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Argyrophilic Proteins of Nucleolar Organizer Regions and Proliferative Activity of Cells in Squamous Cell Carcinoma of the Lung

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Argyrophilic proteins associated with nucleolar organizer regions (Ag-NOR proteins) and Ki-67 antigen were analyzed in 118 samples of squamous cell carcinoma of the lungs. Tumors with low and high content of Ag-NOR proteins and Ki-67 index were selected. It was found that the content of Ag-NOR proteins correlated with some clinical and morphological parameters (indexes T and N, tumor size less and more that 3 cm, stage of the disease, and tumor differentiation degree) and survival rate. High survival was associated with low content of Ag-NOR proteins and Ki-67 index and low survival correlated with high content of Ag-NOR proteins and Ki-67 index, while intermediate survival was associated with opposite values of Ag-NOR protein content and Ki-67 index. The tumor size, parameter N, and the content of Ag-NOR proteins had independent effects on patient's survival. In patients with squamous cell carcinoma of the lung without metastases in lymph nodes, survival correlated with the content of Ag-NOR proteins; in patients without metastases, survival correlated with tumor size.

Key Words: *squamous cell carcinoma of the lung; nucleolar organizer regions; argirophilic proteins; Ki-67*

Delayed treatment results and survival rate in patients with squamous cell carcinoma of the lungs (SCCL) remain unsatisfactory. In this context, the search for new clinical, morphological, and biomolecular markers related to survival of SCCL patients is an urgent problem.

Proliferation is a basic process of tumor development and a prognostic factor of its biological behavior. Immunohistochemical analysis of Ki-67 antigen is a commonly accepted and available method for evaluation of proliferative activity [14]. A correlation of this marker with lung carcinoma prognosis has been demonstrated [3,7].

Argyrophilic proteins associated with nucleolar organizer regions (Ag-NOR proteins) are universally recognized markers of the cell cycle rate. Two main argyrophilic proteins C23 (nucleolin) and B23 (nucleophosmin) play a key role in the synthesis of ribosomal RNA and constitute up to 75% Ag-NOR proteins [13]. These proteins are detected in cell nuclei throughout the cell cycle and their content increases by 1.5-3 times during S and G_2 phases [15]. It has been demonstrated that the content of Ag-NOR proteins negatively correlated with cell cycle duration [4] and tumor doubling time [5].

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Analysis of published data has revealed contradictory associations between the content of Ki-67 and Ag-NOR proteins, on the one side, and clinical and morphological parameters of malignant tumors and patient's survival, on the other [1-3,10-12]. In SCCL these associations were never studied.

Here we analyze the associations of the expression of Ki-67 antigen and Ag-NOR proteins with clinical and morphological parameters of SCCL and patient's survival.

MATERIALS AND METHODS

We analyzed tissue samples (n=118) from patients with SCCL (113 men and 5 women; age 40-75 years) obtained during surgery performed at Altai Regional Cancer Center in 2007-2009 (patients with M1 and multiple tumors were excluded). Pathohistological characteristics of the tumors were determined according to WHO criteria [17].

Tissue samples were fixed in 10% neutral formalin and processed routinely; histological sections were stained with hematoxylin and eosin, with PAS/alcian blue, and by the method of Kreiberg. Antigen Ki-67 (clone MIB-1), High Molecular Weight cytokeratins (clone 34bE12) were analyzed by immunohistochemical methods on a Ventana XT automated stainer. The labeling index (LI) Ki-67 was alciulated as the percent of positively stained cells of the total number of cells. In each case, 1000 cells in 5-7 fields of view were analyzed at ×400. Since the distribution of Ki-67$^+$ cells in SCCL was nonparametric, the central trend was presented as the median; it constituted 30% (interquartile interval 23-45%) and was taken as a threshold value, which agreed with the previous findings [7]. Thus, cases with 30% LI Ki-67 were rated as high (+Ki-67) and <30% – as low (-Ki-67).

