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Analysis of Cell Cycle Regulator Proteins in Encapsulated Thymomas

Alfonso Baldi,[3] Vincenzo Ambrogi,[1] Davide Mineo,[1] Pasquale Mellone,[3] Mara Campioni,[3] Gennaro Citro,[2] and Tommaso Claudio Mineo[1]

Abstract

Purpose: Although survival of encapsulated thymomas is usually good, some patients present a higher incidence rate of recurrence and a shorter long-term survival. Abnormalities in the components of cell cycle checkpoints are extremely common among virtually all neoplasms. In this study, three components of the cell cycle machinery (i.e., p21, p27 and p53) were examined in a series of well-characterized encapsulated thymoma specimens to analyze coregulation and influence on recurrence and survival.

Experimental Design: Sixty-eight consecutive patients with thymoma were operated in our center from 1987 to 2000. Expression of p53, p21, and p27 was studied in specimens from 25 encapsulated thymomas using immunohistochemistry. Generic factors and gene expression influencing the probability of recurrence were studied. Positive expression was dichotomized defining positive when present in more than 5% of tumor cells. Mean follow up was 85.9 months; clinical data about recurrence were recorded.

Results: Univariate analysis suggests that positive p53 ($P < 0.05$), negative p21 ($P = 0.01$), and especially negative p27 expressions ($P = 0.001$) significantly correlate with poor prognosis for disease-free survival. Multivariate Cox regression analysis suggests that negative p27 immunohistology is the only significant variable for poor prognosis ($P = 0.03$; odds ratio, 0.08; 95% confidence interval, 0.01-0.88).

Conclusions: These results show that loss of control of cell cycle checkpoints is a common occurrence in thymomas and support the idea that functional cooperation between different cell cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumor suppression.

Encapsulated thymomas are considered the best prognosis subset (1): surgical resection alone can provide best long-term survival and low recurrence rate. Nevertheless, these tumors present different clinical behavior (2) and variable recurrence pattern (3). For these reasons, even in those good-prognosis neoplasms, a more accurate independent prognostic determinant is highly warranted.

Significant progress has been made in understanding the molecular and cellular pathogenesis. One area that has been the focus of much research is cell cycle control (4). A precise regulation of the cell cycle is a fundamental requirement for the homeostasis of the eukaryotic cell. During the last decade, scientists successfully delved into the molecular machinery devoted to the fine regulation of the cell cycle phases, identifying and characterizing several genes and gene products involved (5). A key role is played by cyclin-dependent kinases, relatively small proteins with an apparent molecular mass between 33 and 43 kDa, of which activity is regulated by the arrangement in a multimeric complex with larger proteins, called "cyclins" after their cyclical expression and degradation during the cell cycle. Different cyclin-dependent kinase/cyclin complexes, formed with clear-cut timing throughout the cell cycle, together with their phosphorylation/dephosphorylation, efficiently regulate the activity of the multimeric holoenzyme. Conversely, cyclin-dependent kinase/cyclin complexes are negatively modulated by the binding of a family of small proteins called cyclin-dependent kinase inhibitors [i.e., the CREB binding protein–interacting protein (p21 and p27) and inhibitor of kinase (p16) families; refs. 4, 5]. The p53 tumor suppressor gene is also involved in cell cycle checkpoints by virtue of its action as a transcription factor for several cell cycle regulatory proteins, including the p21 gene (6).

Few of the factors involved in regulating cell cycle control have been investigated in thymomas; even fewer studies have examined multiple factors in the same tumor series (7, 8). The aim of this study was therefore to evaluate the expression of p53, p21, and p27 proteins in a homogeneous series of same-stage thymomas to assess the integrity of cell cycle checkpoints in these tumors, to evaluate the coexpression of these proteins, and, finally, to examine the relationship between these cell

Authors' Affiliations: [1]Department of Thoracic Surgery, Tor Vergata University; [2]SAFU Department, Regina Elena Cancer Institute, Rome, Italy and [3]Department of Biochemistry and Biophysics, "F. Cedrangolo," Section of Anatomic Pathology, Second University of Naples, Naples, Italy

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Requests for reprints: Alfonso Baldi, Department of Biochemistry, Section of Pathology, Second University of Naples, Via L. Armanni 5, 80138 Naples, Italy. Phone: 39-0815-666-003; Fax: 39-0815-569-693; E-mail: alfonsobaldi@tiscali.it.

