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Thymic epithelial tumours: evaluation of malignant grade by quantification of proliferating cell nuclear antigen and nucleolar organizer regions

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Abstract. Cellular proliferation was studied by quantification of proliferating cell nuclear antigen (PCNA) and argyrophilic nucleolar organizer regions (AgNORs) of cells in 29 thymic epithelial tumours: 8 noninvasive (7 cortical and 1 mixed) thymomas, 11 invasive/metastatic (all cortical) thymomas, and 10 thymic carcinomas. Thymic carcinoma showed the highest percentage of cells positive for PCNA ($14.73 \pm 5.419\%$) and the largest mean number of AgNORs per nucleus (4.89 ± 0.756). The mean percentage of PCNA-positive cells and number of AgNORs in thymoma groups were as follows: in noninvasive thymoma $2.96 \pm 1.256\%$ and 2.73 ± 0.647, respectively, and in invasive/metastatic thymoma $4.41 \pm 1.823\%$ and 3.68 ± 1.148, respectively. The differences in PCNA and AgNORs were statistically significant between thymic carcinoma and each of thymoma groups. The overlap of the values between these tumours was minimal in the PCNA stains, although it was considerable in AgNOR counts as previously noted. However, there was no statistically significant difference in these markers between noninvasive and invasive/metastatic thymomas. These results indicate that thymoma in general is a slow-growing tumour compared with thymic carcinoma and that noninvasive thymoma is similar to invasive/metastatic thymoma with regard to proliferative activity; these latter two tumours may represent an essentially identical type in different stages of progression.

Key words. Thymus – Thymoma – Thymic carcinoma – Proliferating cell nuclear antigen – Nucleolar organizer regions

Introduction

Thymic epithelial tumours are divided into two groups, thymoma and thymic carcinoma, depending on cytological atypia (Marino and Müller-Hermelink 1985; Suster and Rosai 1991). Thymomas show no cytological atypia and are generally well encapsulated, but some exhibit aggressive behaviour such as local invasion or, rarely, distant metastasis. Thus invasive/metastatic thymoma has been called malignant thymoma category I, while malignant thymoma category II is a term which includes thymic carcinomas defined as tumours showing obvious malignant features cytologically (Levine and Rosai 1978). Confusion exists in the term "malignant thymoma", as this designation has also been applied indiscriminately for both invasive/metastatic thymomas and thymic carcinomas. Moreover, since it is very difficult to identify the cytological difference between "benign" noninvasive thymoma and "category I malignant thymoma", macroscopic findings at surgery have to be used to distinguish these tumours (Lewis et al. 1987; Maggi et al. 1986).

Recently several methods for measuring the level of DNA synthesis have been developed to estimate the proliferative activity of a neoplasm. Proliferating cell nuclear antigen (PCNA) appears in the nucleus during the synthetic phase of the cell cycle (Bravo et al. 1987; Celis and Celis 1985; Takasaki et al. 1981). Monoclonal antibodies to PCNA, which can be used on routinely processed tissue, have been produced and have been shown to be useful in the evaluation of cellular proliferation (Ogata et al. 1987; Waseem and Lane 1990). Nucleolar organizer regions (NORs) are DNA loops encoding for the production of ribosomal RNA (Deschenes and Weidner 1990; Hernandez-Verdun 1983; Underwood and Giri 1988) associated with argyrophilic proteins and demonstrated within nuclei by the silver staining technique (Ploton et al. 1986) as black dots called argyrophilic nucleolar organizer regions (AgNORs). Of these two markers PCNA has not been examined, in thymic epithelial tumours although AgNORs were recently

