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Interrelation of Argyrophilic Proteins of Nucleolar Organizer Regions and Ki-67 with Clinical and Morphological Parameters and Survival in Patients with Non-small Cell Lung Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Author DSK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EYB, AMA, IPB, AFL, SAL and NMK managed the analyses and set of materials for the study. Authors AFL, ELL and LMN managed the literature searches, editing manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Argyrophilic proteins associated with nucleolar organizer regions (AgNOR) and Ki-67 were studied at non-small cell lung cancer (NSCLC). Tumors with low and high area index (AI) of AgNOR and label index (LI) Ki-67 were defined. AI AgNOR was related to the key clinical and morphological parameters in accordance with TNM system: values T, N, greatest tumor dimension up to 3 cm and more, disease stage, histogenesis, and tumor differentiation. LI Ki-67 is related to the greatest tumor dimension up to 3 cm and

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more, disease stage, and tumor differentiation. NSCLC patients survival is longer in low AI AgNOR or LI Ki-67 tumors versus high AI AgNOR or LI Ki-67 tumors. NSCLC patients survival is longer in low AI AgNOR and LI Ki-67, shorter in high AI AgNOR and LI Ki-67, and intermediate in opposite AI AgNOR and LI Ki-67. Value N, greatest tumor dimension, histogenesis, AgNOR are independent predictors in NSCLC. NSCLC patients survival without metastases to lymph nodes is related to the greatest tumor dimension and in case of metastases it is related to AI AgNOR and greatest tumor dimension. Combined determination of AgNOR and Ki-67 has prognostic value at NSCLC.

Keywords: Argyrophilic proteins of the nucleolar organizer regions; Ki-67; survival; non-small cell lung cancer.

ABBREVIATIONS

AgNOR - argyrophilic proteins associated with nucleolar organizer regions; NSCLC - non-small cell lung cancer; AI AgNOR - area index of argyrophilic proteins associated with nucleolar organizer regions; LI Ki-67 - label index Ki-67; +AgNOR - high area index of argyrophilic proteins associated with nucleolar organizer regions; -AgNOR - low area index of argyrophilic proteins associated with nucleolar organizer regions; +Ki-67 - high label index Ki-67; -Ki-67 - low label index Ki-67.

1. INTRODUCTION

Lung cancer is a prevailing form among malignant neoplasms 80% of which are accounted for by non-small cell lung cancer (NSCLC). Long-term results of treatment and survival in patients with NSCLC are far from desired ones. Therefore, study of morphological criteria related to the key clinical and morphological parameters and survival in patients with NSCLC and able to predict the course of disease with high probability is actual.

Proliferation is the key process in tumor occurrence and development as well as the predictive factor of its biological behavior. Currently, there are some difficulties in reliable assessment of the tumor proliferative capacity because proliferation includes not only the number of proliferating cells (proliferative activity, growth fraction) but the rate of the cell mitosis (duration of the cell cycle).

Immunohistochemical analysis of Ki-67 is a widely accepted and available method for proliferative activity assessment [1]. Multiple studies show the relation of this marker to prognosis in NSCLC [2-4].

Argyrophilic proteins associated with nucleolar organizer regions (AgNOR) are the markers of the cell cycle rate. Up to 75% of AgNOR staining is accounted for by two main argyrophilic proteins C23 (nucleolin) and B23 (nucleophosmin), which play a very important role in ribosomal RNA synthesis [5]. These proteins are revealed in cell nuclei along the whole cell cycle increasing in S- and G2-stages by 1.5—3 folds [6]. Inverse relationship between AgNOR quantitative content and duration of the cell cycle [7], tumor doubling time [8,9] is shown.

Review of current literature showed controversial character of the Ki-67 and AgNOR relation to clinical and morphological parameters and survival rates in patients with NSCLC [10-15].

Moreover, there are no studies specifying the interrelation of Ki-67 and AgNOR with clinical and morphological parameters and survival in patients with NSCLC.

Based on the above, the aim of the study was to analyze Ki-67 and AgNOR relation to clinical and morphological parameters and survival in patients with NSCLC.

2. MATERIALS AND METHODS

210 surgery samples of NSCLC removed during 2007-2009 in Altai Regional Cancer Dispensary (cases with M1 and multiple tumors were excluded from the study) were examined. Patient's mean age was 59 years old (35 to 75 years old), 177 men (84%) and 33 women (16%) took part in the study. Lobectomy was performed in 148 patients (70%) and pneumonectomy was performed in 62 patients (30%). Preoperative chemotherapy and radiation therapy were not performed. Postoperative chemotherapy was performed in 21 patients (10%), cisplatin and etoposide most often used. Postoperative radiation therapy was performed in 64 patients (30%), dose of 50-60 Gr most often used. Histopathological characteristics of tumors were determined in accordance with TNM classification of 7th edition [16] and provided in the Table 1. The greatest tumor dimension was measured (in cm).

