

# Prognostic Significance of Argyrophilic Nucleolar Organizer Regions in Esophageal Carcinoma

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## ABSTRACT

The argyrophilic nucleolar organizer region (AgNOR) of 100 cancer cells from biopsy specimens of esophageal squamous cell carcinomas in 98 surgically treated cases was examined, using a silver colloid staining technique on biopsy specimens. The number of AgNOR per nucleus (AgNOR number) was higher in the more advanced groups with regard to the length of the tumor ( $P < 0.01$ ), the depth of penetration ( $P < 0.05$ ), and lymph node metastasis ( $P < 0.01$ ). The survival of the patients with a high AgNOR number ( $\geq 6$ ) was significantly poorer than those with either a medium range AgNOR number ( $4 \leq < 6$ ) ( $P < 0.05$ ) or a low AgNOR number ( $< 4$ ) ( $P < 0.01$ ). In the multivariate analysis including conventional clinicopathological factors, the AgNOR number was found to be one of the independent and significant variables ( $P < 0.01$ ). Because the AgNOR method is simple and can be applied to paraffin-embedded sections, the AgNOR number may provide potential benefit in the pretherapeutic assessment of malignant potentiality in esophageal carcinoma.

## INTRODUCTION

The nucleolus plays an important role in the control of cell proliferation and protein synthesis (1). The NORs,<sup>2</sup> which are closely associated with nucleoli, are loop DNA encoded for rRNA production (2, 3). The development of a simple one-step silver staining technique makes it easy to display proteins that are intimately associated with NORs in formalin-fixed tissue (4, 5). They are called AgNORs and have been studied in malignancies of different organs. As a result, it has been suggested that the AgNOR number correlates with both cellular kinetics and the malignant grade of the tumor (6-11). However, there have only been a few reports which evaluated the prognostic significance of the AgNOR number in malignancy (12-15). Furthermore, the AgNOR of esophageal carcinoma has not been reported previously.

In this study, we examined the AgNOR number in 98 cases of esophageal carcinoma undergoing esophageal resection and reconstruction, using routinely processed biopsy specimens. The relation between the AgNOR number and clinicopathological features was analyzed and the prognostic value of the AgNOR number was studied using a multivariate analysis.

## MATERIALS AND METHODS

**Clinical Material.** This study was based on an analysis of 98 patients with carcinoma of the thoracic esophagus. The patients included 84 men and 14 women ranging in age from 43 to 81 years. All of these patients had been diagnosed as cases of squamous cell carcinoma, determined from the results of a biopsy specimen under esophageal endoscopy, and they were all surgically treated from 1972 to 1989 in

our department. There was no evidence of metastasis to any other organs.

Macroscopic and microscopic evaluations were made according to the rules established by the Japanese Society for Esophageal Diseases (16). Analyses of AgNORs were performed, using the biopsy specimens before any treatment.

**Measurements of AgNOR Number.** The tissue was fixed in 10% formalin and processed routinely in paraffin wax. Paraffin sections (4  $\mu$ m) were cut and dewaxed in xylene and hydrated through graded ethanols to deionized water. The AgNOR staining solution was prepared as previously described by Egan *et al.* (12); it was obtained by adding 1 volume of 2% gelatin in 1% formic acid to 2 volumes of 50% aqueous silver nitrate. The tissue sections were inoculated with freshly prepared AgNOR staining solution for 1 h at room temperature in the dark. The silver colloid was then washed off with deionized water and the sections were then dehydrated through graded ethanols to xylene. No counterstain was needed.

The counting sections were examined under an oil immersion lens at  $\times 1000$ . At least 100 cells from each tumor were examined while choosing the fields at random and avoiding any nontumorous areas detected with routine hematoxylin-eosin staining. In each case, the number of NOR dots per nucleus (AgNOR number) was calculated.

**Statistical Analysis.** All data were stored in an IBM (Armonk, NY) 4381 main-frame computer. The BMDP (Los Angeles, CA) was used for all statistical analyses (17). The BMDP P3S program was used for a Kruskal-Wallis one way analysis of variance to compare the NOR number for each clinicopathological feature. The BMDP P1L program was used for survival analysis, with the use of the Kaplan-Meier method, and this program was also used for testing the equality of survival curves according to the Mantel-Cox method. A BMDP P2L program was used for the multivariate adjustment of all covariates, simultaneously, by Cox regression analysis (18).

Prognostic factors analyzed for their influence on survival were age, sex, site of tumor, length of tumor, differentiation of squamous cell carcinoma, depth of penetration, lymph node metastasis, lymphatic invasion, vessel invasion, and AgNOR number. Any deaths resulting from causes other than the primary cancer were censored in the statistical analysis.