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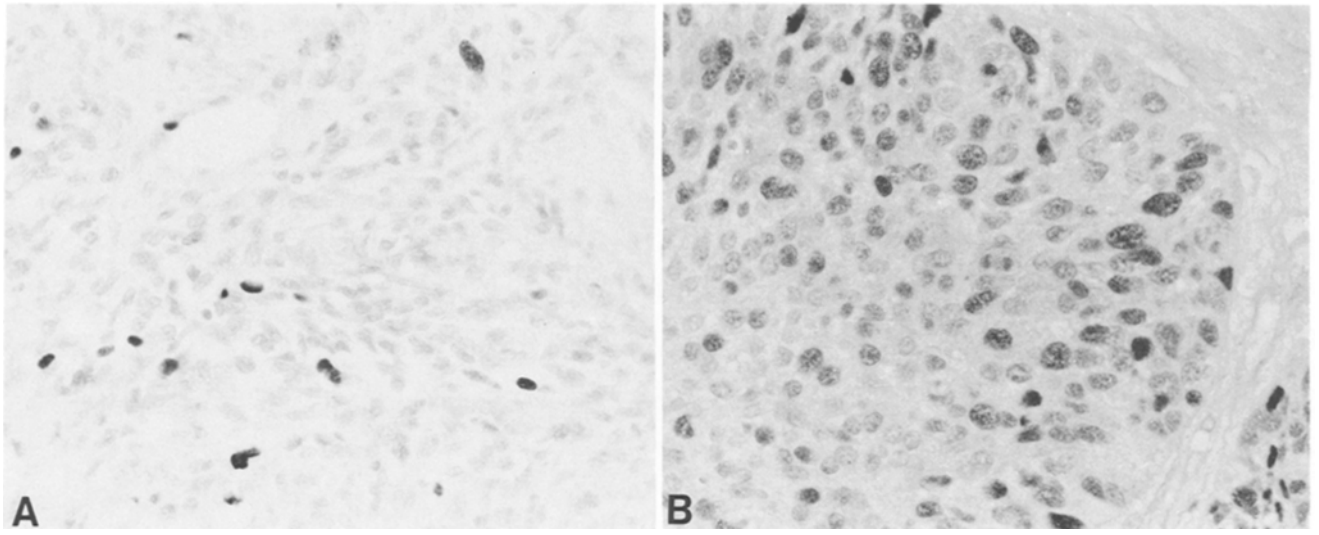

Fig. 1. Immunohistochemical staining for proliferating all nuclear antigen (PCNA) shows predominantly granular positivity in a few nuclei of invasive thymoma (**A**), and in many nuclei of thymic carcinoma (**B**). ABC method with methyl green counterstain, × 500

counted by Rahilly et al. (1991). In this report, we describe both PCNA-positive cells and AgNORs in noninvasive thymoma, invasive/metastatic thymoma, and thymic carcinoma. The results of AgNOR examination were presented at the meeting of the Japanese Society of Pathology in April 1991 (Tateyama et al. 1991) and are comparable to those of Rahilly et al. (1991). The addition of examination of PCNA in the assessment of thymoma and thymic carcinoma made then more clearly separable, while thymomas of different stages showed no change in proliferative activity.

Materials and methods

Twenty-nine thymic epithelial tumours, consisting of 19 thymomas with scanty lymphocyte infiltration and 10 thymic carcinomas with apparent cytological atypia, were used in this study. The criteria for cytological atypia included cellular pleomorphism, nuclear hyperchromasia, nucleolar prominence, and more than an occasional mitosis. The thymomas comprised 8 noninvasive, 7 invasive, and 4 metastatic tumours. According to the cytological criteria of Marino and Müller-Hermelink (1985), all the tumours except 1 were of cortical type; 1 noninvasive tumour was of mixed type. Since one of the purposes of this study was to see the difference in stages of thymomas, tumours of medullary type were excluded. Thymomas with more than moderate lymphocyte infiltration were also excluded because of difficulty of being certain whether the labelled cell was a neoplastic cell or a lymphocyte. The thymic carcinomas, usually having scanty lymphocytes in tumour cell nests, were 6 moderately differentiated squamous cell carcinomas, 2 poorly differentiated squamous cell carcinomas, and 2 lymphoepithelioma-like carcinomas (Hartmann et al. 1990; Suster and Rosai 1991). One tumour was encapsulated, with capsular invasion, 4 were invasive and 5 were metastatic.