For the analysis of Ag-NOR proteins, the sections were stained with silver nitrate by the single-step protocol [16]. Before staining, the sections were autoclaved at 120°C for 20 min in 0.01 M citrate buffer (pH 6.0) [16], the nuclei were not post-stained. In each case, the area of Ag-NOR proteins (μ^2) in nuclei of 100-120 randomly selected cells was measured on 10-15 digital images of the corresponding microscopic fields of view at ×1000. Computer processing of the images was performed using ImageJ 1.42 software. To exclude the errors, the grains <0.1 μ^2 were excluded from the analysis. The area of Ag-NOR proteins in the nuclei of small lymphocytes was used as internal control [6]. The area index (AI) of Ag-NOR proteins was calculated as the ratio of Ag-NOR protein area in the tumor cell and small lymphocyte. Since the distribution of AI of Ag-NOR proteins in SCCL was parametric, the central trend was presented as the mean that constituted 6.96 (standard deviation 1.46). Similar to Ki-67 LI, the cases with AI of Ag-NOR proteins ≥6.96 were interpreted as high and <6.96 as low content of Ag-NOR proteins (+Ag-NOR and -Ag-NOR, respectively).

The data were processed using Statistica 6.0 software. When testing statistical hypotheses, two-way Fisher's exact test for 2×2 contingency tables and Spearman's rank correlation coefficient (r) were used. The overall adjusted survival for the 5-year period after the operation 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

For SCCL, a weak correlation was found between AI of Ag-NOR proteins and LI of Ki-67 (r=0.33, p<0.001). Cross-table distribution of SCCL cases with high and low Ag-NOR protein content and LI of Ki-67 depending on the morphological parameters of the tumor is presented in Table 1. A significant increase in the number of cases with +Ag-NOR was observed in the group of T2 and T3 tumors in comparison with T1: 46 (52%) and 7 (25%) cases, respectively (p=0.02). However, comparison of LI of Ki-67 revealed no significant differences between these groups: 53 (59%) and 12 (43%) cases, respectively (p=0.2).

In primary tumors >3 cm, the number of cases with +Ag-NOR was higher than in tumors <3 cm: 40 (52%) and 13 (31%) cases, respectively (p=0.03). The number of cases with +Ki-67 was also higher in tumors >3 cm in comparison with small tumors: 45 (59%) and 20 (48%) cases, respectively, but the difference was insignificant (p=0.3). The number of cases with +Ag-NOR was significantly higher in the group of tumors with metastases into lymph nodes in comparison with tumors without metastases: 28 (68%) and 25 (32%) cases, respectively (p<0.001). The number of cases with +Ki-67 did not differ significantly in these groups: 26 (63%) and 39 (51%) cases, respectively (p=0.2). The number of cases with +Ag-NOR in tumors of stages II and III was significantly higher than in tumors of stage I: 31 (62%) and 22 (32%) cases, respectively (p=0.002). The number of cases with +Ki-67 in these groups was 33 (66%) and 32 (47%); the differences between the groups did not attain the level of statistical significance (p=0.061). In samples with medium- and low-differentiated SCCL, the number of cases with +Ag-NOR was lower than in samples with highly differentiated SCCL: 44 (55%) and 9 (24%) cases, respectively (p=0.002). The number of cases with +Ki-67 in these groups was 49 (61%) and 16 (42%); the differences between the groups did not attain the level of statistical significance (p=0.074).

TABLE 1. Distribution of Cases with High and Low Content of Ag-NOR Proteins and LI of Ki-67 in SCCL

Characteristic	Number of cases		-Ag-NOR				+Ag-NOR			
			-Ki-67		+Ki-67		-Ki-67		+Ki-67	
	abs.	%	abs.	%	abs.	%	abs.	%	abs.	%
Primary tumor										
T1	28	24	13	46	8	29	3	11	4	14
T2 and T3	90	76	22	24	22	24	15	17	31	35
<3 cm	42	36	17	40	12	29	5	12	8	19
>3 cm	76	64	18	24	18	24	13	17	27	35
Lymph nodes										
N-	77	65	27	35	25	33	11	14	14	18
N+	41	35	8	20	5	12	7	17	21	51
Stage										
I	68	58	25	37	21	31	11	16	11	16
II and III	50	42	10	20	9	18	7	14	24	48
Differentiation degree										
high	38	32	18	47	11	29	4	11	5	13
moderate and low	80	68	17	21	19	24	14	18	30	37

TABLE 2. Content of Ag-NOR proteins, LI of Ki-67, and 5-Year Total Adjusted Survival in SCCL

