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Nucleolar Organizer Regions as a Prognostic Indicator for Stage I Non-Small Cell Lung Cancer[1]

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ABSTRACT

When the number of silver-stained nucleolar organizer regions (Ag-NORs) was counted in 274 patients with non-small cell lung cancer, the mean number per nucleus in patients overall was 5.07 ± 1.92 (SD). With the use of the tumor (T)-nodes (N)-metastasis (M) classification, the mean Ag-NOR count for patients with T_1 and T_2 disease was statistically lower than that for those with T_3 and T_4 disease ($P < 0.01$). The mean Ag-NOR counts were lower in patients with N_0 disease than in those with N_1 and N_2 disease ($P < 0.01$); lower in patients with stage I disease than in those with stage II, IIIA, IIIB, or IV disease ($P < 0.01$); and lower in patients with adenocarcinoma than in those with squamous cell carcinoma ($P < 0.01$) or large-cell carcinoma ($P < 0.05$). In 131 patients with stage I disease, the mean Ag-NOR count was 3.80 ± 1.32, and the 5-year survival rates of patients with Ag-NOR counts of <3.80 and ≥3.80 were 78 and 44%, respectively, including 78% and 25% for adenocarcinoma, respectively ($P < 0.01$). However, there was no statistically significant difference for those in stage II, IIIA, IIIB, or IV, and in stage I (without an adenocarcinoma). Because patients with stage I non-small cell lung cancer and a high number of Ag-NORs had a poor prognosis, Ag-NORs can serve as a pertinent marker of an early recurrence.

INTRODUCTION

NORs[3] are loops of DNA that transcribe rRNA (1) and are found in the nucleoli. These NORs can be visualized by means of the argyrophilia of their associated proteins, using the so-called Ag-NOR technique (2, 3). The number of Ag-NORs per nucleus was suggested to correlate with cellular differentiation and activity and may serve as a possible indicator or a prognostic factor in various malignant neoplasms (4–7). The prognosis of lung cancer strongly depends on the stage of the disease, such as TNM status when the tumor is first detected. The 5-year survival rate is 10–30% of patients with stage IIIA, in contrast to the 50–70% of those with stage I (8, 9). We reported that the frequency of lymph node metastasis in lung cancer was 28.5% in cases of 3 cm or less (10). It is important to clarify the biological nature of rapidly progressing tumors, even in a small-sized lung cancer or one of an early stage. Based on the evidence of DNA flow cytometry of non-small cell lung cancer, patients with the aneuploid pattern had a shorter survival time than did those with the diploid pattern; thus, the DNA ploidy can be an important prognostic factor (11). On the other hand, other workers stated that the DNA ploidy is not useful as a prognostic marker in cases of stage I adenocarcinoma of the lung (12). Inasmuch as the prognostic significance of Ag-NORs in cases of human non-small cell lung cancer has apparently not been reported, we carried out related studies.

The Ag-NOR technique can be used for routine studies on non-small cell lung cancer since the stainability and counts of Ag-NORs are readily feasible.

MATERIALS AND METHODS

Surgical Specimens. We examined tissues that had been surgically excised from 274 patients with a non-small cell lung cancer. All of these Japanese patients had been diagnosed as cases of primary lung cancer, and all operations were performed at the Department of Surgery II, Faculty of Medicine, Kyushu University, from 1976 to 1987. There were 199 men and 75 women with ages ranging from 23 to 85 years (mean, 63 years). For assessment of the TNM classification for lung cancer, we followed the instructions of the International Union Against Cancer (13). T_1 is defined as a tumor of 3.0 cm or less in the greater dimension; T_2 is a tumor with more than 3.0 cm in the greater dimension; T_3 is a tumor that has invaded the chest wall, diaphragm, or the mediastinal pleura or pericardium; and T_4 is a tumor invading the mediastinum or involving the heart, great vessels, trachea, esophagus, or vertebral body, as well as cancerous pleural effusion. N_0 denotes no lymph node metastasis, N_1 indicates metastasis to hilar lymph nodes, N_2 is metastasis to mediastinal lymph nodes, and N_3 is metastasis to the contralateral hilum, mediastinum, and supraclavicular lymph nodes, ipsilateral as well as contralateral. M_0 denotes no distant metastasis, and M_1 denotes distant metastasis. The stages are defined as follows: stage I, $T_{1-2}N_0M_0$; stage II, $T_{1-2}N_1M_0$; stage IIIA, $T_{1-3}N_2M_0$ and $T_3N_{0-2}M_0$; stage IIIB, $T_{1-4}N_3M_0$, $T_4N_{0-3}M_0$; stage IV, $T_{1-4}N_{0-3}M_1$. There were 131 patients with stage I, 27 with stage II, 78 with stage IIIA, 23 with stage IIIB, and 15 with stage IV. Histology of the disease was determined according to the WHO classification (14). There were 159 patients with adenocarcinoma, 85 with squamous cell carcinoma, 25 with large-cell carcinoma, and 5 with adenosquamous cell carcinoma. The resected specimens were fixed in 10% formalin, and paraffin-embedded blocks were prepared. For histological studies, the sections were stained with hematoxylin and eosin.