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cycle regulators and the clinicopathologic features of thymomas, including their ability to predict recurrence, and therefore necessity, of more accurate follow-up and supplemental therapy.

Materials and Methods

Patients and tissue samples. From January 1987 to December 2000, 68 patients with thymoma consecutively underwent surgical resection in the Department of Thoracic Surgery, University of Rome Tor Vergata. Among this group, we selected 25 patients with pathologic stage I according to the classification of Masaoka et al. (1): it implies a microscopically encapsulated thymoma.

Their pathologic and clinical features were retrospectively studied. The morphologic classification of the thymomas was conducted according to the Bernatz et al. (7), Marino and Muller-Hermelink (8), and WHO (9) specifications. Patients' main characteristics were summarized in Table 1. All these patients were approached through a median sternotomy approach and were deemed radically operated without evidence of macroscopic residual disease. Recurrence was defined as any evidence of tumor, such as regrowth of tumor in the mediastinum, pleural dissemination, or pulmonary metastasis, detected by imaging or pathologic examination during follow-up.

Immunohistochemistry. We retrospectively evaluated surgical specimens from 25 encapsulated thymomas. Briefly, sections from each specimen were cut at 3 to 5 μm, mounted on glass, and dried overnight at 37°C. All sections then were deparaffinized in xylene, rehydrated through a graded alcohol series, and washed in PBS. This buffer was used for all subsequent washes and for dilution of the antibodies. Tissue sections were heated twice in a microwave oven for 5 minutes each at 700 W in citrate buffer (pH 6) and then processed with the standard streptavidin-biotin-immunoperoxidase method (DAKO Universal Kit, DAKO Corporation, Carpinteria, CA). Mouse monoclonal antibodies (Santa Cruz biotechnology, Inc., Santa Cruz, CA) specific for p27 (mouse monoclonal) and p21 (mouse monoclonal) were used at a 1:100 dilution, whereas a monoclonal antibody specific for wild-type p53 (DAKO Corporation) and a polyclonal antibody specific for the mutated p53 (Santa Cruz) were used at a 1:500 dilution. All the primary antibodies were incubated for 1 hour at room temperature. Diaminobenzidine was used as the final chromogen and hematoxylin as the nuclear counterstain. Negative controls for each tissue section were done leaving out the primary antibody. Positive controls included in each experiment consisted of tissue previously shown to express the antigen of interest. Two pathologists (A.B. and P.M.) evaluated the staining pattern of the three proteins separately and scored the protein expression in each specimen by scanning the entire section and estimating the percentage of tumor cell nuclei staining. The level of concordance, expressed as the percentage of agreement between the observers, was 92% (23 of 25 specimens). In the remaining specimens, the score was obtained after collegial revision and agreement. All immunoreactive nuclei were regarded as positive, irrespective of the intensity of staining.

Table 1. Characteristics of the thymomas undergoing cell cycle analysis

Total number	25	
Median age (range), y	47.5 (17-72)	
Male vs female	9 vs 16 (36% vs 64%)	
Neoplasm histotype (ref. 7)		*P*
Spindle cell	4 (16%)	0.2 NS
Mixed	9 (36%)	
Lymphocyte predominant	8 (32%)	
Epithelial cell	4 (16%)	
Immunohistochemical (ref. 8)		0.8 NS
Medullary	8 (32%)	
Mixed	7 (28%)	
Cortical	10 (40%)	
WHO (ref. 9)		
A	3 (12%)	0.06 NS
AB	9 (36%)	
B1	8 (32%)	
B2	4 (16%)	
B3	1 (4%)	

Abbreviation: NS, not significant.

Statistical analysis. To carry out statistical analysis, a dichotomized scoring system was used as follows: p53, p21, and p27 expression in more than 5% of tumor cells was defined as positive expression. Fischer's exact test was used to assess relationship between ordinal data (correlation matrix between immunostaining variables).

