

Argyrophilic Nucleolar Organizer Regions in Cells of Thymoma and Thymic Carcinoma*

Correlation With DNA Ploidy and Clinicopathologic Characteristics

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Study objectives: To evaluate the usefulness of argyrophilic nucleolar organizer region (AgNOR) counting and flow cytometric DNA analysis in the differential diagnosis of thymoma and thymic carcinoma, as well as in the differences among various stages and histologic subtypes of these tumors.

Design and interventions: Paraffin-embedded blocks of 64 thymic epithelial tumors (20 noninvasive thymomas, 34 invasive thymomas, and 10 thymic carcinomas) were studied by AgNOR counting and flow cytometric DNA analysis. The thymomas were histologically classified as medullary, cortical, or mixed subtype.

Measurements and results: Invasive thymomas had more AgNORs (\pm SD) than noninvasive thymomas (7.93 ± 2.90 vs 5.97 ± 1.77 ; $p < 0.01$). The number of AgNORs of thymoma increased progressively with advances in stage ($p < 0.01$). Cortical thymomas had the highest number of AgNORs among the three subtypes ($p < 0.05$). Patients with thymoma who presented with myasthenia gravis also had a higher number of AgNORs (8.30 ± 3.12 vs 6.50 ± 2.03 ; $p < 0.01$). The AgNOR number did not correlate with the DNA ploidy of all specimens.

Conclusions: AgNOR counting is useful in differentiating between invasive and noninvasive thymomas, and in predicting the stage of thymomas. A greater number of AgNORs was observed in patients with cortical thymoma and in those who presented with myasthenia gravis.

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Key words: AgNOR; DNA ploidy; thymoma

Abbreviations: AgNOR = argyrophilic nucleolar organizer region; MG = myasthenia gravis; NOR = nucleolar organizer region

According to histologic atypia, thymic epithelial tumors can be divided into thymomas and thymic carcinomas.¹ Although rare, thymomas are the most common neoplasm of the anterior mediastinum in adults.² Differentiation between noninvasive and invasive thymoma is based not on cellular characteristics, but on evidence of microscopic or macroscopic invasion of the capsule or surrounding tissue.³ Inva-

sive thymoma and thymic carcinoma are designated as malignant thymoma type I and type II, respectively.⁴

Nucleolar organizer regions (NORs) are loops of ribosomal DNA on the short arms of five acrocentric chromosomes (chromosomes 13 to 15, 21, and 22) in the nucleoli of cells.⁵ The argyrophilic NOR-related proteins (RNA polymerase I, B23 protein, C23 protein, or nucleolin), encoded by the genes located in the NORs, can be identified as small black dots in the nucleus under a light microscope using the rapid one-step argyrophilic NOR (AgNOR) staining technique.⁶ The number of AgNORs per nucleus has been proved to correlate with the proliferative activity of cells.⁷ In addition, AgNOR count appears to be of value in aiding the cytopathologic diagnosis in a variety of malignancies.⁸⁻¹⁴ Abnormal DNA content, measured by flow cytometry, can also serve as an

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important indicator of malignant potential since DNA ploidy may reflect chromosomal abnormalities.¹⁵

To our knowledge, there is no report of a relationship between AgNOR numbers and DNA ploidy in thymoma and thymic carcinoma. We therefore explored the relationship among AgNOR numbers, DNA ploidy, and clinicopathologic characteristics of thymoma and thymic carcinoma in this study.

MATERIALS AND METHODS

Materials

The surgical pathologic specimens were obtained from the 64 consecutive unselected patients with thymic epithelial tumors, including 54 thymomas and 10 thymic carcinomas from 1987 to 1992 at Veterans General Hospital-Taipei. There were 46 male and 18 female patients, with an age range from 30 to 83 years (median, 60.5 years). Of the 54 thymomas, 20 were noninvasive and 34 were invasive. Each tumor was classified according to surgical and histopathologic findings.

The slides were reviewed independently by an experienced pathologist (C-C.P.) to verify the diagnosis. The 54 specimens of thymoma were histopathologically categorized as cortical (34 cases), medullary (14 cases), and mixed (6 cases) subtypes based on the classification system established by Marino and Müller-Hermelink.¹ The staging system reported by Masaoka et al¹⁶ was adopted. Medical records of patients with thymoma were reviewed to determine the presence of myasthenia gravis (MG).

