Long-Term Disease-Free Survival of Patients with Radically Resected Thymomas

Relevance of Cell-Cycle Protein Expression

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BACKGROUND. Despite radical surgical resection, thymomas often recur. The objective of the current retrospective study was to investigate the prognostic relevance of the expression of cell-cycle proteins in these neoplasms to formulate a possible therapeutic surveillance strategy for the prevention of recurrence.

METHODS. The authors retrospectively reviewed the main clinicopathologic factors, including the World Health Organization (WHO) classification, of patients with thymoma who had undergone radical surgical resection. Specimens were studied using immunohistochemistry and the expression of cell-cycle proteins (i.e., p21, p27, and p53) was assessed. Univariate and multivariate analysis of predicting survival prognostic factors were performed.

RESULTS. The authors analyzed 88 patients with thymoma who underwent radical surgical resection at the study institution. According to the Masaoka staging system, 41 patients had Stage I disease, 31 patients had Stage II disease, and 16 patients had Stage III disease. There were 24 tumor recurrences (27.3%), 4 of which were local, 16 of which were distant intrathoracic, and 4 of which were extrathoracic. The second radical resection provided a disease-free survival rate that was similar to the first. Only Masaoka stage (P=0.001), WHO classification (P=0.001), high expression of p53 (P=0.03), and low expression of p21 (P=0.02) and p27 (P=0.001) were found to be correlated with a reduced disease-free survival. Low p27 expression was found to be the most significant predictive factor of a short disease-free survival (P=0.001), especially when associated with low p21 expression and high p53 expression (P=0.0001).

CONCLUSIONS. Long-term disease-free survival in thymoma patients treated with radical surgical resection was found to be correlated with Masaoka stage, WHO classification, and expression of cell-cycle proteins, with the latter found to be the most significant predictive factor. Functional cooperation between cell-cycle proteins might constitute another level of regulation in tumor growth. More careful surveillance should be adopted whenever there is negative cell-cycle protein expression. ***Cancer*** **2005;104:2063–71.** *© 2005 American Cancer Society.*

KEYWORDS: thymomas, thoracic surgery, Masaoka staging, WHO classification, cell-cycle proteins.

Complete surgical resection is recognized worldwide as the treatment of choice for thymomas[1] and has been reported to be the best predictor of a long disease-free survival.[2–4] Nevertheless, the prevention of postsurgical recurrences remains a heated topic of discussion. The current staging system proposed by Masaoka et al.[5,6] has proven quite effective in predicting survival. However, even early-stage disease occasionally recurs.[7–9] Several histologic classifications for thymoma have been published to date,[10–12] but to our knowledge

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their prognostic significance is still controversial.[13–15] The new World Health Organization (WHO) classification[16] demonstrated that histologic subtype might be an independent prognostic factor.[17,18] Strobel et al.[19] recently proposed a therapeutic algorithm related to tumor stage, WHO histotype, and completeness of surgical removal, thus suggesting adjuvant therapy in the case of Stage III disease or a B2-3 histotype. Nevertheless, postoperative therapeutic strategy continues to be debated,[20,21] and prospective studies are warranted.

Significant progress has been made in understanding the molecular and cellular pathogenesis of neoplasms.[22] One area that has been the focus of much research is the control of the cell-cycle. A key role is played by cell-cycle kinases, relatively small proteins that are regulated by their arrangement in a multimeric complex with larger proteins, called "cyclins" because of their cyclic expression and degradation during the cell-cycle. Cell-cycle kinase/cyclin complexes are negatively modulated through their interaction with a family of small proteins called cell-cycle kinase inhibitors, namely p21 and p27.[23,24] The p53 tumor suppressor gene also is involved in cell-cycle checkpoints, acting as a transcription factor for several cell-cycle regulatory proteins, including the p21 gene.[25] To our knowledge, few of the factors involved in regulating cell-cycle control have been investigated to date in thymomas.

The objective of the current study was to compare the prognostic specific weight of traditional clinicopathologic factors with the expression of p53, p21, and p27 cell-cycle proteins in a large series of patients who underwent radical surgical resection for thymoma and who had an adequate long-term follow-up to select the most suitable patient category for stricter clinical surveillance and incidental adjuvant therapy.