Due to the elevated long-term survival probability of the encapsulated thymomas, our study was aimed only at investigating the probability of recurrence. A univariate survival analysis for each prognostic variable on disease-free survival was estimated according to the Kaplan-Meier method. Free survival was measured from the day of the supposed radical operation until the evidence of recurrence or the last follow-up visit. The statistical significance of the differences in survival distribution among the prognostic groups was evaluated by the log-rank test. The Cox proportional hazards model was applied to the multivariate survival analysis. The prognostic variables on disease-free survival included generic risk factors (sex and age), histology classifications, and expression of p53, p21, and p27. $P < 0.05$ was regarded as statistical significant in two-tailed tests. SPSS software (version 10.00, SPSS, Chicago, IL) was used for statistical analysis.

Results

In Table 2, the correlation matrix between molecular markers and histologic classification in thymoma patients is depicted. There were no significant differences in histotypes distribution. Four (16%) patients relapsed. We first investigate the effect of sex, age (cutoff, 50 years), histologic type, and WHO classification on disease-free survival. Among all factors studied, only WHO classification showed a significant difference on survival ($P = 0.01$; Fig. 1).

As far as the three cell cycle regulators, p53, p21, and p27, are concerned, all of the cell cycle–associated proteins examined were present in the nuclei of tumor cells, although a small proportion of cells displayed cytoplasmic immunoreactivity in addition to nuclear staining. The two antibodies used for the analysis of p53 gave overlapping results. Therefore, we assumed that p53 overexpression corresponded to the presence of a mutated p53 protein. Significant correlations were found between p27-negative and all poorest prognosis categories of all classifications (Table 2). The expression levels of each protein are detailed in Table 3, and examples of positive immunostaining are shown in Fig. 2.

As Fig. 3 showed, both p21- and p27-negative ($P = 0.04$ and $P = 0.001$, respectively) and p53-positive ($P = 0.05$) tumors significantly correlated with disease-free survival, but p27 was the most strictly correlated and the statistical value of this correlation was not modified by combining with the p27-negative cases also the p21-negative and p53-positive cases.

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Table 2. Correlation matrix between molecular markers and histologic classification in thymoma patients

	p53-positive expression	*P*	p21-negative expression	*P*	p27-negative expression	*P*
Neoplasm histotype (ref. 7)						
Spindle	2	NS	1	NS	1	NS
Mixed	2		2		1	
Lymphocytic	3		3		3	
Epithelial	3		3		3	
Immunohistochemical (ref. 8)						
Medullary	0	0.01	0	0.02	0	0.02
Mixed	3		3		2	
Cortical	7		6		6	
WHO classification (ref. 9)						
A	2	NS	1	NS	0	NS
AB	3		6		2	
B1	1		7		2	
B2	3		1		3	
B3	1		0		1	

Afterwards we evaluated the prognostic value of the different cell cycle regulators by multivariate analysis. Interestingly, looking at disease-free survival, p27-negative expression was selected as the only significant prognostic factor (relative risk, 0.08; 95% confidence interval, 0.01-0.88; $P = 0.03$).

Discussion

The ability of a cell to control its own replication is very important for the maintenance of the structure and functions of the organ it belongs to and, in final analysis, of the organism it is a part of. Several pathologies are at the moment connected to an altered control of cellular replication and, among them, cancer is one of the most studied. In particular, the growth of tumors depends on both increased proliferation and deficient cell death. Numerous checkpoint proteins have been examined thus far in thymoma, but few studies have investigated multiple factors in the same tumors (10, 11). We undertook the present study to determine the different expression in a group of thymomas at the same clinicopathologic stage of some key proteins involved in cell cycle checkpoints (i.e., p53, p21, and p27). Even if the management of thymomas is straightforward, with surgical resection being the primary mode of therapy, the literature contains many contradictory points of view on the need for adjuvant therapy or alternative surgical treatments such as video-assisted surgery (12).