Staining Technique. Ag-NOR staining and the modified one-step silver colloid methods were used (15). Briefly, 4-μm-thick sections were cut from paraffin-embedded blocks, dewaxed in xylene, and rehydrated through decreasing concentrations of ethanol to distilled deionized water. The Ag-NOR solution was freshly prepared by dissolving gelatin at a concentration of 2 g/dl in 1 g/dl aqueous formic acid to form the first solution. This solution was combined with 50 g/dl aqueous silver nitrate solution (1:2, v/v) to give the final Ag-NOR solution; the Ag-NOR solution was then immediately poured over the sections, which were then left in the dark, at room temperature, for 40 min. The silver colloid was washed from the sections with distilled deionized water, the sections were dehydrated through a graded series of ethanol to xylene, and coverslips were mounted with synthetic medium.

Counting Procedure. The Ag-NORs appeared as discrete dots within the nuclei of the cells and were counted in 100 tumor cells, from each patient, using a ×1000 magnification and an oil immersion lens. The mean number of Ag-NORs per nucleus was then calculated for each patient.

Statistical Analysis. The data were analyzed by means of Student's *t* test. Statistical comparisons were made with the χ^2 test. The survival rate was calculated by the Kaplan-Meier method (16). For statistical analyses of the survival rate, patients were separated into two groups: for patients with less than the mean number of Ag-NORs in the nucleus, the designation was a low Ag-NOR count; those with a mean number or more of Ag-NORs were considered to have a high Ag-NOR count. Data assessed included factors of sex, TNM stage, and histological cell

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[3] The abbreviations used are: NORs, nucleolar organizer regions; Ag-NORs, silver-stained nucleolar organizer regions; TNM, tumor-nodes-metastasis.

type. Comparisons among survival rates were made by the generalized Wilcoxon test (17). The difference was considered to be significant when $P < 0.05$.

RESULTS

Black silver-stained dots for Ag-NORs were clearly identified in all cell nuclei, as shown in Fig. 1. The number of Ag-NORs assessed according to various clinicopathological factors of sex, TNM status, stage, and histological type is given in Table 1. The mean number of Ag-NORs per nucleus of overall patients was 5.07 ± 1.92. The mean number of Ag-NORs per nucleus of T_1 or T_2 disease was statistically lower than that in cases of T_3 or T_4 disease ($P < 0.01$), the mean number of Ag-NORs per nucleus of N_0 disease was statistically lower than that in cases of N_1 or N_2 disease ($P < 0.01$), and the mean number of Ag-NORs per nucleus of stage I disease was statistically lower than that in cases of stage II, stage IIIA, stage IIIB, or stage IV disease ($P < 0.01$). According to the histological type, the mean number of Ag-NORs per nucleus of adenocarcinoma was statistically lower than that in cases of squamous cell carcinoma ($P < 0.01$) or large-cell carcinoma ($P < 0.05$). There was no statistically significant difference in factors of sex and metastasis.

The 5-year survival rate of patients separated by the mean number of Ag-NORs for the various factors is given in Table 2. In the case of factors such as sex, stage I, adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma there were statistically significant differences in the survival rates of patients with low and high Ag-NOR counts. As shown in Fig. 2, the 5-year overall survival rates of patients with low and high Ag-NOR counts were 56% and 25%, respectively ($P < 0.001$). In those with stage I disease, the 5-year survival rates in those with low and high Ag-NOR counts were 78% and 44%, respectively ($P < 0.01$) (Fig. 3). There were no statistically significant differences between low and high Ag-NOR counts in the cases of stage II, IIIA, IIIB, or IV.