Table 1. Distribution of cases with high and low AI AgNOR and LI Ki-67 in NSCLC (absolute count (in percent))

Parameter	Number of cases	-AgNOR		+AgNOR	
		-Ki-67	+Ki-67	-Ki-67	+Ki-67
Primary tumor					
T 1	54 (26)	25 (46)	16 (30)	6 (11)	7 (13)
T 2-3	156 (74)	36 (23)	28 (18)	30 (19)	62 (40)
Greatest dimension					
< 3 cm	87 (41)	38 (44)	23 (26)	11 (13)	15 (17)
> 3 cm	123 (59)	23 (19)	21 (17)	25 (20)	54 (44)
Lymph nodes					
N 0	134 (64)	45 (33)	32 (24)	20 (15)	37 (28)
N 1-3	76 (36)	16 (21)	12 (16)	16 (21)	32 (42)
Stage					
I	107 (51)	40 (37)	24 (23)	16 (15)	27 (25)
II-III	103 (49)	21 (21)	20 (19)	20 (19)	42 (41)
Histogenesis					
adenocarcinoma	94 (45)	34 (36)	23 (25)	21 (22)	16 (17)
squamous cell	116 (55)	27 (23)	21 (18)	15 (13)	53 (46)
Differentiation					
high	54 (26)	29 (54)	8 (15)	8 (15)	9 (16)
moderate-low	156 (74)	32 (21)	36 (23)	28 (18)	60 (38)

Tissue fragments were fixed for 18-24 hours in 10% neutral buffered formalin. After standard preparation of the surgical specimens 4µm histological slices were prepared. Specimens were stained with hematoxylin and eosin, Schiff-reactive/alcian blue, by Kreiberg. Ki-67 (MIB-1 clone), cytokeratins 7 (SP52 clone), 20 (SP33 clone), High Molecular Weight (34βE12 clone) were defined by immunohistochemical method in automatic stainer Ventana XT. Ki-67 label index (LI) was defined - positive stained cells count of the total number of counted cells (in percent). In every case 1,000 cells were examined in 5-7 fields of vision,

per power x400. Due to LI Ki-67 distribution in NSCLC was nonparametric central tendency value was presented as median, which was 25% (interquartile interval 18-42%). This value was considered to be threshold that meets literature data [2,11]. Consequently, cases with LI Ki-67 of 25% and more were considered as with high LI Ki-67 (+Ki-67), up to 25% - with lower one (-Ki-67).

For examination AgNOR sections were stained by silver nitrate by one-step method [17]. Sections were autoclaved at 120 °C for 20 minutes, in 0.01 M citrate buffer (pH=6.0) before staining. Counterstaining of nuclei was not performed; sections were put in Canadian balsam. In every case AgNOR area (in μm^2) was determined in nuclei of 100-120 randomly selected cells with 10-15 digital images of the relevant microscope fields of vision per power x1,000 (lens x100, 1.25, oil). Computer analysis of images was performed by the program ImageJ 1.42. Granules of less than 0.1 μm^2 in size were excluded for error avoiding. AgNOR area in small lymphocyte nuclei was used as the internal control [18]. AgNOR area index (AI) - quotient of division of AgNOR areas in the tumor cell and small lymphocyte - was defined. As AI AgNOR in NSCLC was parametric, central tendency value was presented as an average value, which was 6.51 (standard deviation 1.68). Similar to LI Ki-67 assessment, cases with AI AgNOR of 6.51 and more was considered as with high AI AgNOR (+AgNOR), up to 6.51 - with lower one (-AgNOR).

Statistical analysis of obtained data was performed in STATISTICA 6.0. At statistical hypotheses testing, two-sided Fisher's exact test was used for tables 2x2 and chi-square test for identifying correlations. Total adjusted 5-years postsurgical survival rate in patients was defined; Kaplan-Meier method, log-rank test, Cox regression model were used. Reliability of obtained criteria was assessed at $p < 0.05$.

3. RESULTS AND DISCUSSION

Loose correlation was found between AI AgNOR and LI Ki-67 in NSCLC ($r=0.33$, $p < 0.001$). Cross-tabulated distribution of NSCLC cases with high and lower AI AgNOR and LI Ki-67 depending on morphological parameters of tumor is provided in the Table 1.

Statistically significant increase of cases number with +AgNOR in the T2 and T3 tumors group in comparison with T1 - 92 (59%) and 13 (24%) cases, respectively ($p < 0.001$) - was defined. Although at LI Ki-67 examination no statistically significant difference was found in these groups - 90 (58%) and 23 (43%) cases, respectively ($p=0.06$). At NSCLC with greatest tumor dimension more than 3 cm, number of cases with +AgNOR and +Ki-67 is more, than if greatest tumor dimension is less than 3 cm: for AgNOR - 79 (64%) and 26 (30%) cases, respectively ($p < 0.001$); for Ki-67 - 75 (61%) and 38 (43%) cases, respectively ($p=0.02$). Number of cases with +AgNOR is significantly higher in the group of tumors with metastases in lymph nodes in comparison with tumors without metastases - 48 (63%) and 57 (43%) cases, respectively ($p < 0.01$). Number of cases with +Ki-67 doesn't significantly differ between these groups - 44 (58%) and 69 (52%) cases, respectively ($p=0.4$). Number of cases with +AgNOR is significantly higher in the II-III stage of disease in comparison with I stage - 62 (60%) and 43 (40%) cases, respectively ($p=0.006$). Number of cases with +Ki-67 doesn't significantly differ between II-III and I stages of disease - 62 (60%) and 51 (48%) cases, respectively ($p=0.07$). Number of cases with +AgNOR and +Ki-67 is significantly higher in squamous cell lung carcinoma than in adenocarcinoma: for AgNOR - 68 (59%) and 37 (39%) cases, respectively ($p=0.008$); for Ki-67 - 74 (64%) and 39 (42%) cases, respectively ($p=0.001$). Number of cases with +AgNOR and +Ki-67 is significantly higher in the group of moderate and low differentiated tumors in comparison with high differentiated