Characteristic	Number of cases		5-year total adjusted survival, %
	abs.	%	
Content of Ag-NOR proteins			
low	65	55	68.9±6.3
high	53	45	19.0±5.9
LI of Ki-67			
low	53	45	57.7±7.9
high	65	55	38.4±6.4
Type of the tumor by Ag-NOR protein content of LI of Ki-67			
type 1: -Ag-NOR/-Ki-67	35	30	72.2±9.6
type 2: -Ag-NOR/+Ki-67	30	25	60.6±9.5
type 3: +Ag-NOR/-Ki-67	18	15	25.8±10.9
type 4: +Ag-NOR/+Ki-67	35	30	15.5±6.7
«intermediate» type	48	40	49.3±7.7

The content of Ag-NOR proteins in SCCL samples weakly correlated with parameter T (r=0.30, p=0.008), size of primary tumor >3 cm or larger (r=0.29, p=0.03), parameter N (r=0.37, p=0.002), stage of the disease (r=0.35, p<0.001), and differentiation degree (r=0.36, p<0.001). LI of Ki-67 did not correlate with these parameters. At early stages of SCCL development (T1, tumor size <3cm, N0, stage I, highly differentiated tumor), the content of Ag-NOR proteins in tumor cells was higher than at the late stage of the tumor process. Thus, a correlation was found between activity of nucleolar organizers and clinical and morphological parameters of SCCL (in contrast to Ki-67 antigen that did not correlate with these parameters), which suggests that tumor progression is related to ribosome synthesis.

Based on these data, four types of SCCL depending on the content of Ag-NOR proteins and LI of Ki-67 were distinguished: -Ag-NOR/-Ki-67 (type 1), -Ag-NOR/+Ki-67 (type 2), +Ag-NOR/-Ki-67 (type 3; Fig. 1), +Ag-NOR/+Ki-67 (type 4).

The total adjusted survival of patients with SCCL over 5 years after surgery was 47.6±4.7%. Survival of patients significantly correlated with the content of Ag-NOR proteins and LI of Ki-67 (Table 2; Fig. 2, *a*, *b*). Patient's survival gradually decreased in the following series: types 1, 2, 3, and 4 (Table 2; Fig. 2, *c*). In cases with high or low content of Ag-NOR proteins, LI of Ki-67 did not affect patient's survival (no differences were found between -Ag-NOR/-Ki-67 and -Ag-NOR/+Ki-67 and between +Ag-NOR/-Ki-67 and

