

Immunohistochemical analysis of Ki-67 antigen is a commonly accepted and available method for evaluation of proliferative activity. Ki-67 antigen is expressed in cells during the late G_1-phase, and during S-, G_2-, and M-phases of the cell cycle, but the role of this nuclear protein in proliferative processes remains poorly understood [10]. Argyrophilic proteins associated with nucleolar organizer regions (Ag-NOR proteins) are the markers of the cell cycle rate: in particular, C23 (nucleolin) and B23 (nucleophosmin). These proteins are involved in **RNA (в оригинале рРНК)** synthesis and are detected in cell nuclei throughout the cell cycle; during S- and G_2-phases, their content increases by 1.5-3 times [11]. The content of Ag-NOR proteins negatively correlated with cell cycle duration [4] and tumor doubling time [8].

A method of double staining for Ki-67 antigen and Ag-NOR proteins was proposed that allows evaluation of nucleolar organizer activity (cell cycle duration) in proliferating cells [7]. Evaluation of tumor proliferative potential with the use of double staining for Ki-67 antigen and Ag-NOR proteins was used in few papers [1-3,5,6,13,15]. However, in none studies the results of double staining for Ki-67 antigen and Ag-NOR proteins were evaluated by computer imaging analysis and in correlation with clinical and morphological parameters of the tumor and survival of patients with lung adenocarcinoma.

Here we studied the correlation between the area of Ag-NOR proteins in Ki-67$^+$ cells and clinical and

Altai Branch of N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Barnaul; *Research Institute of Regional Pathology and Pathomorphology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** pathol@soramn.ru. A. F. Lazarev

morphological parameters and survival of patients with lung adenocarcinoma.

MATERIALS AND METHODS

We examined 94 samples of lung adenocarcinoma obtained during surgeries in 2007-2009 in Altai Regional Oncological Center (M1 cases and patients with multiple tumors were excluded from the study). The study included 68 male (72%) and 26 female (28%) patients; mean age 59 years (35-75 years). Lobectomy and pneumonectomy were performed in 76 (81%) and 18 (19%) patients, respectively. No chemotherapy and radiotherapy were performed prior to surgery. Pathohistological characteristic of the tumors was evaluated by the TNM system (7th edition) [12] (Table 1).

The tissue samples were fixed in 10% buffered formalin and processed routinely; 4-μ histological sections were stained with hematoxylin and eosin, with PAS/alcian blue, and by the method of Kreiberg. For more precise evaluation of tumor histogenesis and for differential diagnostic purposes, cytokeratin 7 (clone SP52), cytokeratin 20 (clone SP33), high-molecular-weight cytokeratin (clone 34bE12), and cytokeratins 5/6 (clone D5/16B4) were assayed in a Ventana XT automatic stainer (staining control were skin epidermis and gastric mucosa).

Based on the analysis of histological sections, tissue columns were taken from the paraffin blocks with a puncher (inner diameter 1.5 mm). To exclude staining heterogeneity, a tissue matrix was made and 4-μ histological sections were prepared. The matrix was stained by manual immunohistochemical method according to manufacturer's instruction: streptavidin–avidin staining with primary antibodies to Ki-67 (clone MIB-1, Dako) and chromogen (new fuchsin).

Before staining, the sections were autoclaved at 120°C for 20 min in 0.01 M citrate buffer (pH 6.0). After incubation with the chromogen, the sections were washed in bidistilled water and stained with silver nitrate (single step technique) [7,14] in a humid chamber at 37°C for 19 min. Nuclei were not post-stained; the sections were embedded in Faramount aqueous medium (Dako). In each case, the area of Ag-NOR proteins (μ^2) in nuclei of 100-120 randomly selected Ki-67$^+$ cells was measured on 10-30 digital images of the corresponding microscopic fields of view at ×1000 (objective ×100, 1.25, oil). Computer processing of the

TABLE 1. Area of Ag-NOR Proteins in Ki-67$^+$ Cells in Lung Adenocarcinoma

No.	Tumor characteristics	Number of cases		Area of Ag-NOR proteins in Ki-67$^+$ cells, μ^2	
		abs.	%		
	Primary tumor				
1	T1	31	33	9.08±2.21	p_{1-2}=0.003
2	T2	50	53	11.05±3.11	p_{1-3}<0.001
3	T3	13	14	12.32±2.49	p_{2-3}=0.1
	Greatest diameter				
4	<3 cm	43	46	9.17±2.55	p_{4-5}<0.001
5	>3 cm	51	54	11.76±2.77	
	Lymph nodes				
6	N0	60	64	9.76±2.60	p_{6-7}<0.001
7	N1-3	34	36	12.03±3.03	
	Stage				
10	I	51	54	9.63±2.45	p_{10-11}=0.005
11	II	24	26	11.77±3.19	p_{10-12}=0.01
12	III	19	20	11.62±3.14	p_{11-12}=0.8
	Differentiation				
13	High	14	15	8.25±1.99	p_{13-14}<0.001
14	Moderate	46	49	11.28±3.02	p_{13-15}=0.003
15	Low	34	36	10.59±2.77	p_{14-15}=0.3

images was performed using ImageJ 1.42 software. To exclude measurement errors, the grains <0.1 μ² were not included in the analysis.