MATERIALS AND METHODS

Patients

Between January 1987 and December 2004, 97 consecutive patients with thymoma underwent surgery in the Department of Thoracic Surgery at the University of Rome Tor Vergata. Several clinicopathologic features were studied retrospectively: patient gender and age (cutoff age of $\geq$ 50 yrs), the presence of myasthenia gravis (MG), surgical notes (including surgical approach and radical surgical resection), postoperative complications, pathologic issues, postoperative therapy, pattern of disease recurrence, and available long-term follow-up information.

Staging was based on surgical and pathologic criteria as described by Masaoka et al.[5,6] and was retrospectively performed at the time of the review of the surgeon's pathology and surgical notes. The morphologic classification of the thymomas was conducted according to the specifications of Bernatz et al.,[11] Marino and Muller-Hermelink,[12] and the WHO.[16] All specimens were reevaluated retrospectively by the same pathologist (A.B.).

An essential prerequisite for inclusion in the study was the complete surgical resection of the thymoma, implying total thymectomy with excision of the mediastinal fatty tissue between both phrenic nerves and evidence of a free surgical resection margin, with no macroscopic or microscopic residual tumor. Invasion of the great mediastinal vessels resulted in absolute exclusion from the study. Disease recurrence was defined as any evidence of tumor, such as regrowth of tumor in the mediastinum, pleural dissemination, or pulmonary metastasis, as detected by imaging or biopsy during follow-up. Patients were contacted by telephone. In cases in which patients could not be reached, their primary care physician or neurologist was contacted to update their care. Patients with incomplete follow-up information were excluded from the study.

Immunohistochemistry

We were able to retrieve surgical specimens from 79 patients in the study group in which cell-cycle analysis was performed. Briefly, sections from each specimen were cut at 3–5 μm, mounted on glass, and dried overnight at 37 °C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series, and washed in phosphate-buffered saline. 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 watts in citrate buffer (pH of 6.0) and we then processed with the standard streptavidin-biotin-immunoperoxidase method (Dako Universal Kit; Dako Corporation, Carpinteria, CA). Mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), specific for p27 (mouse monoclonal antibody sc-1641) and p21 (mouse monoclonal antibody sc-6246), were used at a 1:100 dilution, while a monoclonal antibody specific for p53 (D01; Dako Corporation) was 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 was used as the nuclear counterstain. Negative controls for each tissue section were performed, leaving out the primary antibody. Positive controls included in each experiment consisted of tissue previously shown to express the antigen of interest. One pathologist (A.B.) evaluated the staining pattern of the three proteins separately and scored the

TABLE 1
Epidemiology and Main Clinico-pathogic Features

	No. of patients (%)	*P* value	Median age in yrs (range)	*P* value	Gender ratio M/F	*P* value	Presence of MG (%)	*P* value	Median follow-up in mos (range)	*P* value
Staging system (Masaoka et al.[5,6])										
I	41 (46.65)	NS	50 (16–72)	NS	15/20	0.05	10 (24.3)	0.01	79 (3–204)	NS
II	31 (35.2)		48 (18–72)		17/20		13 (41.9)		78 (7–215)	
III	16 (18.42)		43.5 (25–78)		10/6		11 (68.87)		55.5 (7–120)	
Neoplasm histotype (Bernatz et al.[11])										
Spindle	11 (12.5)	NS	53 (21–72)	NS	4/7	NS	6 (17.6)	NS	66 (18–159)	NS
Mixed	35 (39.87)		54 (16–78)		15/20		15 (42.98)		91 (2–215)	
Lymphocytic	27 (30.69)		44 (17–73)		14/13		9 (33.3)		67 (3–180)	
Epithelial	15 (17.40)		46 (15–72)		9/6		4 (26.7)		54 (7–166)	
Immunohistochemical (Marino and Muller-Hermelink[12])										
Medullary	19 (21.46)	NS	44 (21–71)	NS	8/11	NS	7 (36.8)	NS	118 (3–199)	NS
Mixed	21 (23.8)		38 (16–71)		9/12		8 (38.1)		79 (12–215)	
Cortical	48 (54.5)		50.5 (17–78)		25/23		19 (39.56)		59 (7–166)	
WHO classification (Rosai et al.[16])										
A	9 (10.2)	NS	33 (21–72)	NS	3/6	0.03	3 (33.3)	NS	62 (3–144)	NS
AB	35 (397.89)		54 (16–78)		16/19		14 (40.0)		91 (7–215)	
B1	27 (30.67)		50 (18–73)		12/15		10 (37.0)		79 (12–180)	
B2	15 (17.1)		46 (17–72)		10/5		6 (40.0)		54 (7–166)	
B3	2 (2.3)		43.5 (42–45)		1/1		1 (50)		41 (17–65)	
Total	88		48.5 (16–78)		42/46		34 (38.6)		71 (3–215)	