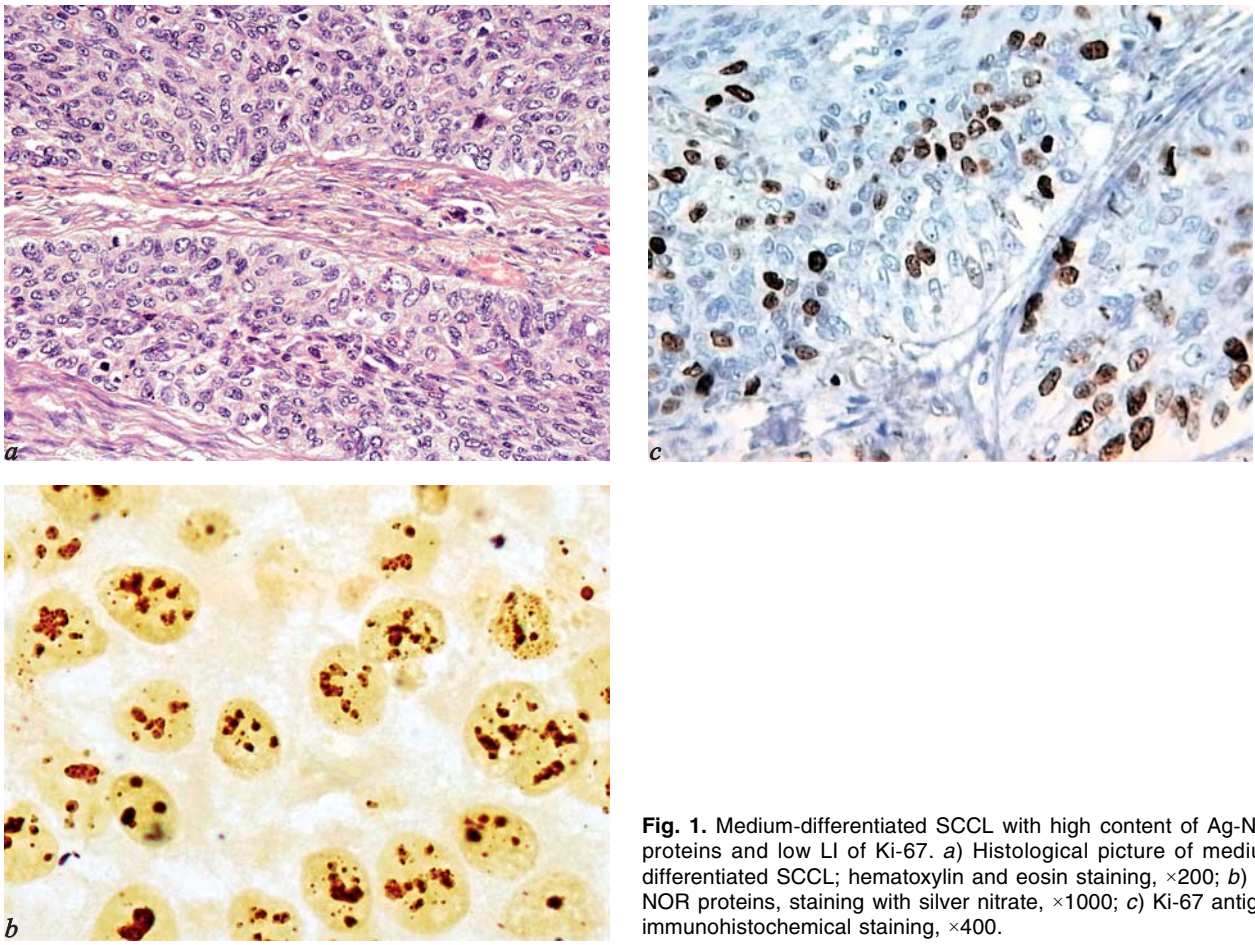

Fig. 1. Medium-differentiated SCCL with high content of Ag-NOR proteins and low LI of Ki-67. *a*) Histological picture of medium-differentiated SCCL; hematoxylin and eosin staining, ×200; *b*) Ag-NOR proteins, staining with silver nitrate, ×1000; *c*) Ki-67 antigen, immunohistochemical staining, ×400.

+Ag-NOR/+Ki-67). Survival of patients with tumors with low LI of Ki-67 and low content of Ag-NOR proteins (-Ag-NOR/-Ki-67) was higher than in patients with tumors with high content of Ag-NOR proteins and low LI of Ki-67 (+Ag-NOR/-Ki-67).

Similarly, in patients with tumors with high LI of Ki-67, survival in cases with low content of Ag-NOR proteins (-Ag-NOR/+Ki-67) was higher than in cases with low content of Ag-NOR proteins (+Ag-NOR/+Ki-67). Significant differences in survival were observed for patients tumor phenotypes -Ag-NOR/-Ki-67 and +Ag-NOR/+Ki-67 (types 1 and 4) and -Ag-NOR/+Ki-67 and +Ag-NOR/-Ki-67 (types 2 and 3). Taking these data into account, SCCL types 2 and 3 were united in an intermediate type characterized by opposite contents of Ag-NOR proteins and LI of Ki-67 (-Ag-NOR/+Ki-67 and +Ag-NOR/-Ki-67). Survival of patients with this intermediate type of tumors significantly differed from that in patients with tumor types 1 and 4 (Table 2; Fig. 2, *d*).

Multivariate regression analysis showed that parameter T, disease stage, differentiation degree, LI of Ki-67, and tumor types 1-4 (by the content Ag-NOR proteins and LI of Ki-67) did not correlate with survival of SCCL patients. Tumor size had more pronounced effect on patient's survival than parameter T (χ^2=72.9 and χ^2=67.9, respectively), the same can be said about the combination of tumor size and parameter N in comparison with stage of the disease (χ^2=72.9 and χ^2=61.9, respectively).