Follow-up data were avialable in 6 cases of noninvasive thymomas, 9 invasive/metastatic thymomas, and 9 thymic carcinomas. All patients except for 1 with noninvasive thymoma are alive with no evidence of recurrence during the follow-up period which ranged from 2 to 8 years. One died of unrelated disease (carcinoma of the colon) at 5 years. Three patients with invasive thymoma and 1 patient with metastatic thymoma are alive, free from disease, over a period ranging from 3 to 14 years. One patient with metastatic thymoma is alive with diesease at 30 months. Four patients in this group died with the mean survival period of 22 months. Six patients with thymic carcinoma are alive with no evidence of disease during the period ranging from 8 months to 7 years. Two patients died at 10 months and 1 died at 17 months.

Portions of each tumour were fixed in 10% buffered formalin for an average of 24 h. Cases in which the time of fixation may have been longer than 48 h were excluded. Immunohistochemical staining for PCNA was performed on paraffin sections by the avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981) using the monoclonal anti-PCNA antibody (Novocastra Laboratories, Burlingame, Calif.). The sections were counterstained with methyl green. For negative control, the anti-PCNA antibody was replaced by phosphate-buffered saline. The percentage of PCNA-positive cells was determined by counting at least 300 tumour cells.

AgNOR staining was performed on sections of paraffin-embedded tissues as described previously (Ploton et al. 1986). Briefly, deparaffinized and hydrated sections were exposed for 30 min,

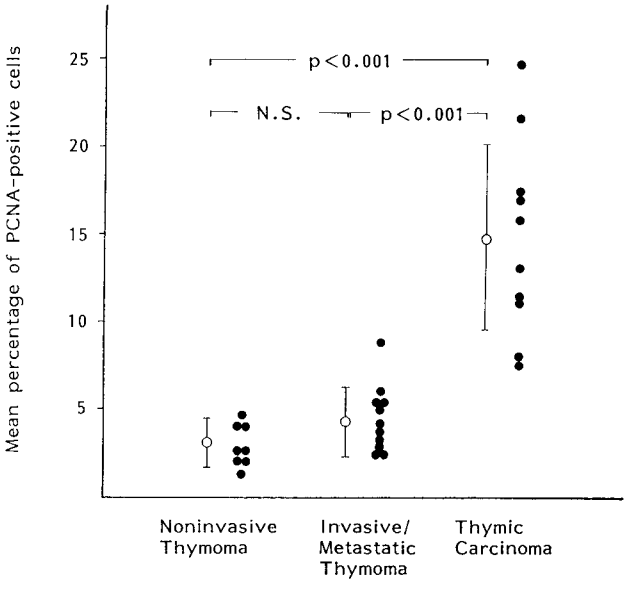

Fig. 2. Scattergram showing the mean percentages of PCNA-positive cells; *N.S.*, not significant