The data were processed using Statistica 6.0 software. Since the data obtained in the samples met the criteria for normal distribution (Shapiro–Wilk test; W=0.98, p>0.05), the central tendency in the groups was presented as the mean (M) and the dispersion as standard deviation (SD). Statistical hypotheses were verified using nonparametric methods: Mann–Whitney and Kruskal–Wallis U tests and Spearman's rank correlation test. The overall adjusted survival rate for the 5-year period after surgery was determined using the Kaplan–Meier estimator, log-rank test, Cox regression model. Significance of the obtained criteria was evaluated at p<0.05.

RESULTS

Products immunohistochemical reaction with primary antibody to Ki-67 (clone MIB-1) followed by silver nitrate staining were seen on sections as round black granules (Ag-NOR proteins) above red nucleus (in Ki-67⁺ cells) or above brown nucleolus or pale yellow nucleus (in Ki-67⁻ cells; Fig. 1).

In lung adenocarcinoma, the area of Ag-NOR proteins in Ki-67⁺ cells was 10.56±2.96 μ². In groups T1, T2, and T3, gradual increase in the area of Ag-NOR proteins in Ki-67⁺ cells was noted, but only the differences of T1 from T2 and T3 reached statistical significance (Table 1). In primary tumors <3 cm, the area of Ag-NOR proteins in Ki-67⁺ cells was lower than in tumors >3 cm. In lung adenocarcinoma with metastases in regional lymph nodes, the area of Ag-NOR proteins in Ki-67⁺ cells was significantly higher than in tumor without metastases (Fig. 1, *c*, *d*). The area of Ag-NOR proteins in Ki-67⁺ cells was significantly higher in stage II and III tumors in comparison with stage I and in moderately and low-differentiated carcinomas in comparison with highly differentiated tumors (Fig. 1, *a*, *b*). The area Ag-NOR proteins in Ki-67⁺ cells moderately correlated with parameter