Thus, three criteria – tumor size (< **(less?)** or >3 cm), parameter N (absence or presence of metastases), and content of Ag-NOR proteins (low or high) – independently correlated with survival of SCCL patients; it should be noted that parameter N more substantially affected patient's survival (Table 3). Then, we studied the effect of tumor size on the content of Ag-NOR proteins in tumors with and without metastases in lymph nodes. In the absence of metastases, patient's survival correlated only with the content of Ag-NOR proteins (χ^2=16.5, p<0.001); on the contrary, in the presence of metastases, this parameter correlated only with tumor size (χ^2=14.7, p<0.001; Table 3).

A correlation was found between the content of Ag-NOR proteins and LI of Ki-67 in SCCL, which agreed with published data [9]. However, the content of Ag-NOR proteins in SCCL cells was related with a number of clinical and morphological parameters

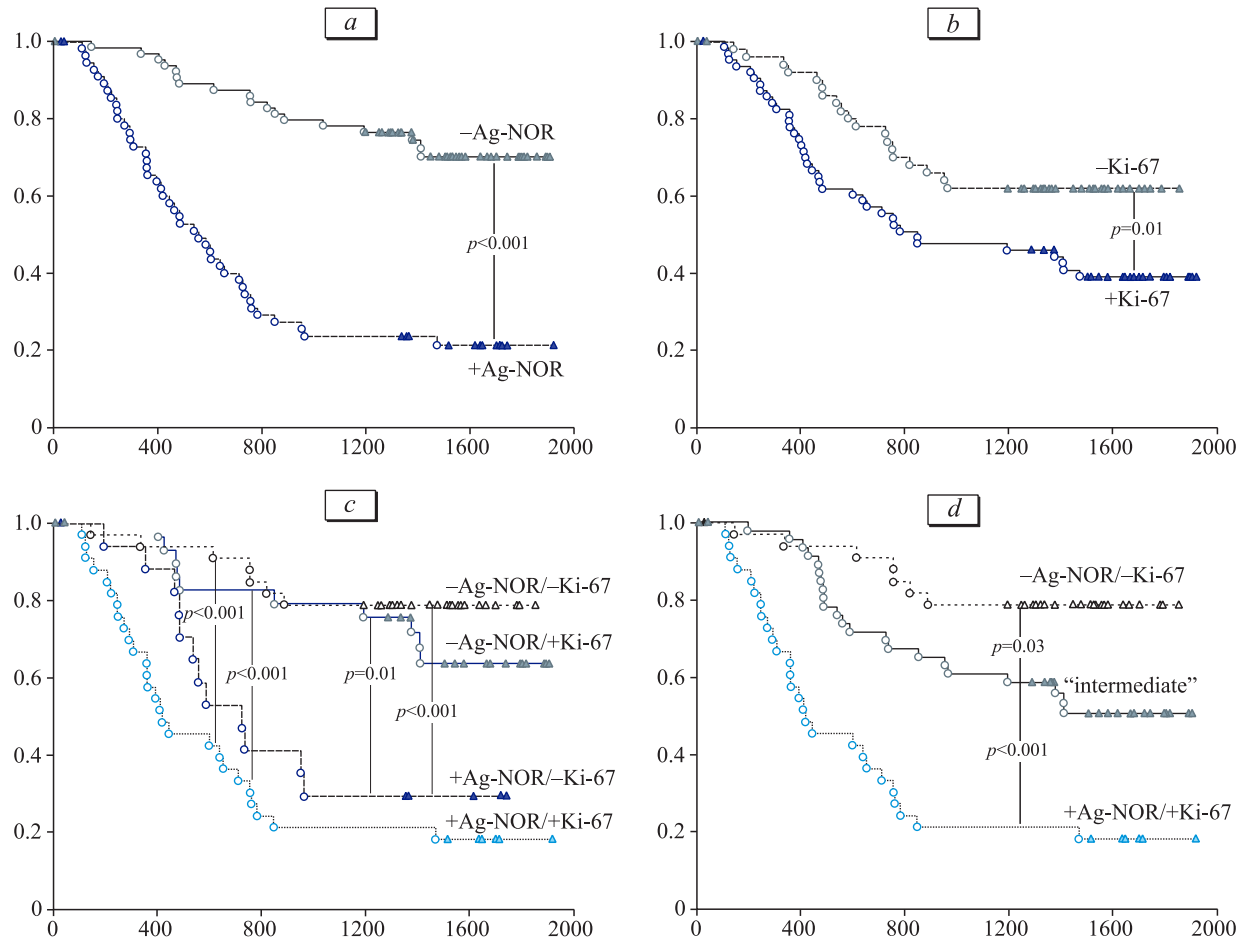

Fig. 2. Survival of patients with SCCL. Kaplan–Meier curves. *a*) With low and high content of Ag-NOR proteins; *b*) with low and high LI of Ki-67; *c*) by four tumor types (by Ag-NOR proteins and LI of Ki-67); by three tumor types (by Ag-NOR proteins and LI of Ki-67). Abscissa: lifespan, days. Ordinate: fraction of survivors.

according to TNM system: parameters T, N, primary tumor size (< 3 cm or > 3 cm), stage of the disease, and tumor differentiation degree. Similar data were obtained by other researchers studying nucleolar organizers in lung cancer [8].

Survival of SCCL patients with -Ag-NOR or -Ki-67 tumors was significantly higher than in patients with +Ag-NOR or +Ki-67 tumors. This interrelationship of nucleolar organizers and LI of Ki-67 with patient's survival was also observed in other studies [2,3,11,12]. According to published data, the correlation of nucleolar organizers with survival of patients with carcinomas of different histogenesis in different organs was more often detected in studies where the area of Ag-NOR proteins was evaluated by computer-assisted image analysis than in studies with visual recording of Ag-NOR proteins.

Four types of SCCL were distinguished depending on the content of Ag-NOR proteins and LI of Ki-67 and patient's survival decreased in the following order: -Ag-NOR/-Ki-67, -Ag-NOR/+Ki-67, +Ag-