M/F: male to female ratio; MG: myasthenia gravis; NS: not significant; WHO: World Health Organization.

protein expression in each specimen by scanning the entire section and estimating the percentage of tumor cell nuclei staining. All immunoreactive nuclei were regarded as positive, regardless of the intensity of staining.

Statistical Analysis

All data were statistically analyzed using SPSS software (SPSS® 9.05 for Windows; SPSS Inc., Chicago IL). Preliminary descriptive analysis was performed for main epidemiologic and pathologic variables to identify anomalous data.

Interdependence among factors (clinicopathologic parameters and cell-cycle protein expression) was assessed using the chi-square test and the Fisher exact test. A P value < 0.05 was considered to be statistically significant in two-tailed tests.

Thereafter, every prognostic variable was correlated for risk of disease recurrence and of death. According to our previous report,[26] a dichotomized scoring system was used, with p53, p21, and p27 expression in $> 5\%$ of the tumor cells defined as high expression and used as a cutoff point. Survival analysis was performed according to the Kaplan–Meier method. Thymoma recurrence and death attributable to cancer or noncancer causes were considered as the final event. 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 only for those factors found to be significant on univariate analysis.

RESULTS

Clinicopathologic Findings

The study population consisted of 88 patients (42 men and 46 women), with a median age of 48.5 years (range, 16–78 yrs). The main clinicopathologic characteristics of the patients are summarized in Table 1. All patients underwent a surgical approach through a total longitudinal median sternotomy; in 5 patients (6%) we were obliged to perform a second access through a lateral thoracotomy to permit radical surgical resection. Four procedures that began for diagnostic purposes as videothoracoscopy were converted into open access. Radical thymectomy also included infiltrated areas of the pericardium ($n = 10$ patients), lung ($n = 4$ patients), and phrenic nerve ($n = 2$ patients). The mean surgical time was 118 ± 37 minutes. No intraoperative or postoperative mortality occurred. Ten patients (11.3%) developed postoperative complications: pneumonia ($n = 4$ patients), respiratory failure because of MG crisis ($n = 2$ patients), surgical wound infections ($n = 5$ patients), and prolonged air

TABLE 2
Interdependence between Clinico-pathologic Features and Cell-Cycle Protein Expression

	High *p53* expression	*P* value	Low *p21* expression	*P* value	Low *p27* expression	*P* value
Staging system (Masaoka- et al.[5,6])						
I	18	NS	16	NS	14	0.03
II	7		10		12	
III	6		8		9	
Neoplasm histotype (Bernatz et al.[11])						
Spindle	3	NS	3	NS	4	NS
Mixed	15		17		15	
Lymphocytic	6		7		8	
Epithelial	7		7		8	
Immunohistochemical (Marino and Muller-Hermelink[12])						
Medullary	4	NS	5	NS	3	0.05
Mixed	11		10		11	
Cortical	16		19		21	
WHO classification (Rosai et al.[16])						
A	3	NS	3	NS	2	0.03
AB	16		18		17	
B1	4		5		6	
B2	7		7		9	
B3	1		1		1	

NS: not significant; WHO: World Health Organization.