tumors: for AgNOR - 88 (56%) and 17 (31%) cases, respectively ($p < 0.01$); for Ki-67 - 96 (61%) and 17 (31%) cases, respectively ($p < 0.001$).

AI AgNOR in NSCLC had correlation with value T ($p < 0.001$), greatest tumor dimension up to 3 cm and more ($p < 0.001$), value N ($p < 0.01$), disease stage ($p < 0.01$), histogenesis ($p < 0.01$) and tumor differentiation ($p < 0.01$). LI Ki-67 was related to the greatest tumor dimension up to 3 cm and more ($p = 0.01$), histogenesis ($p = 0.001$), and tumor differentiation ($p < 0.001$).

On the basis of obtained data four types of NSCLC was revealed depending on AgNOR and Ki-67: -AgNOR/-Ki-67 - 1 type, -AgNOR/+Ki-67 - 2 type Fig. 1 a,b,c, +AgNOR/-Ki-67 - 3 type Fig. 1 d,e,f, and +AgNOR/+Ki-67 - 4 type.

General adjusted 5-years postsurgical survival in patients with NSCLC was $40.3 \pm 3.7\%$. Survival in patients with NSCLC had statistically significant difference depending on AgNOR and Ki-67 Table 2, Fig. 2a,b. Also, relationship between patients survival and mutual AgNOR and Ki-67 was observed in these groups Table 2, Fig. 2c. Consequent decrease of survival was observed in the row: 1 type, 2 type, 3 type, and 4 type of tumor. In the group of tumors with lower or high AgNOR survival did not differ at lower or high Ki-67 (between -AgNOR/-Ki-67 and -AgNOR/+Ki-67, between +AgNOR/-Ki-67 and +AgNOR/+Ki-67). In the group of tumors with -Ki-67 survival was longer in tumors with -AgNOR (-AgNOR/-Ki-67) in comparison with tumors with +AgNOR (+AgNOR/-Ki-67). Similarly, in the group of tumors with +Ki-67 survival was longer in tumors with -AgNOR (-AgNOR/+Ki-67) in comparison with tumors with +AgNOR (+AgNOR/+Ki-67). Also, no statistically significant difference was found in survival in patients with types -AgNOR/+Ki-67 and +AgNOR/-Ki-67. According to obtained data, types -AgNOR/+Ki-67 and +AgNOR/-Ki-67 of NSCLC are combined in one intermediate type where AgNOR and Ki-67 had converse values. Survival in patients with intermediate type of tumors significantly differs from that of tumors of types -AgNOR/-Ki-67 and +AgNOR/+Ki-67 and has intermediate value Table 2, Fig. 2d.

Table 2. AI AgNOR, LI Ki-67 and survival in NSCLC

Parameter	Number of cases (abs.(%))	Five-year total adjusted survival (%)
AI AgNOR		
low	105 (50)	51.9 \pm 5.4
high	105 (50)	28.9 \pm 4.7
LI Ki-67		
low	97 (46)	50.3 \pm 5.5
high	113 (54)	31.6 \pm 4.7
Tumor type according to AI AgNOR and LI Ki-67		
1 type: -AgNOR/-Ki-67	61 (29)	56.8 \pm 7.4
2 type: -AgNOR/+Ki-67	44 (21)	43.9 \pm 8.1
3 type: +AgNOR/-Ki-67	36 (17)	33.8 \pm 8.7
4 type: +AgNOR/+Ki-67	69 (33)	23.8 \pm 5.6
"intermediate" type	80 (38)	41.0 \pm 5.9

At multivariate regression analysis type of surgery, postoperative chemoradiotherapy, value T, disease stage, differentiation, Ki-67, data on reveal of four or three types of tumor (by mutual AgNOR and Ki-67) had no influence on the survival in patients with NSCLC. Four parameters - value N (with or without metastases), greatest tumor dimension (up to 3 cm or more), histogenesis (adenocarcinoma or squamous cell carcinoma), AgNOR (low or high) -

had independent influence on survival in patients with NSCLC, among them value N had the largest impact Table 3. So, the influence on the survival of greatest tumor dimension, histogenesis and AgNOR in tumors with or without lymph nodes metastases was studied. At NSCLC without lymph nodes metastases survival in patients was related to greatest tumor dimension and histogenesis ($\chi^2=25.0$, $p<0.001$), in case with metastases - with greatest tumor dimension and AgNOR ($\chi^2=19.0$, $p<0.001$) Table 3.