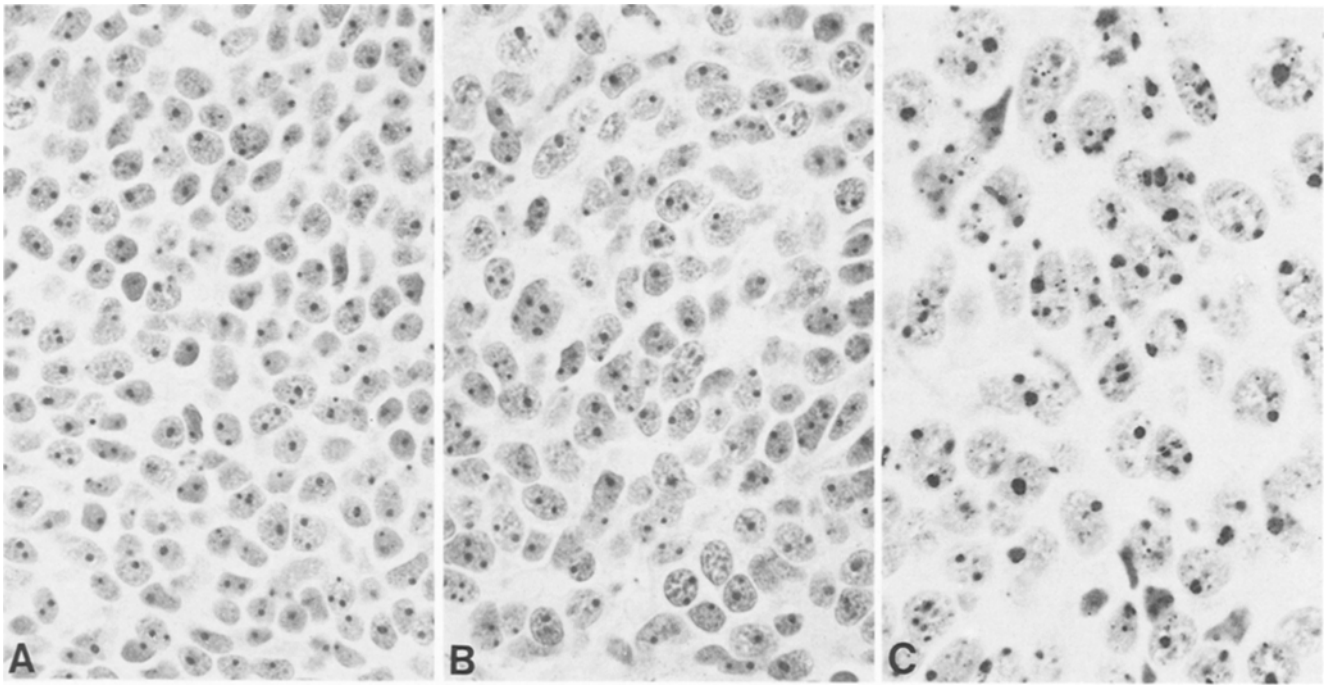

Fig. 3. Nuclear organizer regions (AgNORs) in noninvasive thymoma (**A**) and invasive/metastatic thymoma (**B**). The dots are small and regular. AgNORs in thymic carcinoma (**C**). The dots are more numerous, larger in size, and irregular in shape. The nuclear size is also larger than that of thymomas. **A–C** AgNOR stain, ×870

under darkroom conditions, to a silver staining solution which was prepared by dissolving 2% gelatin in 1% aqueous formic acid and mixed in a ratio of 1:2 by volume with 50% aqueous silver nitrate. AgNORs were visualized as distinct intranuclear black dots of varying sizes. In each case, 100 tumour cells taken at random were examined using a x100 oil-immersion objective lens and a x10 ocular lens. Single AgNORs and individual AgNORs within clumps were counted by carefully focusing to visualize all AgNORs within the section according to the method of Crocker et al. (1989).

To eliminate bias, both PCNA-positive cells and AgNORs were counted without prior knowledge of the diagnosis of the tumours. Student's t-test was used for statistical analysis. The difference was considered to be significant when the P value was less than 0.05.

Results

The positive cells for PCNA showed nuclear staining with granular or diffuse pattern and varied in intensity of staining, but all identifiable staining was regarded as positive (Fig. 1A, B). The percentages of PCNA-positive cells for each group are shown in Fig. 2. The mean values for each group were as follows; noninvasive thymoma ($2.96 \pm 1.256\%$), invasive/metastatic thymoma ($4.41 \pm 1.823\%$), and thymic carcinoma ($14.73 \pm 5.419\%$). There was a considerable variation in the number of positive cells from case to case, particularly in the thymic carcinoma group, but thymic carcinomas as a whole showed the highest percentage of positivity and the differences between this group and each of thymoma groups were statistically significant ($P < 0.001$). Moreover, there was very little overlap between them. The difference between noninvasive thymoma and invasive/metastatic thymoma showed no statistical significance.