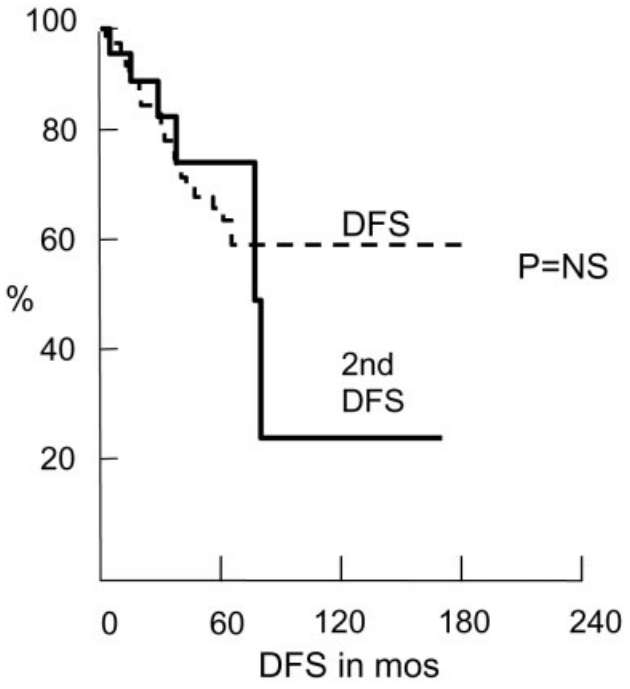

FIGURE 1. Disease-free survival (DFS) after the first recurrence versus global disease-free survival. NS: not significant.

leak (n = 3 patients). The mean hospital stay was 5.3 $\pm$ 0.7 days. Adjuvant therapy was administered in all patients with Stage III disease (according to the Masaoka staging system[5,6]). The median follow-up period was 70 months (range, 3–216 mos).

All the cell-cycle-associated proteins examined were present in the nuclei of tumor cells, although a small proportion of the cells displayed cytoplasmic immunoreactivity in addition to nuclear staining.

Interdependence Analysis

Clinicopathologic factors were distributed in the study group according to findings in the literature. No significant differences were noted with regard to length of follow-up or patient age and gender distribution within the different categories of the study group. No significant interdependence was found between stage of disease and histotypes (data not shown). The cell-cycle checkpoint proteins were analyzed with respect to the detailed clinicopathologic information available for all patients in this cohort. No correlations were found with the other clinical features, such as patient age, patient gender, clinical tumor stage, and all histologic classifications. A mild but significant correlation has been identified between p27 expression and traditional prognostic factors. These results are summarized in Table 2.

Pattern of Disease Recurrence

Only late clinical stage and WHO classification were found to be correlated with the probability of disease recurrence and a shorter overall survival, whereas different histologic classifications, the male-to-female ratio, patient age $\geq$50 years, and the surgical approach used (sternotomy vs. thoracotomy) were not. The presence of MG was found to be correlated significantly with a higher rate of disease recurrence (P=0.01), without affecting the survival. There were 24 (27.3%) tumor recurrences: local in 4 patients, distant intrathoracic in16 patients, and extrathoracic in 4 patients. The median time to disease recurrence was 36 months (range, 3–78 mos). Treatment of the recurrence consisted of 14 radical and 2 incomplete surgical reresections, whereas 8 patients were treated with other therapies (chemotherapy alone in 4 patients and combined chemoradiotherapy in 4 patients). The overall 5-year survival rate from the time of disease recurrence was 26%. Radical surgical reresection was associated with a significantly higher survival compared with patients treated with other therapies (5-year survival rates of 73% and 0%, respectively). The second disease-free interval was not found to be statistically significantly different from that reported after the first surgery (Fig. 1).

Four patients died from thymoma spread after first intrathoracic disease recurrence (three patients)

or distant recurrence (one patient); two of these patients had MG. The median time from disease recurrence to death was 20.5 months.