Table 3. Cox regression analysis and prognostic factors in NSCLC

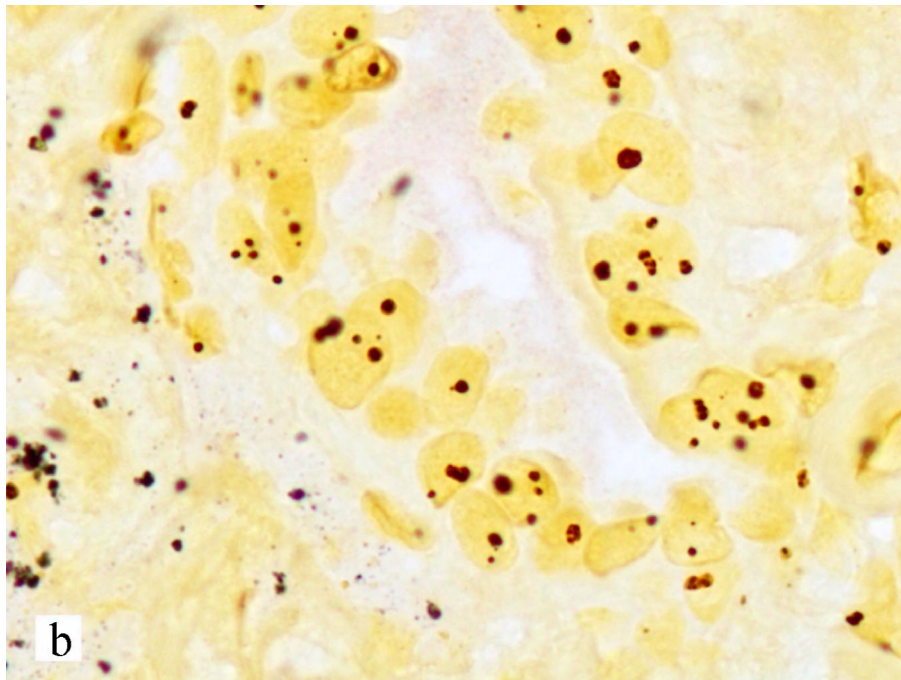
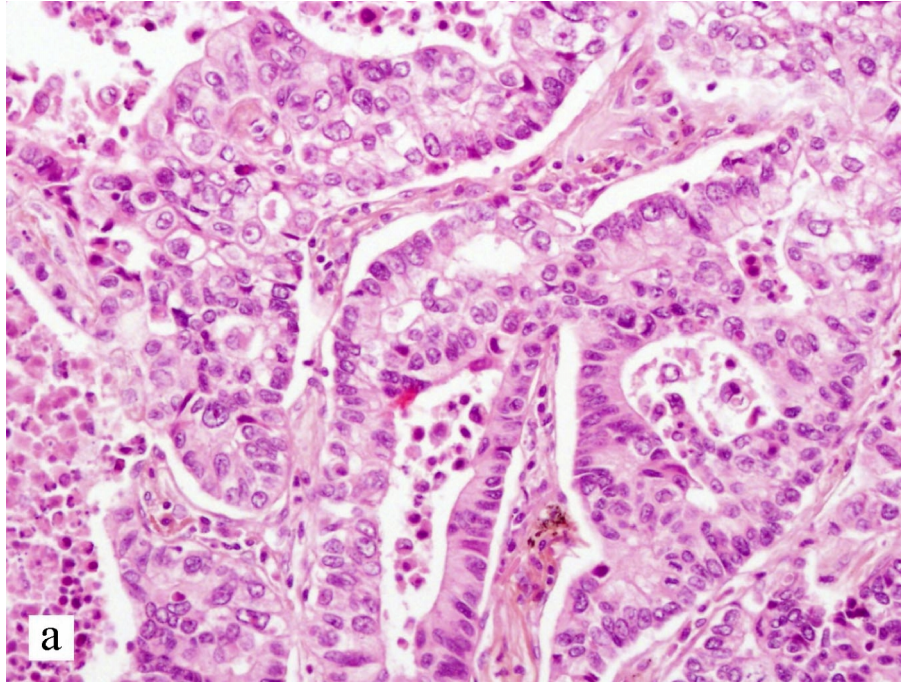
Prognostic factor	β	Standard deviation	p
Value N	1.41	0.20	<0.001
Greatest tumor dimension	0.88	0.21	<0.001
Histogenesis	0.63	0.19	<0.001
AI AgNOR	0.51	0.21	0.01
Without metastases			
Greatest tumor dimension	1.08	0.32	<0.001
Histogenesis	0.96	0.29	<0.001
AI AgNOR	0.45	0.29	0.1
With metastases			
Greatest tumor dimension	0.73	0.29	0.01
Histogenesis	0.36	0.26	0.2
AI AgNOR	0.70	0.31	0.02