## RESULTS

**AgNOR Number and Clinicopathological Features.** The AgNOR number in all 98 cases averaged  $4.93 \pm 1.49$  (SD). The AgNOR number was larger in cases where the tumors were longer than 5 cm ( $P < 0.01$ ); however, there was no statistically significant difference in AgNOR number regarding the patient's other clinical background, such as sex, age, and site of tumor (Table 1). As for the pathological features, there was a statistically significant difference in AgNOR number with regard to the depth of penetration ( $P < 0.05$ ) and lymph node metastasis ( $P < 0.01$ ). The AgNOR number was thus larger in the more advanced groups in view of these features. However, there was no statistically significant difference in the AgNOR number according to other pathological factors (Table 2).

**Relationship between AgNOR Number and Prognosis.** Patients were tentatively divided into three groups according to the AgNOR number; high (AgNOR number,  $\geq 6$ ); medium range ( $4 \leq$  AgNOR number  $< 6$ ); and low (AgNOR number,  $< 4$ )

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<sup>2</sup> The abbreviations used are: NORs, nucleolar organizer regions; AgNOR, argyrophilic nucleolar organizer region; AgNOR number, number of AgNOR per nucleus; BMDP, Biomedical Computer Program.

AgNOR number (Fig. 1). The borders between the groups were based on the fact that the mean AgNOR number of all cases was nearly 5. The survival time of patients with a high AgNOR number was significantly shorter than both those with a medium range AgNOR number ( $P < 0.05$ ) and those with a low AgNOR number ( $P < 0.01$ ). The 5-year survival rates for each group were 11.0, 30.8, and 44.8%, respectively (Fig. 2).

Table 3 summarizes the results of the multivariate analysis

Table 1 Clinical features and AgNOR number

Clinical features	No. of cases	AgNOR no. (mean ± SD)	$P^a$
All	98	4.93 ± 1.49	
Sex			
Male	84	4.92 ± 1.39	NS <sup>b</sup>
Female	14	5.01 ± 2.05	
Age (yr)			
30–49	6	5.15 ± 1.41	NS
50–69	69	4.94 ± 1.53	
≥70	23	4.85 ± 1.44	
Site of tumor			
Upper esophagus	14	4.95 ± 2.06	NS
Midesophagus	64	4.92 ± 1.36	
Lower esophagus	20	4.93 ± 1.53	
Length of tumor (cm)			
<5.0	29	4.09 ± 1.28	<0.01
≥5.0	69	5.23 ± 1.44	

<sup>a</sup> Based on the Kruskal-Wallis test.

<sup>b</sup> NS, not significant.

Table 2 Pathological features and AgNOR number

Histological features	No. of cases	AgNOR no. (mean ± SD)	$P^a$
Differentiation of SCC <sup>b</sup>			
Well	27	5.06 ± 1.55	NS
Moderately	45	5.11 ± 1.43	
Poor	26	4.48 ± 1.48	
Depth of penetration			
No invasion into the adventitia	35	4.38 ± 1.31	<0.05
Invasion into the adventitia	32	4.97 ± 1.35	
Invasion into the neighboring areas	31	5.51 ± 1.62	
Lymph node metastasis			
Negative	55	4.46 ± 1.44	<0.01
Positive	43	5.53 ± 1.34	
Lymphatic invasion			
Negative	51	4.78 ± 1.53	NS
Positive	47	5.10 ± 1.44	
Vessel invasion			
Negative	80	4.94 ± 1.48	NS
Positive	18	4.91 ± 1.54	

<sup>a</sup> Based on the Kruskal-Wallis test.

<sup>b</sup> SCC, squamous cell carcinoma; NS, not significant.

for prognostic factors. Regarding the prognostic factors analyzed for their influence on survival, the AgNOR number ( $P < 0.01$ ) along with lymph node metastasis ( $P < 0.001$ ), vessel invasion ( $P < 0.01$ ), and depth of penetration ( $P < 0.05$ ) were all observed to be significantly correlated with the prognosis.

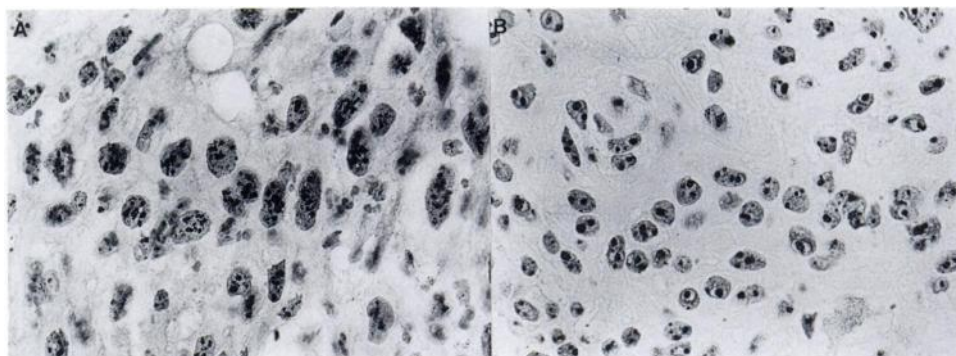
DISCUSSION

Recent improvement in the staining techniques of NORs have enabled us to more accurately visualize the NORs in conventionally fixed and processed paraffin sections. It has been suggested that the AgNOR number is significantly different in both benign and malignant tumors of different organ systems such as the breast and bronchus; the AgNOR number has been proposed as a diagnostic parameter for the grade of malignancy (19, 20). With regard to the cancers in the alimentary tract, it has been reported that the AgNOR number can reflect the prognosis of colon cancer (14, 15), it is higher in malignant lesions than in benign lesions of the stomach (11).