Generally, AgNORs in the tumour cells of thymomas of both noninvasive and invasive/metastatic types were small, round, and regular, and almost all confined to the nucleoli (Fig. 3A, B). In only a few invasive/metastatic thymomas did the tumour cells show AgNORS which were more variable in configuration. However, in the tumour cells of thymic carcinomas, AgNORs were often large, pleomorphic, and irregular and distributed throughout the nuclei (Fig. 3C). The mean numbers of

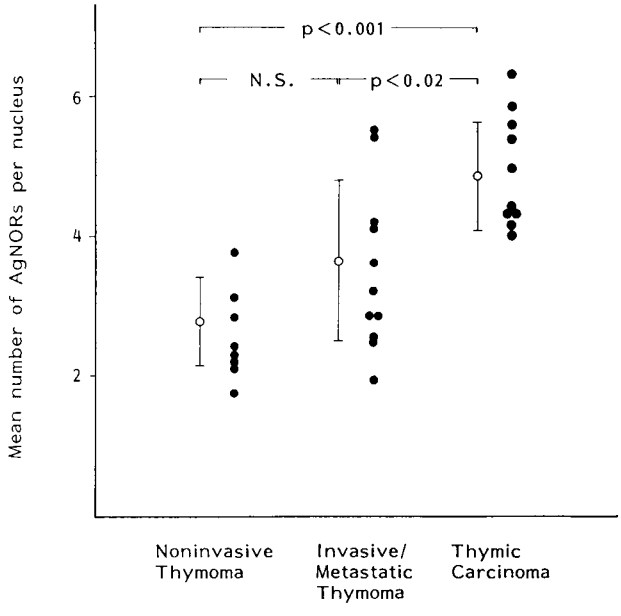

Fig. 4. Scattergram showing the mean numbers of AgNORs per nucleus; *N.S.*, not significant

AgNORs per nucleus for each group are shown in Fig. 4. The mean numbers for each group were as follows; noninvasive thymoma (2.73 ± 0.647), invasive/metastatic thymoma (3.68 ± 1.148), and thymic carcinoma (4.89 ± 0.756). The difference in the mean numbers of AgNORs per nucleus was significant between noninvasive thymoma and thymic carcinoma ($P < 0.001$), and between invasive/metastatic thymoma and thymic carcinoma ($P < 0.02$), but not significant between noninvasive thymoma and invasive/metastatic thymoma. However, there was considerable overlap between the three groups.

The differences in the percentage of PCNA-positive cells and the number of AgNORs between squamous cell carcinoma ($15.3 \pm 5.796\%$, 4.95 ± 0.881) and lymphoepithelioma-like carcinoma ($12.5 \pm 6.788\%$, 4.66 ± 0.395) were not statistically significant, although the cases of the latter carcinoma were small in number. The difference between invasive thymoma and metastatic thymoma showed no statistical significance in the percentage of PCNA-positive cells or the number of AgNORs.

Discussion

In thymic epithelial tumours, the most important prognostic factor is their clinical stage (Masaoka et al. 1981). Thus stage I or II thymoma is called "benign" thymoma and stage III or IV "malignant" thymoma, despite the fact that there is no histologcial difference between them. Thymic carcinoma is defined as a primary thymic epithelial neoplasm exhibiting obvious cytologically malignant features (Levine and Rosai 1978; Shimosato et al. 1977). However, the terminology is confusing, since on occasions the designation of "malignant thymoma" has been applied indiscriminately for both invasive/metastatic thymoma and thymic carcinoma. In this study, we examined the PCNA and AgNORs in 29 thymic epithelial tumours to see the difference in cellular proliferation between noninvasive thymoma, invasive/metastatic thymoma, and thymic carcinoma.