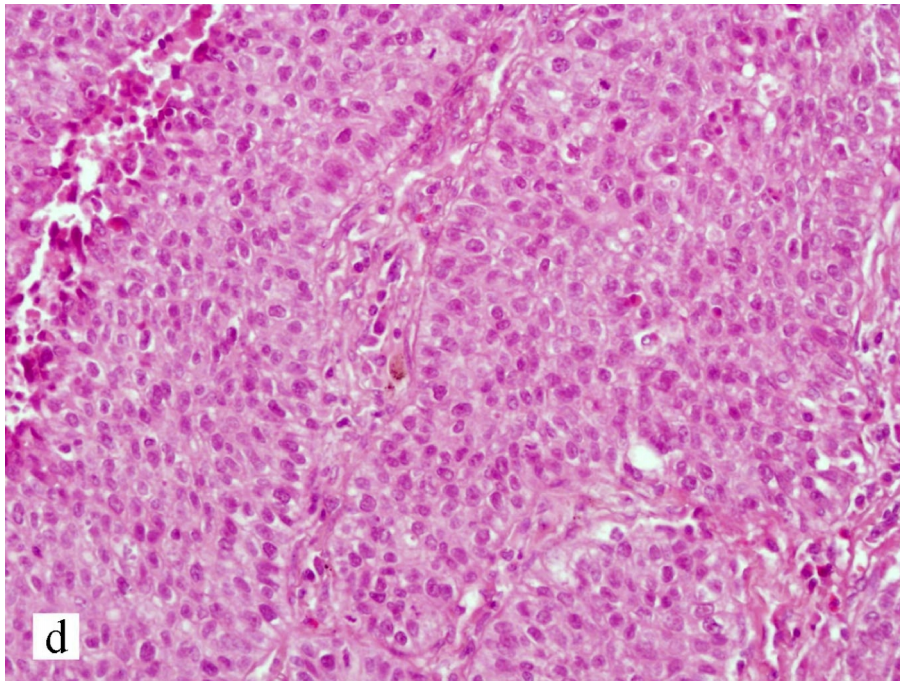
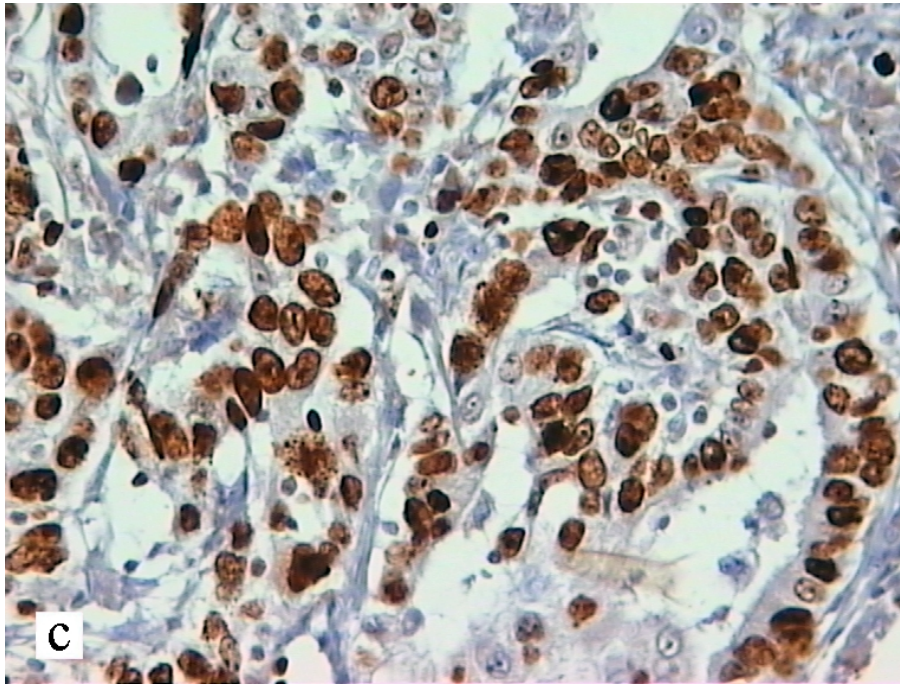
Correlation of AgNOR and Ki-67 was found in our study, and that corresponds to literature data [19]. AgNOR in cells of NSCLC was related to several clinical and morphological parameters in accordance with TNM system: value T, N, greatest tumor dimension up to 3 cm and more, disease stage, histogenesis, and tumor differentiation. Kaneko S. et al. received analogous conclusions at study of AgNOR in lung cancer [20]. Ki-67 in NSCLC is related to the greatest tumor dimension up to 3 cm and more, histogenesis and tumor differentiation.

Survival in patients with NSCLC with -AgNOR or -Ki-67 is significantly longer in comparison with that in patients with tumors with +AgNOR or +Ki-67. Such interrelation of AgNOR and LI Ki-67 with survival in patients with NSCLC was also observed in other studies [2- 4,13-15]. According to literature data, interrelation of AgNOR with survival in patients with carcinoma of different organs and histogenesis is more frequently observed at AgNOR area determination by computer analysis of images than by visual counting of AgNOR.