Survival According to the Pathologic Staging

According to the Masaoka pathologic staging system,[5,6] 41 patients had Stage I disease, 31 patients had Stage II disease, and 16 patients had Stage III disease. The 5-year and 10-year disease-free survival rates were 91% and 87%, respectively, for patients with Stage I disease; 91% and 65%, respectively, for patients with Stage II disease; and 23% and 21%, respectively, for patients with Stage III disease (P=0.001). At the same time, the 10-year overall survival rate was 100% for patients with Stage I disease, 90% for patients with Stage II disease, and 75% for patients with Stage III disease (Fig. 2).

Survival by Histologic Classification

Using the system of Bernatz et al.,[11] we classified 11 spindle, 35 mixed, 27 lymphocytic, and 15 epithelial histotypes; using the system of Marino and Muller-Hermelink,[12] we classified 19 medullary, 21 mixed, and 48 cortical thymomas. No classification disclosed a statistically significant difference in survival for either overall or disease-free survival.

According to WHO criteria, there were 9 patients (10%) with subtype A tumors, 35 patients (40%) with subtype AB tumors, 27 patients (31%) with subtype B1 tumors, 15 patients (17%) with subtype B2 tumors, and 2 patients (2%) with subtype B3 tumors. The 10-year disease-free survival rate was 100% for patients with subtype A tumors, 73% for those with subtype AB tumors, 65% for those with subtype B1 tumors, 43% for patients with subtype B2 tumors, and 0% for patients with subtype B3 tumors, with a significant difference noted among the 5 patient populations (P=0.001). Conversely, the 10-year overall survival revealed only a mild difference (Fig. 3).

Survival by Cell-Cycle Protein Expression

p53 was found to be highly expressed in 31 cases out of 79 available samples (39.2%), and p21 and p27 were low-expressed in 34 (43.0%) and 35 samples (44.3%), respectively. No correlation was noted between cell-cycle protein expression and disease recurrence (data not shown). Disease-free survival appeared to be influenced by high expression of p53, and low expression of p21 and p27. The 5-year and 10-year disease-free survival rates were 61% and 19%, respectively, for patients with high expression of p53 (P=0.03), 77% and 40%, respectively, for patients with low expression of p21 (P=0.02), and 71% and 33%, respectively, for patients with Masaoka Stage III disease (P=0.001) (Fig. 4).