In order to evaluate the malignant potentiality of the lesion, DNA flow cytometry and immunohistochemical staining with monoclonal antibodies are applied. Unlike these methods, in silver colloid staining, no special or expensive reagents or apparatuses are required and it is simple to carry out. Furthermore, this method can be applied to routinely processed paraffin-embedded sections, even when the specimen is only a minute biopsy. The AgNOR number has been proved to be closely related with the result of immunohistochemical staining with Ki67 and monoclonal antibody to bromodeoxyuridine, which reflects cellular proliferation (8–10). Furthermore, it correlates well with the number of cells in S phase by means of DNA flow cytometry (6). These results suggest that the AgNOR number may reflect the cellular kinetics of carcinoma.

The clinicopathological and prognostic role of the AgNOR number for esophageal carcinoma has not been reported previously. In this study, the prognosis of patients with a high AgNOR number was significantly poorer. However, the AgNOR number was found to be closely correlated to lymph node metastasis, the depth of penetration, and the length of the tumor. These clinicopathological factors apparently play an important role in the prognosis of patients with esophageal carcinoma. In order to accurately evaluate the prognostic significance of the AgNOR number, it is essential to remove the effects of such clinicopathological factors on the prognosis. Thus, a multivariate analysis was applied with various prognostic factors, including conventional clinicopathological factors and AgNOR number. As a result, the AgNOR number proved to be one of prognostic factors independent from other factors and to be a good indicator of malignant potentiality of esophageal cancer.

Fig. 1. AgNORs of esophageal carcinoma. A, case with a high AgNOR number. AgNORs are heterogeneous in size, shape, and number. Many nuclei contain a large number of AgNOR dots. B, case with a low AgNOR number. Tumor nuclei contain mostly a few undispersed AgNOR dots. Silver colloid staining, × 1040.



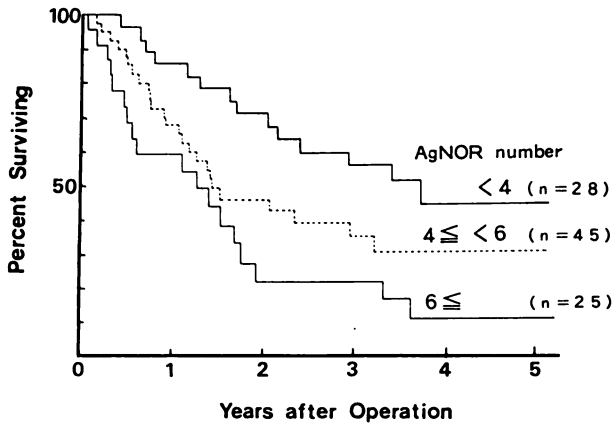


Fig. 2. Survival curve of patients with esophageal carcinoma according to AgNOR number. The survival time of patients with a high AgNOR number (AgNOR number,  $\geq 6$ ) is significantly shorter than both those with a medium range AgNOR number ( $4 \leq$  AgNOR number  $< 6$ ) ( $P < 0.05$ ) and those with a low AgNOR number (AgNOR number,  $< 4$ ) ( $P < 0.01$ ).

Table 3 Multivariate analysis of Cox's proposional hazards model in patient's with esophageal cancer

Variables	$\chi^2$	P
Age	0.23	NS <sup>a</sup>
Sex	0.02	NS
Site of tumor	2.32	NS
Length of tumor	1.56	NS
Differentiation of SCC	3.39	NS
Depth of penetration	5.21	$< 0.05$
Lymph node metastasis	13.65	$< 0.001$
Lymphatic invasion	0.20	NS
Vessel invasion	9.64	$< 0.01$
AgNOR number	9.29	$< 0.01$

<sup>a</sup> NS, not significant; SCC, squamous cell carcinoma.

In general, the prognosis of esophageal carcinoma is poor (21). Radical operation (21) and pre- and postoperative combined therapy (22) play an important role in the treatment of this disease. The AgNOR number of a preoperative biopsy is a good indicator of the malignant potentiality of esophageal carcinoma. Characterization of esophageal carcinoma with AgNOR number may be of potential use in the pretherapeutic assessment of the malignant potentiality of esophageal carcinoma and as a guide line for selecting the therapeutic strategy, including operative radicality and pre- and postoperative combined therapy.

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