PCNA, also known as cyclin or auxiliary protein for polymerase-delta, is a 36 kDa nuclear protein which is directly involved in DNA synthesis (Bravo et al. 1987; Celis and Celis 1985; Garcia et al. 1989; Takasaki et al. 1981). The monoclonal anti-PCNA antibody is applicable for formalin-fixed paraffin-embedded tissues (Robbins et al. 1987), although the time of fixation can markedly affect the intensity of PCNA immunoreactivity (Hall et al. 1990). The percentage of PCNA-positive cells is correlated with tumour grades in some tumours (Garcia et al. 1989; Robbins et al. 1987), but this antigen has not been examined in thymic epithelial neoplasms.

AgNORs are considered to reflect cell and nuclear activity and a correlation between the mean number of AgNORs and cellular proliferation has been reported in various lesions (Egan and Crocker 1992). In general, malignant cells show considerably more AgNORs than benign cells (Dervan et al. 1989; Deschenes and Weidner 1990; Raymond and Leong 1989). Rahilly et al. (1991) recently studied this marker in 37 cases of thymoma and 3 cases of thymic carcinoma and concluded that the AgNOR counts of medullary type thymomas were significantly lower than those of the cortical and mixed type tumours. Our results are similar to theirs for the cortical type tumours. The similarity of AgNOR values in our two studies despite the use of different techniques in identifying the tumour cells indicates that the counting of AgNORs is fairly consistent between different laboratories.

In this study the mean number of AgNORs in the tumour cells in thymic carcinoma was significantly higher than those in thymomas of noninvasive or invasive/metastatic type, but there was considerable overlap between thymic carcinoma and two thymoma groups as also pointed out by Rahilly et al. (1991). However, AgNORs in thymic carcinoma were larger and more irregular when compared with those of thymomas (Fig. 3). The size and shape as well as number of AgNORs may be indicators of cellular proliferation (Hansen and Ostergart 1990). In addition, immunostaining of PCNA demonstrate a statistically significant difference in the percentages of positive cells between thymic carcinoma and thymomas of both stages, with minimal overlap between them. The detection of PCNA seems to be superior to counting of AgNORs as a marker of aggressive behaviour in thymic epithelial neoplasms. Combining these two examinations certainly discriminates thymic carcinoma from thymoma.

Nevertheless the percentage of PCNA-positive cells or AgNOR counts showed no significant difference between noninvasive thymomas and invasive/metastatic thymomas. These results indicate that noninvasive thymoma and invasive/metastatic thymoma are not different in cellular proliferation characteristics or in cytological features of the tumour cells as previously thought. Although Rahilly et al. (1991) described a significant difference of the AgNORS counts between stage I (noninvasive) and stage II and III (invasive) thymomas, this may be due to the lower AgNOR counts in medullary tumours which were mostly stage I lesions in their study. Their series, unlike ours, contained tumours with variable lymphocyte infiltration which apparently did not affect the AgNOR values.

It is conceivable that noninvasive thymoma may progress to invasive/metastatic thymoma if left untreated. Although none of the 6 noninvasive tumours in the present series shows recurrence, the 5-year and 10-year survival rates of the patients with stage I tumour have been reported as 96.2% and 66.7%, respectively (Masaoka et al. 1981). Therefore, it is misleading to use the name "benign" thymoma for noninvasive thymoma, because it may have ability to be aggressive like invasive/metastatic thymoma. A benign clinical course is the rule after surgical treatment of many malignant tumours in their early stages. In conclusion, this cellular proliferation study supports the view that thymoma is essentially a low-grade malignant lesion regardless of the stages. We suggest that the designation "benign" or "malignant" should be discarded; both are misleading and the latter causes confusion with thymic carcinoma.

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