We focused on a subgroup of 25 encapsulated thymomas. In this particular subset, the most important problem is to

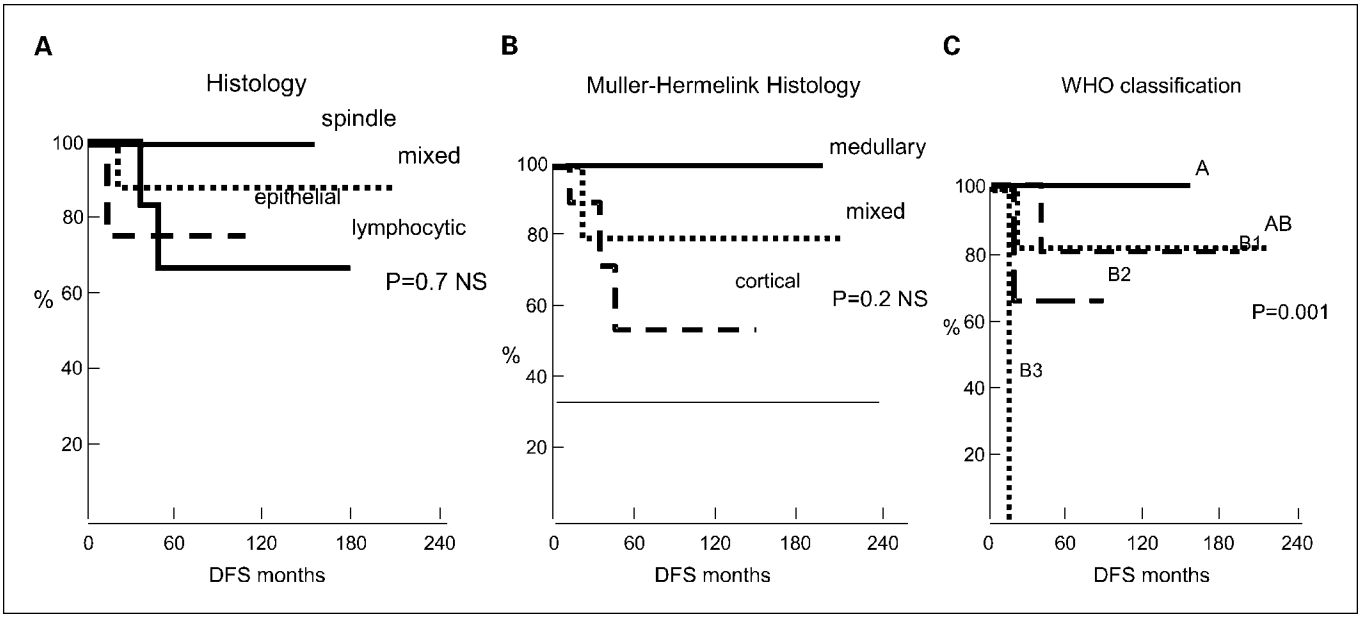

Fig. 1. Kaplan-Meier disease-free survival of the study group according to the histology classifications. Only WHO classification showed a significant difference on survival ($P = 0.01$). *A,* Bernatz and Hollmann (7); *B,* Marino and Muller-Hermelink (8); *C,* WHO (9).

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Table 3. Dichotomized expression levels of cell cycle control proteins in thymoma

Patient	Age (y)	Sex	p53	p21	p27
1	71	F	–	+	+
2	50	M	–	+	+
3	21	F	–	+	+
4	41	F	–	+	+
5	72	F	+	–	+
6	61	F	+	+	+
7	50	M	–	+	+
8	53	F	–	+	+
9	64	F	–	+	–
10	44	F	–	+	+
11	20	F	+	–	–
12	28	F	+	+	+
13	59	M	–	+	+
14	54	M	+	–	–
15	33	F	–	–	+
16	25	M	+	–	–
17	67	F	–	+	+
18	43	M	+	–	–
19	68	M	–	+	+
20	54	M	+	–	–
21	54	F	+	–	–
22	56	F	–	+	+
23	57	F	–	+	+
24	26	F	–	+	+
25	17	M	+	–	–

select the cases with higher chances of recurrence. Nakagawa et al. (13) have recently affirmed that capsular invasion in thymoma has no prognostic significance, and that the prognosis of patients is also affected by the histologic subtype, and it is not the simple matter of tumor progression according to stage.