TABLE 3. Cox Regression Analysis and Prognostic Factors in SCCL

Prognostic factor	β	Standard error	*p*
Parameter N	1.37	0.27	<0.001
Content of Ag-NOR proteins	1.19	0.30	<0.001
Tumor size	0.75	0.28	0.008
Absence of metastases			
Content of Ag-NOR proteins	1.40	0.40	<0.001
Tumor size	0.49	0.41	0.2
Presence of metastases			
Content of Ag-NOR proteins	0.84	0.44	0.06
Tumor size	1.04	0.39	0.008

NOR/-Ki-67, and +Ag-NOR/+Ki-67. Similar actuarial survival curves constructed with consideration to the content of Ag-NOR proteins and LI-of Ki-67 were obtained by other researchers for breast cancer patients [9]. In a previous analysis of 20 "minor" lung cancers (<3 cm) [1], 3-5-year and <2 year survival was demonstrated for tumors with phenotypes -Ag-NOR/+Ki-67 and +Ag-NOR/-Ki-67, respectively. In our study, significant differences in survival of SCCL patients with -Ag-NOR/+Ki-67 and +Ag-NOR/-Ki-67 tumor types was found.

Multivariate regression analysis revealed three criteria (tumor size, state of lymph nodes, and content of Ag-NOR proteins) that independently correlated with patient's survival. Numerous studies of nucleolar organizer activity in malignant tumors also suggest that the content of Ag-NOR proteins is an independent prognostic factor [2,11]. In patients with SCCL without metastases in lymph nodes, survival correlated with the content of Ag-NOR proteins; in patients with metastases, survival correlated with tumor size.

The content of Ag-NOR protein and LI-Ki-67 in SCCL are of diagnostic and prognostic significance. The content of Ag-NOR proteins in SCCL cells is related with clinical and morphological TNM parameters. Patients with SCCL and low content of Ag-NOR proteins or LI of Ki-67 has higher survival in comparison with patients with tumors characterized by high content of Ag-NOR proteins or LI of Ki-67.

High survival was observed in SCLL patients with -Ag-NOR/-Ki-67 tumors, low survival in patients with +Ag-NOR/+Ki-67 tumors, and intermediate in patients with -Ag-NOR/+Ki-67 and +Ag-NOR/-Ki-67 tumors. The tumor size, state of lymph nodes, and content of Ag-NOR proteins are independent prognostic factors in SCCL. In patients with SCCL without metastases in lymph nodes, survival correlated with the content of Ag-NOR proteins; in patients without metastases, survival correlated with tumor size.

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