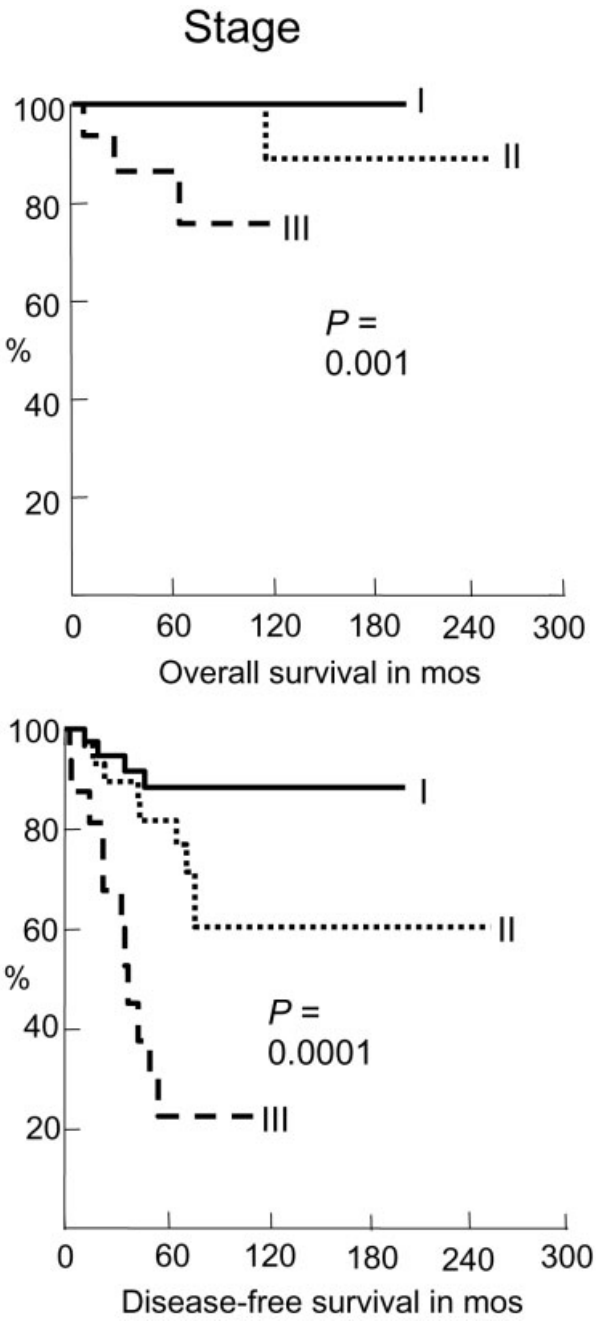

FIGURE 2. Overall survival and disease-free survival according to the stage of disease.

Multivariate Analysis of Survival

Using multivariate analysis, we matched Stage I patients versus Stage non-I, A-AB-B1 versus B2-B3 WHO histotypes, and single cell-cycle protein expression. The most relevant negative predictor of

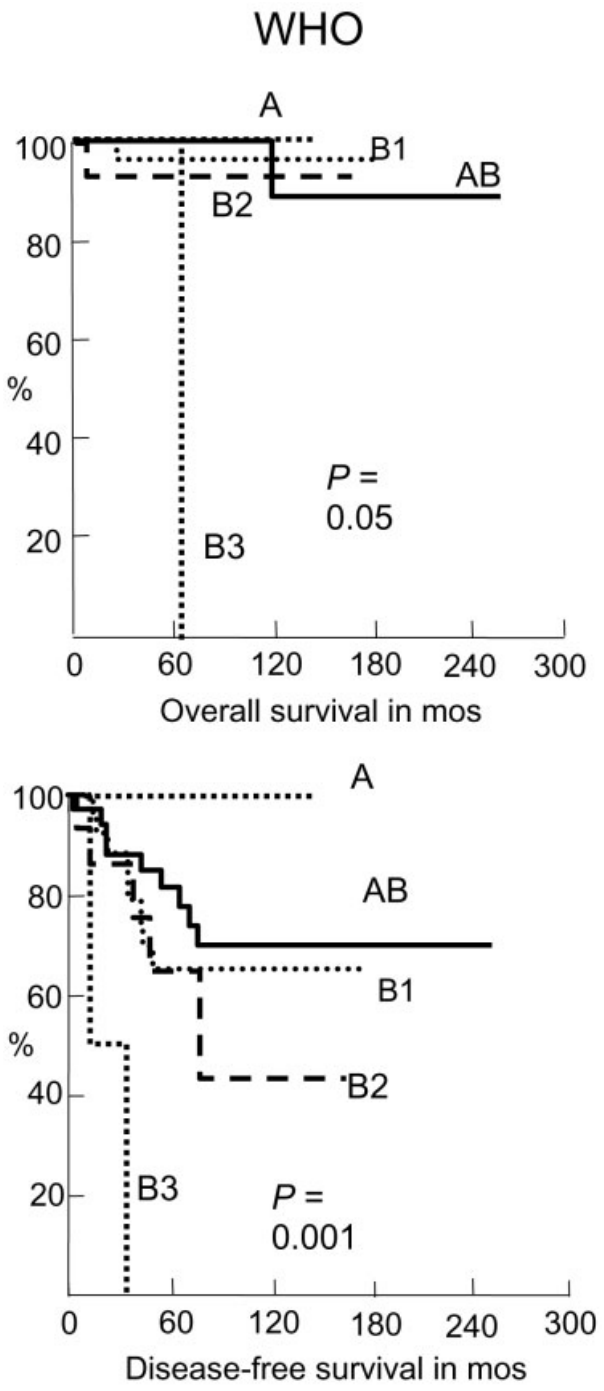

FIGURE 3. Overall survival and disease-free survival according to the World Health Organization (WHO) classification.

disease-free survival was found to be low p27 expression (P=0.001). Furthermore, dichotomizing the most negative expression of cell-cycle protein expression (i.e., the combination of low p27 expression with high p53 and low p21 expression vs. all other combinations), we obtained an even more significant result (P = 0.0001). The data are reported in Table 3.