When we looked at the correlation between pathologic classification and disease-free survival in the study population, we found that only WHO classification was significantly correlated (9, 14–17).

When we investigated by univariate analysis the correlation between different protein expressions and disease-free survival, we found that all the cell cycle markers analyzed had a statistically significant correlation with survival. To note, we obtained overlapping results with the two antibodies specific for the wild-type and the mutated p53 protein. Therefore, we assumed that high p53 expression was indicative of a mutated p53 protein. This result is in agreement, at least for p21 and p53, with numerous data published about the cell cycle checkpoints investigated in this article (5–8, 10). Surprisingly, when we grouped the thymoma specimens, collecting the p27- and p21-negative samples and the p53-positive samples, we found that this group of thymoma specimens did not display a significant shorter overall disease-free survival with respect to the specimens with only p27-negative expression.

Numerous data from the literature suggest an important role for p27 in the pathogenesis and progression of several tumors (18, 19). Moreover, it has been recently shown a potential role for p27 also in the risk of recurrent disease in several neoplasms (20–22). This is in accordance with the data presented in this manuscript underlying an important role for p27 in thymoma recurrence.

To the best of our knowledge, this is the first report underlying a role for p27 in the pathogenesis of thymoma. Nevertheless, it is not possible to exclude that other cell cycle regulators play an important role. In fact, it has been proposed that functional cooperation between different cell cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumor suppression (23). Taking into account the complicated functional network constituted by the

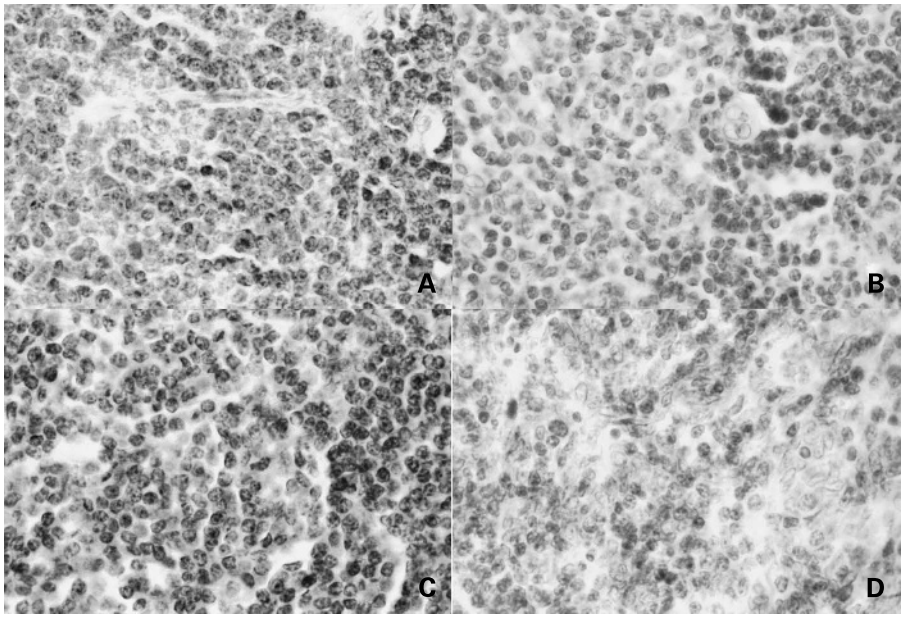

Fig. 2. Immunohistochemical staining of cell cycle proteins in thymoma specimens. (*A*) p53 positive expression >5%, (*B*) p53 positive expression <5%, (*C*) p21 positive expression >5%, (*D*) p27 positive expression >5%.