Depending on mutual AgNOR and Ki-67, four types of NSCLC were revealed, and sequential survival decrease was found in the row: -AgNOR/-Ki-67, -AgNOR/+Ki-67, +AgNOR/-Ki-67, +AgNOR/+Ki-67. Similar actuarial survival curves based on mutual AgNOR and Ki-67 was received by Lorenzato M. et al. [19] at breast cancer study. Raykhlin N.T. et al. [12] in the study of 20 minimal lung cancers (up to 3 cm in size) showed that the type -AgNOR/+Ki-67 is common in patients with life expectancy of 3-5 years and the type +AgNOR/-Ki-67 is common in patients with life expectancy of up to 2 years. In our study no statistically significant difference in survival in patients with NSCLC of types -AgNOR/+Ki-67 and +AgNOR/-Ki-67 is found. Thus, these two types were combined in one type (with converse values of AgNOR and Ki-67) where survival significantly differed from types -AgNOR/-Ki-67 and +AgNOR/+Ki-67 and had intermediate value. Thus, our research shows that the combined determination of AI AgNOR and LI Ki-67 provides more accurate actuarial survival curves of NSCLC patients: both with identical contents of AI AgNOR and LI Ki-67

(-AgNOR/-Ki-67 and +AgNOR/+Ki-67) and, especially, with opposite values of AI AgNOR and LI Ki-67 (-AgNOR/+Ki-67 and +AgNOR/-Ki-67).