Table 1 *Mean numbers of Ag-NORs per cell nucleus in patients with non-small cell lung cancer*

Variables	No. of patients	No. of Ag-NORs
Sex		
Male	199	5.00 ± 1.85[a]
Female	75	5.27 ± 2.12
Tumor		
1	91	4.53 ± 1.98
2	126	4.97 ± 1.74
3	33	6.12 ± 1.98
4	24	6.28 ± 1.54
Nodes		
0	162	4.22 ± 1.66
1	36	6.19 ± 1.57
2	76	6.34 ± 1.62
Metastasis		
0	259	5.05 ± 1.96
1	15	5.47 ± 1.13
Stage		
I	131	3.80 ± 1.32
II	27	6.20 ± 1.60
IIIA	78	6.37 ± 1.74
IIIB	23	6.29 ± 1.58
IV	15	5.47 ± 1.13
Histology		
Adenocarcinoma	159	4.71 ± 1.99
Squamous cell carcinoma	85	5.51 ± 1.74
Large-cell carcinoma	25	5.72 ± 1.54
Adenosquamous carcinoma	5	6.11 ± 2.22
Total	274	5.07 ± 1.92

[a] Mean ± SD.
[b] $P < 0.01$.
[c] $P < 0.05$.

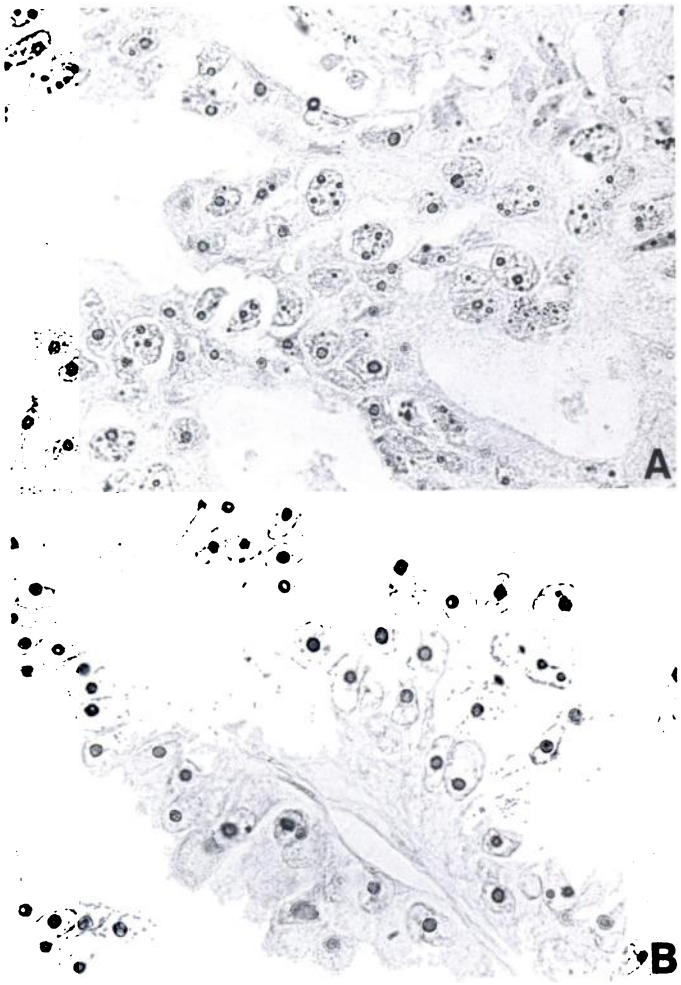

Fig. 1. Ag-NOR staining in adenocarcinoma of the human lung. *A*, high Ag-NOR counts (mean number, 3.84), multiple irregular dots in the nuclei. Ag-NOR method, × 850. *B*, low Ag-NOR counts (mean number, 1.12), mostly single regular dots in the nuclei. Ag-NOR method, × 850.