AgNOR Staining and Counting

AgNOR staining was performed according to the modified method reported by Ploton et al.⁶ The staining solution was prepared by mixing one part of 0.5% gelatin and 1% formic acid with two parts of 45% aqueous silver nitrate. All sections were cut to 3 μm in thickness from routinely processed paraffin blocks. These were dewaxed in xylene and then rehydrated through graded ethanols to distilled deionized water. The sections were covered with the AgNOR staining solution at room temperature in the dark for 16 min to reach the optimal results. The specimens were then washed with distilled deionized water, dehydrated through graded ethanol to xylene, and mounted in synthetic medium.

All discernible AgNORs, which appeared as dots both outside and within the nucleoli, were counted according to the description of Crocker et al.⁹ Two hundred nuclei of epithelial cells from each specimen were evaluated randomly. All specimens were studied independently by two observers on a photomicroscope (VANOX-S; Olympus; Huntington Station, NY) with an oil immersion lens at a magnification of $\times 1,000$.

Analysis of DNA Content

DNA flow cytometric analysis was performed, with minor modification, according to the method from the report of Hedley et al¹⁷ using samples prepared from archival paraffin blocks. Briefly, two 100- μm -thick sections were dewaxed in xylene, rehydrated in a series of graded ethanols, washed in Dulbecco's phosphate-buffered saline solution, and then digested in 0.5% pepsin for 30 min at a pH of 1.5. The resulting suspension was then filtered through a nylon gauze, washed, centrifuged, and

incubated with RNase at 37°C for 20 min. The cells were then stained with propidium iodide for at least 1 h and analyzed by a flow cytometer (Epics Profile II; Coulter Electronics; Hialeah, FL).

On average, 1×10^6 nuclei per specimen were analyzed and the DNA content of each nucleus was expressed as a histogram. The stromal cells in the same tumor were used as internal reference cells for diploidy. Diploid samples were defined as having a single G_0G_1 peak as the reference cells, while aneuploid tumors were considered when multiple peaks were present in addition to the diploid G_0G_1 peak.

Statistical Analysis

The total number of AgNORs from 200 nuclei per specimen was determined and the mean value and SD of each case were established. Two-tailed independent Student's *t* test was used to compare data between groups, whereas one-way analysis of variance test was utilized to compare among groups. χ^2 test was used to check the correlation of two independent items. The trend of correlation between AgNORs and staging was assessed by Spearman rank correlation coefficient. The interobserver variation was evaluated by Pearson's correlation coefficient (SPSS for Windows version 6.0; SPSS; Chicago, IL).

RESULTS

AgNORs in the nucleoli and nucleoplasm of thymic epithelial tumor cells appeared as round or irregular dark brown dots within the tan background of the nuclei (Figs 1–3). The relationships between the number of AgNORs and clinicopathologic variables of all cases are summarized in Table 1.

Invasive thymomas had more AgNORs than noninvasive thymomas ($p < 0.01$), while there was no significant difference in AgNORs between invasive thymomas and thymic carcinomas. Stages III and IV thymomas were considered as late-stage thymomas. A significant difference in AgNORs was noted be-

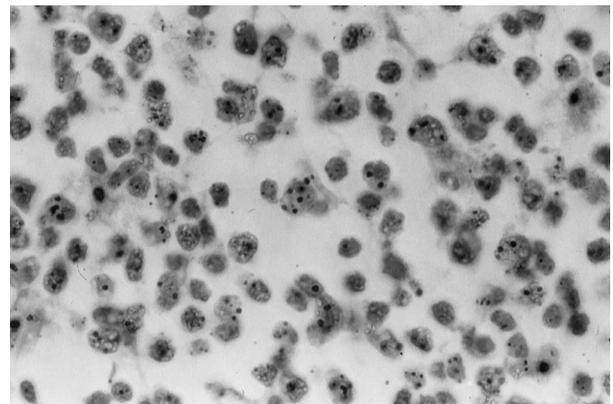


FIGURE 1. AgNORs in a cortical thymoma specimen. The nuclei of epithelial cells are larger than those of lymphocytes, with more than five AgNORs per nucleus, in contrast to the nuclei of lymphocytes that are smaller and have dense chromatin (AgNOR stain, original magnification $\times 1,000$).