DISCUSSION

The natural history of thymomas remains unpredictable; they may recur independently of classic clinicopathologic factors and despite radical surgical resection, which is still considered the treatment of choice.[1,9] This difficulty complicates the planning of a structured postoperative therapeutic strategy. In fact, to our knowledge multiple studies have failed to demonstrate the precise role of adjuvant therapy, especially after radical surgical resection at early stages of disease. Since 1985, adjuvant radiotherapy has been performed by Monden et al.,[27] who proposed it even for patients with Stage I disease. The results with this procedure generally were good but not completely satisfactory; after total surgical resection, approximately 5% of the irradiated patients still developed disease recurrence. Dziuba and Curran[28] recommended postoperative radiation therapy for Stage II disease in the case of transgression of the tumor through the capsule, despite macroscopically complete surgical resection. Conversely, two recent studies[21,29] found that the addition of adjuvant radiotherapy did not significantly alter rates of local or distant disease recurrence in patients with Stage II thymomas who had undergone radical surgical resection. Furthermore, even after complete surgical resection, postoperative irradiation has demonstrated only marginal benefits in patients with Stage III disease, with significant morbidity reported.[20]

Many studies to date have demonstrated that the probability of disease recurrence is not a simple matter of tumor progression according to stage of disease, but histologic subtypes also may represent an independent prognostic factor.[9,17–19] Therefore, the therapeutic strategy should be redefined also according the histologic subtype of the tumor.

The WHO histologic typing of tumors of the thymus first was published in 1999.[16] Some studies have reported that the WHO classification appears to have clinical and prognostic value.[17,18,30] More recently, Strobel et al.[19] proposed a therapeutic algorithm to be tested prospectively that includes WHO classification, radicalness of the surgical resection, and clinical stage of disease. Furthermore, Inoue et al.[22] suggested that the WHO histotypes are somewhat correlated with genetic alterations; A-AB histotypes are biologically distinct from the others and B2-B3 thymomas form a continuum, with evidence of tumor progression.

On the basis of analogous studies performed in patients with lung carcinoma,[26] we hypothesized an active role of genomic expression for thymoma growth

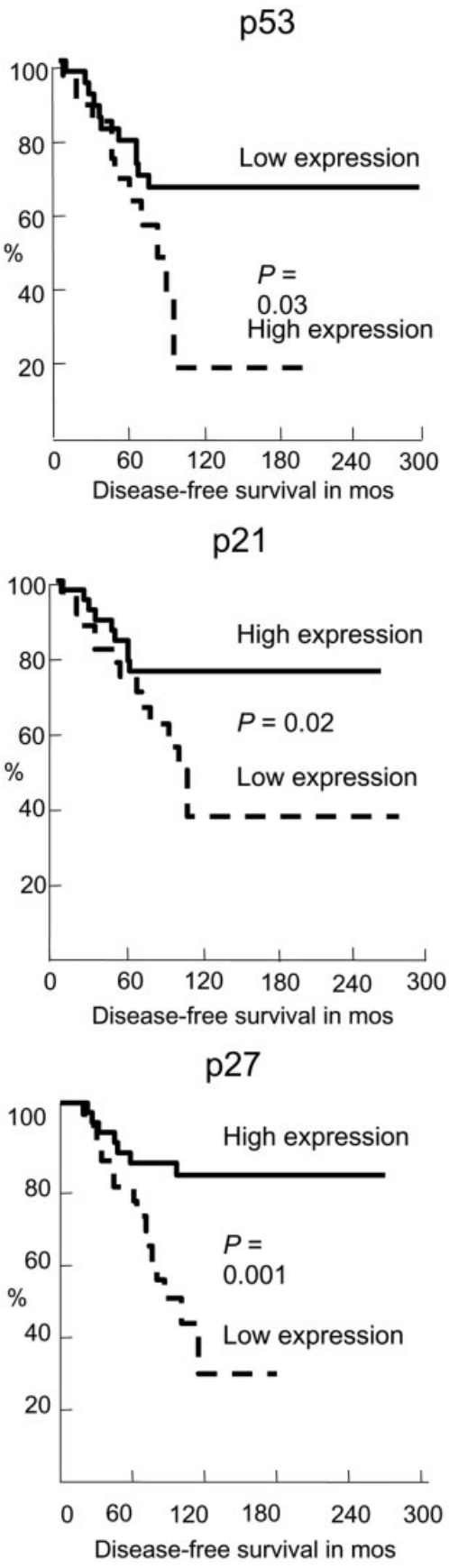

TABLE 3
Multivariate Cox Regression Analysis of Disease-Free Survival in Thymomas Patients