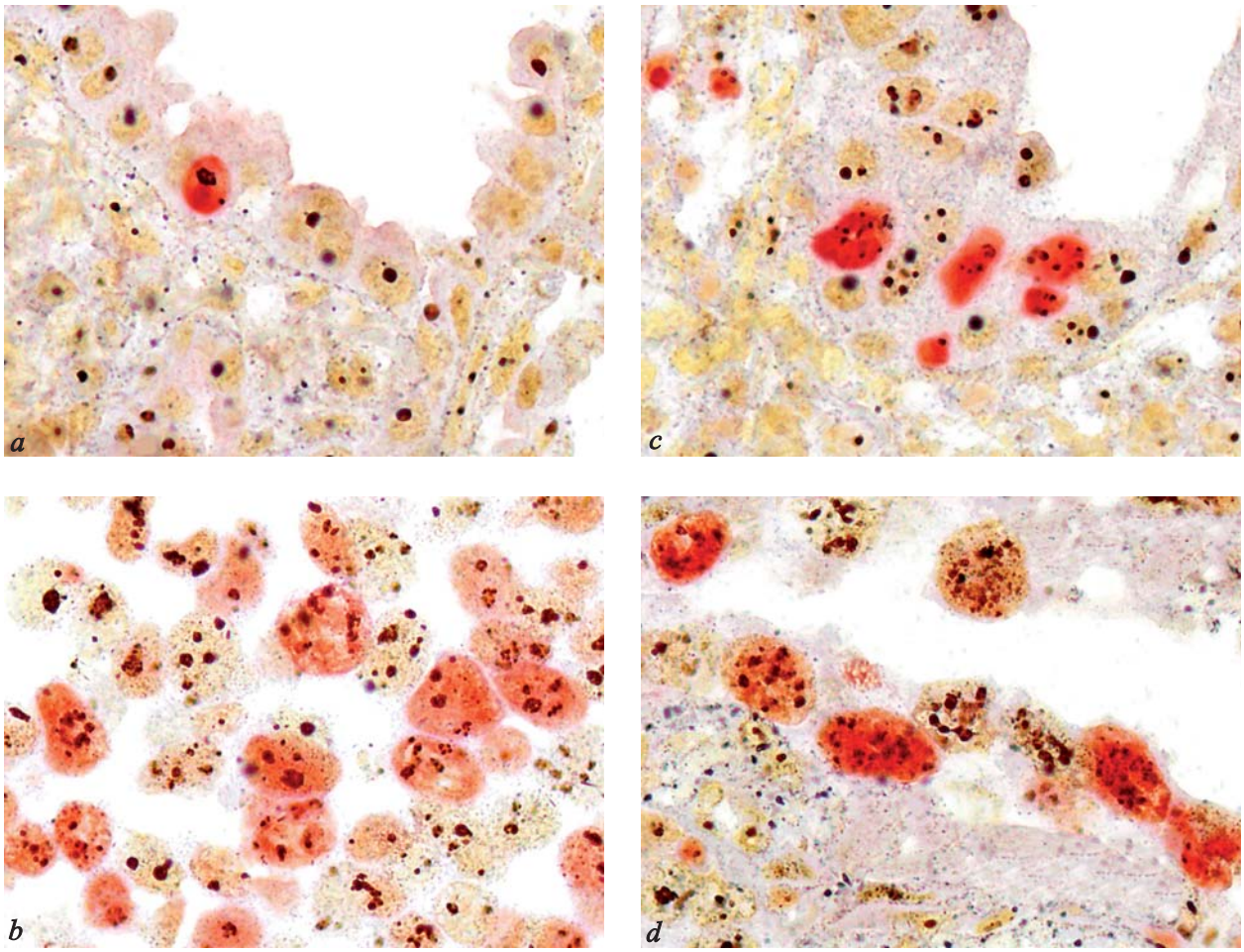

Fig. 1. Ag-NOR proteins in Ki-67⁺ and Ki-67⁻ lung adenocarcinoma cells. Double staining for Ki-67 (clone MIB-1) by histochemical method and for Ag-NOR proteins with silver nitrate, chromogen (new fuchsin), ×1000. Highly (*a*), low (*b*), moderately (*c*) differentiated adenocarcinoma without metastases; *d*) adenocarcinoma with metastases to lymph nodes.

T (r=0.40; p<0.001), the greatest tumor diameter (r=0.46; p<0.001), parameter N (r=0.35; p<0.001), stage of the process (r=0.33; p=0.001) and tumor differentiation (r=0.36; p<0.001).

The area occupied by Ag-NOR proteins in Ki-67$^+$ cells ≥10.56 mm^2 was considered great (50 cases, 53%) and are <10.56 mm^2 was considered small (44 cases, 47%). The total adjusted survival of patients with lung adenocarcinoma over 5 years after surgery was 29.8±5.6%. A significant correlation (p<0.001) was found between the area of Ag-NOR proteins in Ki-67$^+$ cells and survival rate: 45.0±8.9 and 7.9±4.8% in cases with small and great area, respectively (Fig. 2). Univariate regression analysis showed that the area of Ag-NOR proteins in Ki-67$^+$ lung adenocarcinoma cells was associated with patient survival (χ^2=25.5, β=1.43, SD=0.29, p<0.001). Multivariate regression analysis (χ^2=49.9) revealed correlations of patient survival rate with the area of Ag-NOR proteins in Ki-67$^+$ cells (β=0.95, SD=0.33, p=0.004), stage of the process (β=1.00, SD=0.46, p=0.03), and the greatest tumor diameter (β=0.96, SD=0.40, p=0.02).

A correlation was found between Ag-NOR protein area in Ki-67$^+$ cells with clinical and morphological parameters according to the TNM system; however, significant differences were obtained only at the initial stages of tumor growth: T1, maximum tumor diameter <3 cm; N0, stage I and high differentiation. Other studies also have demonstrated the relationship between some clinical and morphological parameters by the TNM system and Ag-NOR proteins in Ki-67$^+$ cells. In non-small cell lung cancer, increased area of Ag-NOR proteins in Ki-67$^+$ cells was found in T4 in comparison with T1-3 and in N2 and N3 in comparison with N0 and N1 [15]. In breast cancer, increased content of Ag-NOR proteins in Ki-67$^+$ cells was found in tumors >4 cm in comparison with tumors <4 cm [5]. In bladder cancer, gradual increase in the content of Ag-NOR proteins in Ki-67$^+$ cells was found in T1, T2, and T3 groups, the difference was significant only for T1 [13]. However, these findings were obtained in visual counting of Ag-NOR proteins, while in our study, computer image analysis was used.