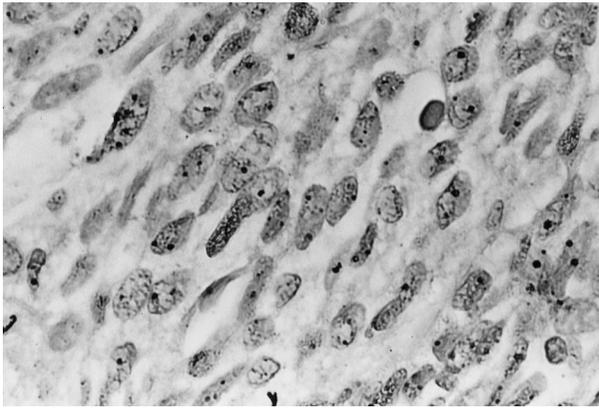


FIGURE 2. AgNORs in medullary thymoma. The nuclei of epithelial cells are spindle-shaped (AgNOR stain, original magnification $\times 1,000$).

tween stage I and late-stage thymomas ($p < 0.01$). Although there was no significant difference in AgNORs between stage II and late-stage thymomas, a trend toward an increased number of AgNORs was observed with more advanced stage (Spearman rank correlation coefficient, $\gamma = 0.8$, $p < 0.01$).

The cortical subtype of thymomas had a significantly higher number of AgNORs than the other two subtypes ($p < 0.05$). The thymoma patients with MG had a significantly higher number of AgNORs than those without MG ($p < 0.01$). No statistically significant difference was noticed between aneuploid and diploid AgNORs number. The interobserver agreement of the AgNORs scoring was excellent ($\gamma = 0.9$, $p < 0.001$).

Table 2 shows the relationship between DNA ploidy and clinicopathologic variables in our cases. DNA ploidy was not associated with invasiveness,

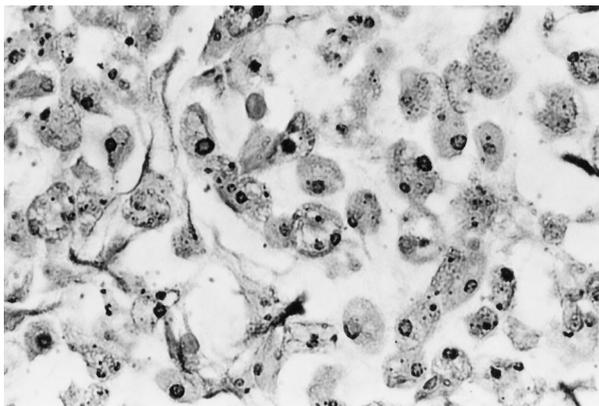


FIGURE 3. AgNORs in thymic carcinoma. The nuclei are larger and more pleomorphic than those in thymoma (AgNOR stain, original magnification $\times 1,000$).

Table 1—Relationship Between Mean Number of AgNORs and Clinicopathologic Variables of Thymoma and Thymic Carcinoma

Variable	No.	AgNORs (Mean \pm SD)	p Value
Histology			
Thymic carcinoma	10	7.22 \pm 2.42	
Thymoma	54	7.20 \pm 2.70	> 0.05*
Noninvasive	20	5.97 \pm 1.77	
Invasive	34	7.93 \pm 2.90	< 0.01*†
Stage of thymomas			
I	20	5.97 \pm 1.77	
II	13	6.74 \pm 2.10	
III + IV	21	8.67 \pm 3.13	< 0.01‡§
Classification of thymomas			
Cortical	34	8.17 \pm 2.90	
Medullary	14	5.68 \pm 1.17	
Mixed	6	5.27 \pm 0.75	< 0.05‡
MG			
With	25	8.30 \pm 3.12	
Without	39	6.50 \pm 2.03	< 0.01*
DNA ploidies			
Aneuploidy	14	7.93 \pm 3.82	
Diploidy	50	7.00 \pm 1.21	> 0.05*

*Two-tailed Student's *t* test.

† $p < 0.01$ between noninvasive thymoma and invasive thymoma.

‡One-way analysis of variance test.

§ $p < 0.01$ between stage I and III + IV.

|| $p < 0.05$ between cortical and the other two subtypes.

stage, classification of the thymic tumors, or the presence or absence of MG in our cases.