When classification was restricted to those with stage I disease, data on various clinicopathological factors were obtained, as shown in Table 3. The patients were divided into two groups at a cutoff point of 3.80 for the mean number of Ag-NORs for stage I. In those with an adenocarcinoma, the 5-year survival rate was 78% in cases of low Ag-NOR counts, a statistically significant value compared with the 25% in those with high Ag-NOR counts ($P < 0.01$) (Fig. 4). However, in cases of squamous cell carcinoma, large-cell carcinoma, and adenosquamous cell carcinoma, there was no significant difference between low and high Ag-NOR counts.

DISCUSSION

The Ag-NOR technique is in use in cytology studies to evaluate various genetic disorders (18). The standard chromosome banding technique has enabled trisomies to be detected in metaphase spreads (19). Recent modifications of this method have enabled a one-step sequence to be applied to paraffin sections, by incubation at 20°C without interfering background

Table 2 *Five-year survival rates of patients with non-small cell lung cancer, based on Ag-NOR counts*

Variables	Ag-NOR counts	No. of patients	5-yr survival rate (%)	P
Sex				
Male	Low[a]	101	57	<0.001
	High[b]	98	29	
Female	Low	42	57	<0.001
	High	33	16	
Stage				
I	Low	74	78	<0.01
	High	57	44	
II	Low	14	29	NS[c]
	High	13	19	
IIIA	Low	39	25	NS
	High	39	25	
IIIB	Low	8	29	NS
	High	15	18	
IV	Low	12	10	NS
	High	3	33	
Histology				
Adenocarcinoma	Low	86	66	<0.001
	High	73	15	
Squamous cell carcinoma	Low	48	53	<0.05
	High	37	42	
Large-cell carcinoma	Low	15	41	<0.01
	High	10	10	
Adenosquamous carcinoma	Low	3	30	NS
	High	2	0	
Total	Low	146	56	<0.001
	High	128	25	

[a] The number of Ag-NORs in the nucleus is less than the mean number of Ag-NORs, according to each variable factor.
[b] The number of Ag-NORs in the nucleus is the mean number or more than the mean number of Ag-NORs, according to each variable factor.
[c] NS, not significant.

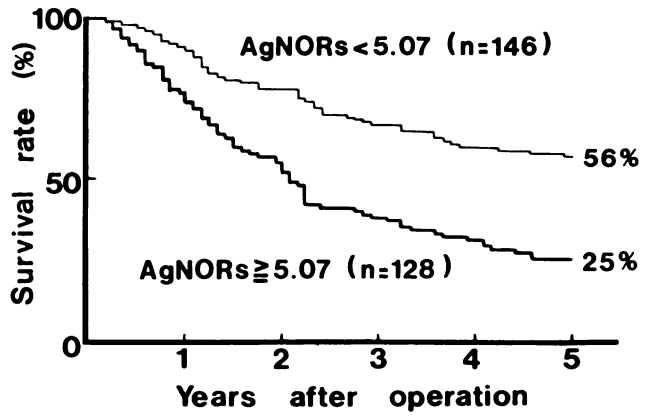

Fig. 2. Survival curves of patients with non-small cell lung cancer, according to Ag-NOR counts. The difference is significant between low and high Ag-NOR counts ($P < 0.001$).

deposits that would be present in cases of incubation at 60°C (15).

Other investigators reported that Ag-NOR correlated with the proliferative activity of certain human malignant tumors. Crocker and Nar (4) found a significant difference between the number of Ag-NORs of low-grade and high-grade lymphomas. Crocker and McGovern (5) applied the Ag-NOR method to normal, cirrhotic, and carcinomatous livers and found significant differences in their numbers. Smith and Crocker (6) used the Ag-NOR method to study normal breast tissues and benign and malignant breast tumors, and they stated that the number of Ag-NORs was diagnostically useful (6). Derenzini *et al.* (7) made use of the Ag-NOR method to examine epithelial tumors of the human intestine. They found that the number of Ag-NORs in the malignant lesions was significantly higher than in benign lesions. We obtained evidence for a close correlation between the stage of lung cancer and increase in the Ag-NOR counts. Therefore, a high number of Ag-NORs can serve as an indicator or prognostic factor in various malignant neoplasms, together with DNA flow cytometry and labeling with proliferating cell markers such as the antibody Ki-67 (11, 20).