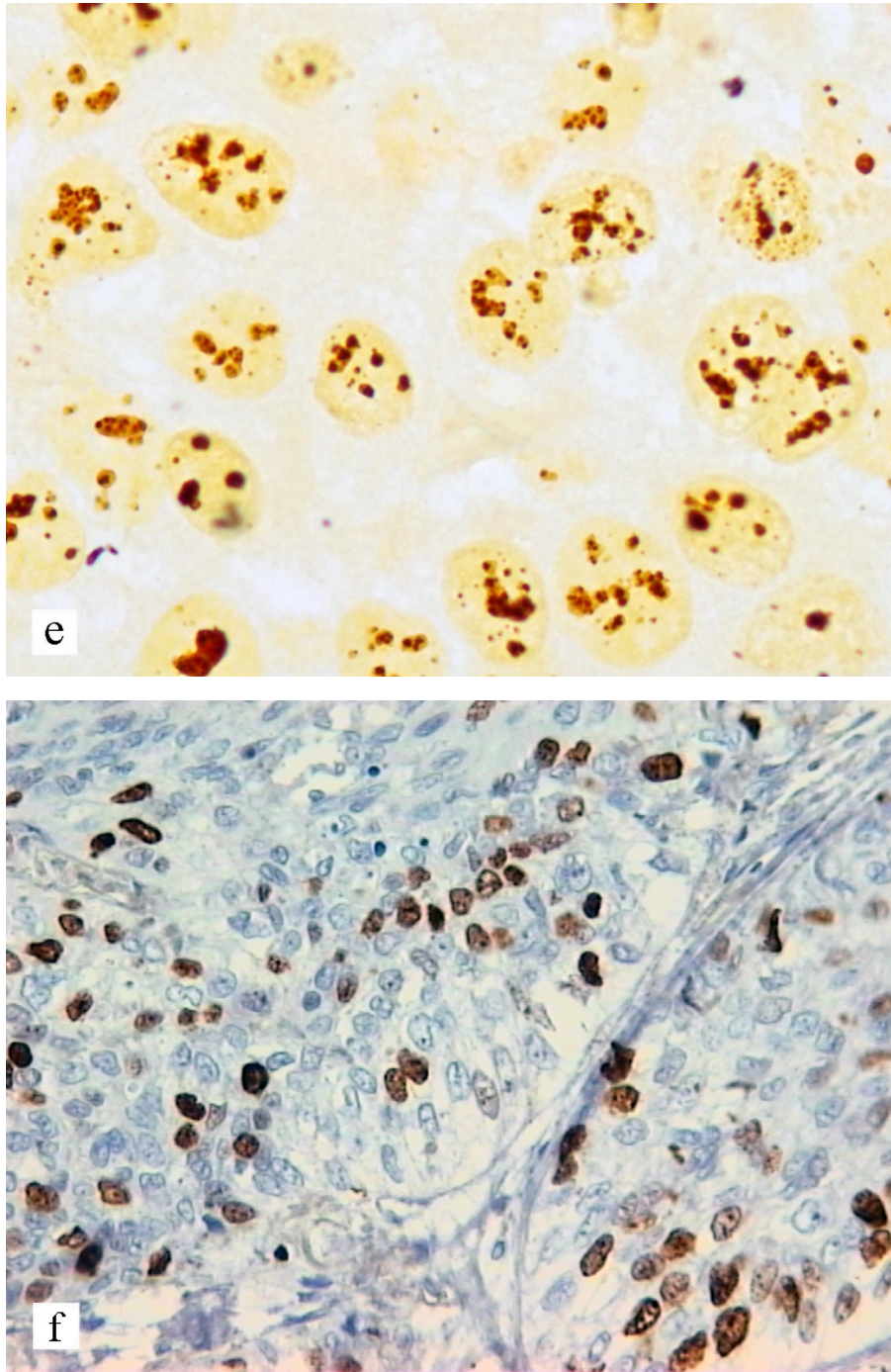
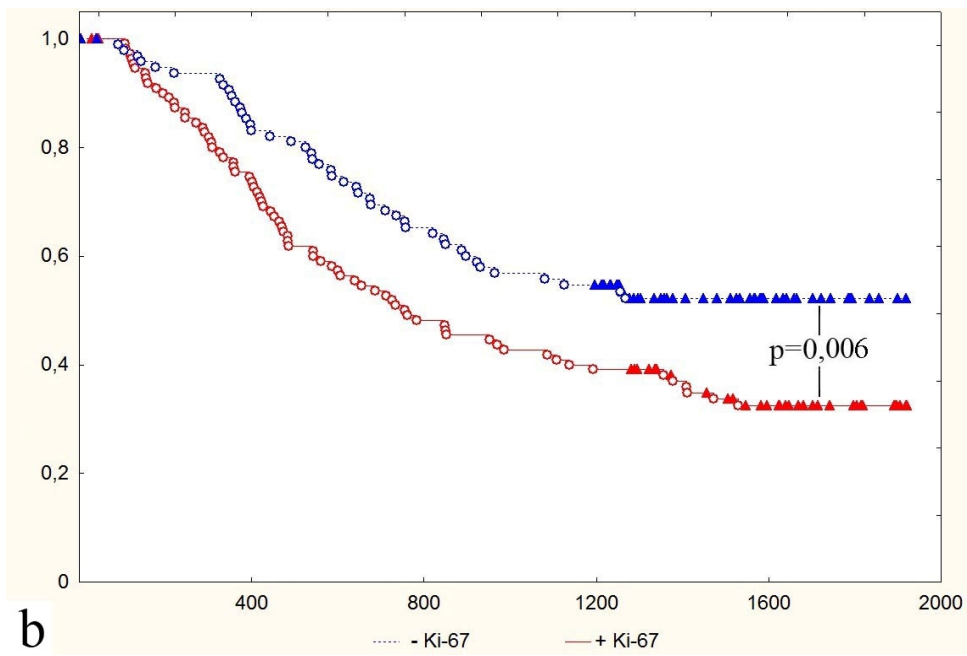
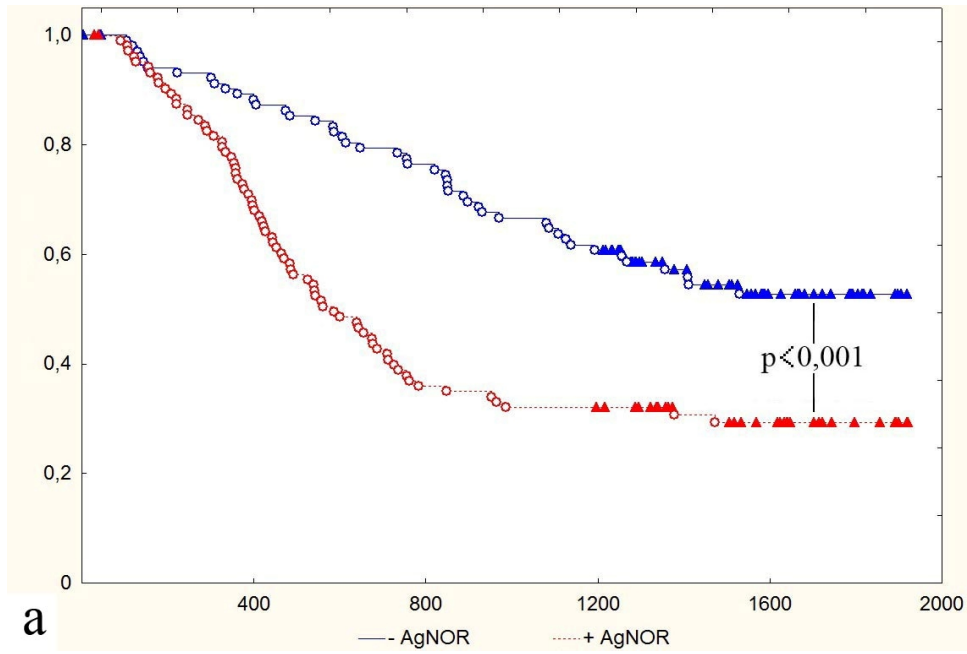


Fig. 1. Non-small cell lung cancer: a, b, c – moderate differentiated adenocarcinoma with low AgNOR and high Ki-67; d, e, f – moderate differentiated squamous cell carcinoma with high AgNOR and lower Ki-67. a, d - staining by hematoxylin and eosin, x200. b, e - argyrophilic proteins associated with nucleolar organizer regions (AgNOR), staining by silver nitrate, x1000. c, f - Ki-67, immunohistochemical method, x400