	RR of recurrence	95% CI	*P* value
Masaoka stage			
Stage I	1	—	
Stages II and III	1.7	0.796–2.007	0.05
WHO classification			
A-AB-B1	1	—	
B2–B3	12.8	1.17–140.7	0.03
p27 expression			
High	1	—	
Low	3.49	1.44–6.73	0.001
High p53 expression, low p21 expression, and low p27 expression			
Absent	1	—	
Present	2.18	1.01–5.61	0.0001

RR: relative risk; 95% CI: 95% confidence interval; WHO: World Health Organization.

and progression. 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. Currently, several pathologies are connected to an altered control of cellular replication and, among these, cancer is one of the most studied. We therefore analyzed the expression of three key proteins involved in cell-cycle checkpoints in a large series of well characterized thymoma patients.

When we examined the correlation between clinicopathologic data and the expression of cell-cycle proteins, we found a slightly positive correlation between classic disease recurrence prognosticators and p27 expression, suggesting a possible role for this protein in the progression of this disease.

When we investigated, using univariate analysis, the correlation between the expression of different proteins and survival, we found that all the cell-cycle proteins analyzed had a statistically significant correlation with disease-free survival. On the multivariate analysis, the most significant parameter found to influence the disease-free survival was p27 and this significance became even more evident when we use the combination of high p53 expression and low p21 and p27 expression.

Taking into account the complicated functional network constituted by the cell-cycle regulator pro-

FIGURE 4. Disease-free survival according to expression of cell-cycle proteins.

teins, it appears evident that knowledge of the level of expression of these factors, and their coregulation, may be important in predicting patient clinical response to simple surgical therapy. Nevertheless, targeting multiple checkpoint proteins may represent a good therapeutic strategy for the development of new molecular treatments for lung carcinoma. In fact, it is clear that functional cooperation between different cell-cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumor suppression. Therefore, it could be possible to hypothesize that silencing more then one cell-cycle regulator in the same tumor cell, acting at either the RNA or protein level, could achieve more effective results. This could be obtained through the use of several technologies such as antisense oligonucleotides, dominant-negative constructs, or antibodies able to block the action of a specific protein. The data presented in the current study support this hypothesis and strongly suggest further research aimed at investigating the simultaneous expression of numerous cell-cycle regulators in patients with thymoma.

We acknowledge several limitations to the current study. First, the retrospective nature of the study, which is necessary because of the relative rarity of the disease, unless performing multicentric analysis. Second, the length of follow-up in the current study was not long enough for a disease with low malignancy. Thymoma has a known potential to recur beyond 10 years and our median follow-up time was 70 months; however, this follow-up was similar to that used in many recent studies.[19,21,29] Another potential concern is that the selection of patients to receive radiotherapy was based only on their having Stage III disease, regardless of the histologic classification of the tumor. This bias restricted potential adjuvant benefit to patients per se affected with a more aggressive disease, which is one of the purposes of postoperative therapy.

Long-term disease-free survival in patients with thymoma who are treated with radical surgical resection appears to be correlated with the Masaoka stage of disease, the WHO classification, and cell-cycle gene expression proteins, with the latter found to be the prevalent factor on multivariate analysis. This supports the theory that functional cooperation between different cell-cycle inhibitor proteins constitutes another level of regulation in cell growth control and tumor suppression. Finally, analysis of cell-cycle regulators, such as p27, should be included in ongoing prospective studies to determine a more precise predictor of disease recurrence and to adopt a stricter surveillance program.

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