We examined the number of Ag-NORs and prognosis in

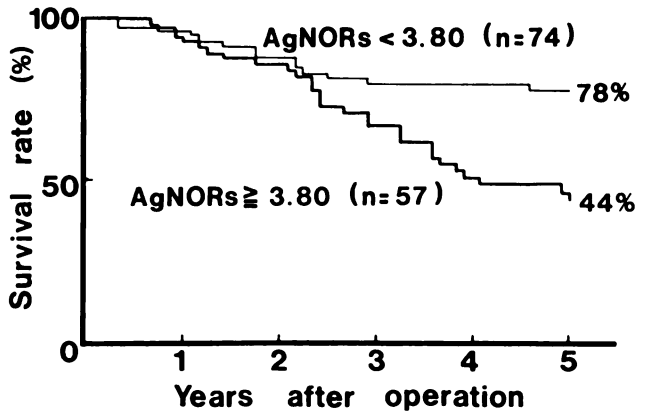

Fig. 3. Survival curves of patients with stage I non-small cell lung cancer according to Ag-NOR counts. The difference is significant between low and high Ag-NOR counts ($P < 0.01$).

Table 3 *Relationship between the Ag-NORs and various clinicopathological factors in patients with stage I non-small cell lung cancer*

Factors	Low Ag-NOR counts[a]	High Ag-NOR counts[b]	
Sex			
Male	51 (53.7)[c]	44 (46.3)	
Female	23 (63.9)	13 (36.1)	
Tumors			
1	43 (64.2)	24 (35.8)	
2	31 (48.4)	33 (51.6)	
Histology			
Adenocarcinoma	59 (72.0)	23 (28.0)	[d]
Squamous cell carcinoma	12 (30.8)	27 (69.2)	[d]
Large-cell carcinoma	3 (37.5)	5 (62.5)	
Adenosquamous carcinoma	0	2 (100)	
Total	74 (56.5)	57 (43.5)	

[a] The number of Ag-NORs is <3.80/nucleus.
[b] The number of Ag-NORs is ≥3.80/nucleus.
[c] Numbers in parentheses, percentage.
[d] $P < 0.01$.

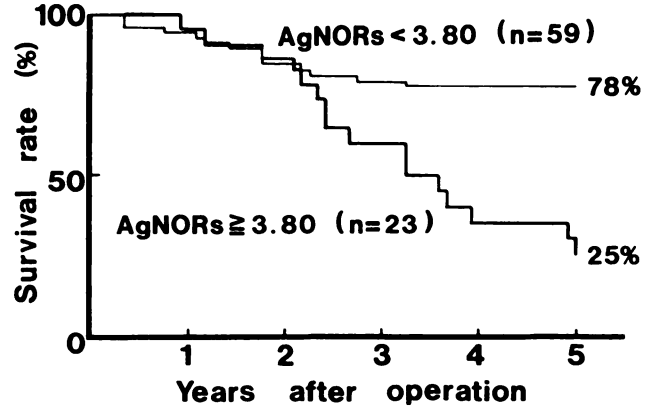

Fig. 4. Survival curves of patients with stage I adenocarcinoma of the lung according to Ag-NOR counts. The difference is significant between low and high Ag-NOR counts ($P < 0.01$).

cases of non-small cell lung cancer and found an increase in patients in the advanced stage. The mean number of Ag-NORs per cell of adenocarcinoma was lower than that of squamous cell carcinoma or large-cell carcinoma. In cases of stage I adenocarcinoma, the prognosis of patients with a large number of Ag-NORs was worse. Ag-NOR counting is tedious and does not lend itself to widespread use, even though the staining method is relatively easy, the preparation of solutions is economically feasible, and no special equipment need be purchased. One of the most outstanding features of this technique is that it can be used on routinely processed histological sections, for specimens obtained at preoperative biopsies. Predicting the prognosis in individual cases will greatly influence related therapeutic planning.

In conclusion, the present study shows that there is a close correlation between Ag-NOR counts and various prognostic factors in lung cancers. Even in stage I disease, the Ag-NOR counts reflected the survival time. We wish to emphasize that Ag-NOR staining can serve as a pertinent marker of an early recurrence in patients with a malignant lung tumor. Patients with a large number of Ag-NORs in the tumor will need systemic adjuvant therapy.

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