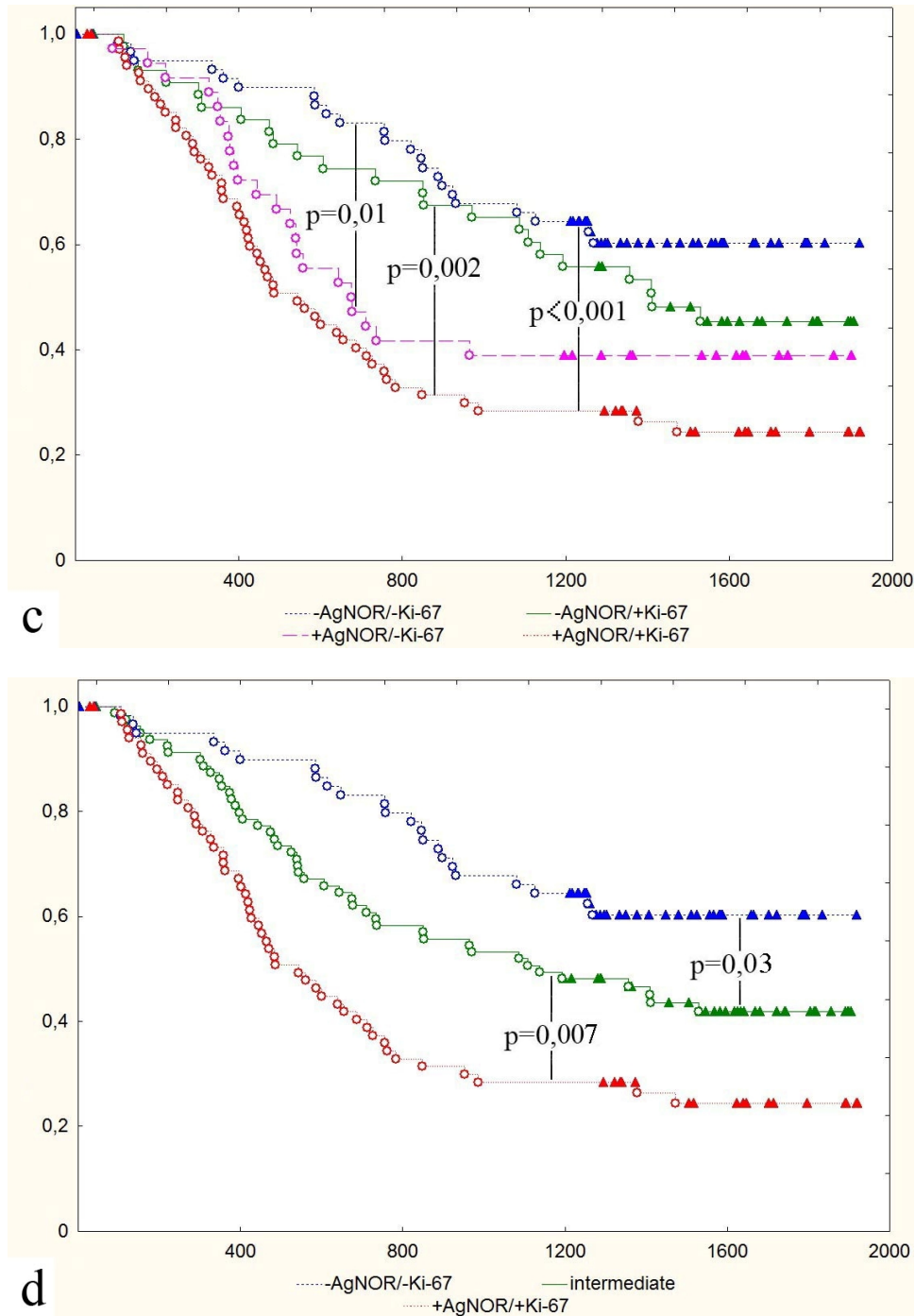


Fig. 2. Kaplan-Meier curve for patients with non-small cell lung cancer: With low and high AgNOR (a); with low and high Ki-67 (b); four (c) and three (d) types of tumor (by mutual AgNOR and Ki-67). The X-axis shows duration of survival (days), the Y-axis shows the proportion of surviving patients

At multivariate regression analysis four parameters - value N, greatest tumor dimension, histogenesis, AgNOR - had independent influence on survival in patients. Multiple studies devoted to AgNOR examination in malignant tumors also indicate that AgNOR is an independent predictive factor [15]. Survival in patients with NSCLC without lymph nodes metastases was related to greatest tumor dimension and histogenesis and in case with metastases - to greatest tumor dimension and AgNOR. Probably, survival in patients with NSCLC without lymph nodes metastases is related to cancer cells histogenetic origin and primary tumor growth rate (local size increase), and at metastatic potential appearance - to primary tumor growth rate and cancer cells cycle rate both in primary tumor and metastases.

Thus, combined determination of AgNOR and Ki-67 has prognostic value at NSCLC.

4. CONCLUSION

In NSCLC, clinical and morphological parameters on the TNM system are interconnected with molecular biological parameters - AI AgNOR and LI Ki-67. NSCLC patient survival depends on both separate and combined contents of AI AgNOR and LI Ki-67. Clinical and morphological (value N, greatest tumor dimension, histogenesis) and molecular biological (AI AgNOR) parameters are independent predictive factors at NSCLC.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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