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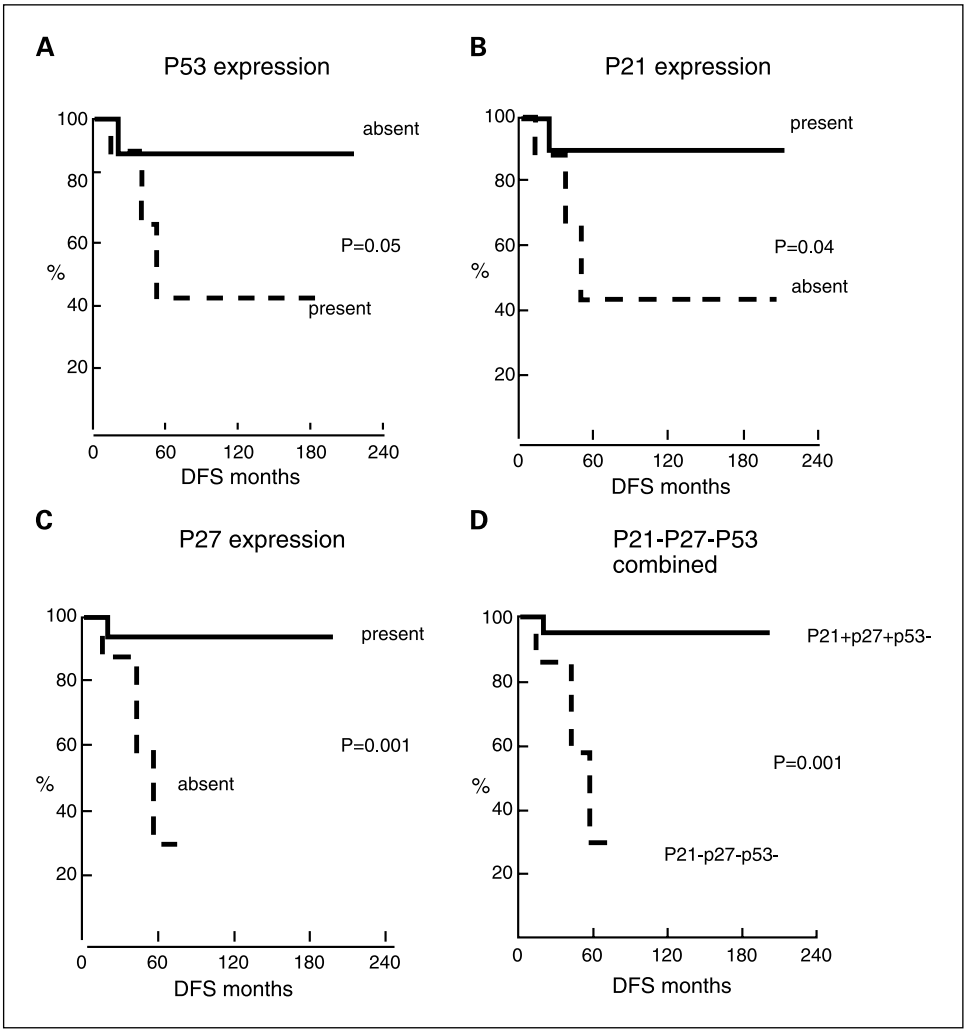

Fig. 3. Kaplan-Meier survival curves showing the effects of cell cycle proteins on disease-free survival of thymoma patients. *A,* positive expression of p53 was associated with shorter patient survival. *B,* positive expression of p21 was correlated with improved outcome. *C,* positive expression of p27 was associated with improved outcome. *D,* patients lacking both p21 and p27 expression and with positive p53 expression did not have a significant shorter disease-free survival with respect to patients in *C* (p27-negative patients).

cell cycle regulator proteins, it seems evident that knowledge of the level of expression of these factors, and their coregulation, may be important in predicting patient clinical response to therapy. Nevertheless, targeting multiple checkpoint proteins may represent a good therapeutic strategy for the development of new molecular treatments for thymoma. The data presented in this manuscript support this hypothesis and strongly suggest further works aimed at investigating the simultaneous expression of numerous cell cycle regulators in thymomas.

Therefore, this article supports the idea that in determining the therapeutic strategy of patients with thymoma, the histologic subtype along with stage should be always considered. In particular, for the encapsulated thymomas, the staging system also should be further refined according to the findings described. Analysis of cell cycle regulators, such as p27, should be included in the staging system to have more precise indication on the possibility of recurrence.

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