DISCUSSION

Since the number of AgNORs highly correlates with increased protein synthesis and cell prolifera-

Table 2—Relationship Between DNA Ploidy and Clinicopathologic Variables of Thymoma and Thymic Carcinoma

Variable	Aneuploid, No. (%)	Diploid, No. (%)	p Value*
Histology			
Thymic carcinoma	2 (20)	8 (80)	
Thymoma	12 (22)	42 (78)	NS
Noninvasive	5 (25)	15 (75)	
Invasive	7 (21)	27 (79)	NS
Stage of thymomas			
I	5 (25)	15 (75)	
II	1 (8)	12 (92)	
III + IV	6 (29)	15 (71)	NS
Classification of thymomas			
Cortical	9 (26)	25 (74)	NS
Medullary	1 (7)	13 (93)	
Mixed	2 (33)	4 (67)	
MG			
With	6 (24)	19 (76)	
Without	8 (21)	31 (79)	NS

*NS = not significant (χ^2 test).

tion, AgNORs are considered to represent cell and nuclear activity.⁷ It is therefore believed that malignant cells would have more AgNORs than benign ones. The usefulness of the AgNOR staining method in histopathologic diagnosis of malignancies was initially demonstrated by Ploton et al⁶ in 1986. Later studies, which have assessed the usefulness of AgNOR counting in assisting cytopathologic diagnosis of a variety of malignancies,⁸⁻¹⁴ have proved that malignant tumors generally have more AgNORs than benign tumors or nonneoplastic tissues. Our study confirmed these results and showed that malignant thymomas, either type I or type II, have more AgNORs than noninvasive thymomas.

Rahilly et al¹⁸ first reported a significant difference in AgNOR counts between noninvasive and invasive thymomas (n = 37). Tateyama and coworkers¹⁹ later reported a converse result (n = 19) and explained that the discrepancy might result because most noninvasive tumors were medullary subtypes that had lower AgNOR counts in previous study.¹⁸ However, in a larger series (n = 60), Pich et al²⁰ reported that AgNOR counts were significantly higher in invasive than in noninvasive thymomas. Our study, which included a similar number of cases (n = 64) as the study of Pich et al,²⁰ did support this finding. Since the histologic features do not predict the biological behavior of thymomas,²¹ the finding that invasive thymomas had a significantly higher AgNOR count than noninvasive ones may imply that the AgNOR count is useful in distinguishing benign from malignant lesions both pathologically and clinically.

In the reports of both Rahilly et al¹⁸ and Tateyama et al,¹⁹ the AgNOR counts of thymic carcinomas were significantly higher than those of invasive thymomas. At variance with these reports, our results showed no significant difference in AgNOR counts between these two neoplasms. Since the histologic and clinical features of invasive thymoma and thymic carcinoma are different, the malignant potential of these two neoplasms needs further study.

Despite the fact that there was no difference in AgNOR counts between stage II and late-stage thymomas, a trend of increased AgNOR counts with more advanced stage was noted in this series. Our finding was in agreement with the results of thymoma reported by Pich et al²⁰ and the results reported in other tumors.²²⁻²⁴

Our study showed that the cortical subtype of thymoma had higher numbers of AgNORs. It has been reported that cortical thymomas have more invasive tendencies.²⁵ The invasiveness of thymomas was previously reported to be related to the distribution of fibril substances.²⁶ In this series, we demonstrated that the invasiveness of thymomas may be

related to their nuclear activity, which was reflected by a higher number of AgNORs.

Our data indicated that thymoma patients with MG had higher numbers of AgNORs than those without MG. This finding may be explained by the close association between myasthenic thymoma and cortical subtype of thymoma,²⁵ which tends to have more AgNORs.

In this study, we showed that the DNA ploidy of thymic epithelial tumors did not correlate with the number of AgNORs. This finding coincided with comparable results of studies of breast,¹⁰ prostate,²⁷ and non-Hodgkin's lymphoma.²⁸

The impaired nucleolar association in an actively proliferating cell may produce a greater number of AgNORs irrespective of its ploidy state.¹⁰ However, malignant potential exists in thymomas even if their histologic and clinical findings are benign since there is no significant difference in DNA ploidy between noninvasive and invasive thymomas. Our data also showed that DNA ploidy, unlike AgNORs, has no value in predicting invasiveness, stage of histopathologic classification of thymoma, and the presence of MG in thymoma patients.

We concluded that the AgNOR staining is useful in differentiating between invasive and noninvasive thymomas and in predicting early or late stage of thymomas. A greater number of AgNORs was observed in patients with cortical thymoma and in those presenting with MG. This rapid, simple, and inexpensive technique may be of value when applied to routine processing of paraffin-embedded blocks of thymomas and thymic carcinomas.

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