The survival rate in patients with primary lung adenocarcinoma node <3 cm and area of Ag-NOR proteins in Ki-67$^+$ cells was higher than in patients with greater area of these proteins. Univariate and multivariate regression analysis showed that the area occupied by Ag-NOR proteins in Ki-67$^+$ cells is a prognostically valuable (significant) parameter for evaluation of survival in patients with lung adenocarcinoma. Similar results were reported for non-small **lung [3], breast [1,2,5,6], and bladder cancer** [13]. Numerous studies of nucleolar organizer activity in malignant tumors have demonstrated that the content of Ag-NOR proteins is an independent prognostic factor [9]. The prognostic value of the content of Ag-NOR proteins in Ki-67$^+$ cells is related to different rate of proliferation of lung adenocarcinoma cells: short cycle of proliferating cells and high proliferation rate in tumors with great area of Ag-NOR proteins in Ki-67$^+$ cells and otherwise, long cell cycle of proliferating cells and low proliferation rate in small area.

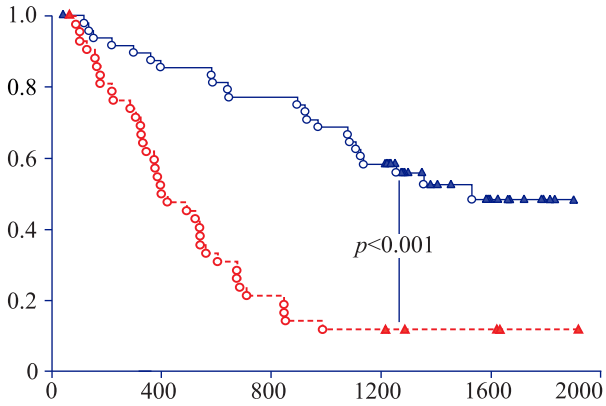

Fig. 2. Survival rate of patients with lung adenocarcinoma with great (interrupted line) and small (solid line) area of Ag-NOR proteins in Ki-67$^+$ cells (Kaplan–Meier curves). Abscissa: lifespan, days. Ordinate: fraction of survivors.

Thus, the area of Ag-NOR proteins in Ki-67$^+$ lung adenocarcinoma cells correlates with clinical and morphological parameters by the TNM system and survival rate: parameter T, greatest tumor diameter, parameter N, stage of the disease and differentiation. The survival rate in patients with lung adenocarcinoma with small area of Ag-NOR proteins in Ki-67$^+$ cells is higher than in patients with greater area of these proteins in the section. The area of Ag-NOR proteins in Ki-67$^+$ cells is an independent prognostic factor in lung adenocarcinoma.

REFERENCES

1. P. Abboud, M. Lorenzato, D. Joly, *et al.*, *Am. J. Obstet. Gynecol.*, **199**, No. 2, 146.e1-7 (2008).
2. S. Biesterfeld, F. Farokhzad, D. Kluppel, *et al.*, *Virchows Arch.*, **438**, No. 5, 478-484 (2001).
3. G. Bigras, R. Marcelpoil, E. Brambilla, and G. Brugal, *Anal. Cell. Pathol.*, **11**, No. 3, 183-198 (1996).
4. V. Canet, M. P. Montmasson, Y. Usson, *et al.*, *Cytometry*, **43**, No. 2, 110-116 (2001).
5. H. Kidogawa, A. Nanashima, H. Yano, *et al.*, *Anticancer. Res.*, **25**, No. 6B, 3957-3962 (2005).
6. M. Lorenzato, P. Abboud, C. Lechki, *et al.*, *Micron.*, **31**, No. 2, 151-159 (2000).
7. S. Munakata and J. B. Hendricks, *J. Histochem. Cytochem.*, **42**, No. 6, 789-793 (1994).
8. S. Ogura, S. Abe, N. Sukoh, *et al.*, *Cancer*, **70**, No. 1, 63-68 (1992).

9. A. Pich, L. Chiusa, and E. Margaria, *Micron*., **31**, No. 2, 133-141 (2000).
10. T. Scholzen and J. Gerdes, *J. Cell. Physiol*., **182**, No. 3, 311-322 (2000).
11. V. Sirri, P. Roussel, and D. Hernandez-Verdun, *Micron*., **31**, No. 2, 121-126 (2000).
12. *TNM Classification of Malignant Tumours*, Eds. L. Sobin, *et al*., Oxford (2009), pp. 138-146.
13. M. Tomobe, T. Shimazui, K. Uchida, *et al*., *J. Urol*., **162**, No. 1, 63-68 (1999).
14. D. Trere, *Micron*., **31**, No. 2, 127-131 (2000).
15. S. Yamaguchi, *Acta Med. Nagasaki*, **39**, 147-152 (1994)