

Poor Prognostic Factors in Surgically Resected Stage I Non-small Cell Lung Cancer: Histopathologic and Immunohistochemical Analysis

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Background: A better understanding of the histopathology and molecular biology of lung cancer might improve our capability to predict the outcome for any individual patient. The purpose of this study was to evaluate several histopathologic and molecular markers in order to assess their prognostic value in stage I non-small cell lung cancer.

Materials and Methods: One hundred ten patients at the Kyungpook National University Hospital were enrolled in the study. Histopathologic factors and molecular markers were selected. **Results:** Univariate analysis showed that the T stage, differentiation, visceral pleural invasion, and survivin expression were significantly associated with recurrence. Multivariate analysis demonstrated that differentiation and survivin overexpression emerged as independent prognostic factors of recurrence. **Conclusion:** In resected stage I non-small cell lung cancer, poor differentiation and survivin overexpression have been identified as independent predictors of poor disease-free survival.

Key words: 1. Lung neoplasms
2. Prognosis
3. Pathology
4. Immunohistochemistry

INTRODUCTION

Lung cancer is the most common cause of cancer mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer cases and pathologic stage I represents the fastest growing segment due to the use of low-dose computed tomography for screening. Despite the potential benefits of surgical resection, the 5-year survival rate is only 60% to 70% in stage I patients, predominantly as a result of the development of distant metastasis [1,2]. Variation in survival largely reflects the heterogeneity of tu-

mor biology, with some tumors having more aggressive growth and greater metastatic potential than others; therefore, current tumor stage alone cannot exactly establish the prognosis for these patients. New prognostic factors must be identified to help clinicians better assess the probability of survival and to optimize therapeutic strategies for each individual patient with pathologic stage I lung cancer. Several studies have already demonstrated possible prognostic roles for several biological factors in NSCLC and have found helpful tools for identifying patients with a poor prognosis [3-6]. Among those factors, thyroid transcription factor 1 (TTF-1)

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expression, especially in adenocarcinoma, was known to be a prognostic factor as well as a differential diagnostic factor between primary lung cancer and other adenocarcinoma [7]. Nuclear survivin expression might be an independent biomarker for disease recurrence and survival for NSCLC [8]. Epidermal growth factor receptor (EGFR) overexpression may predict shorter survival in patients with resected stage I-III NSCLC, although this is under debate [9]. E-cadherin is known to play a role in tumor progression and distant metastasis; therefore, reduced E-cadherin expression could potentially affect tumor differentiation and prognosis [10,11]. As a result, the stratification of patients without lymph node involvement, according to prognostic risk, might aid in selecting a group of high-risk patients who would benefit from adjuvant therapy.

The purpose of this study is to evaluate several histopathologic variables and a panel of molecular markers-TTF-1, survivin, EGFR, and E-cadherin expression in order to assess their prognostic value and their combined effects on recurrence in patients with resected stage I NSCLC.

MATERIALS AND METHODS

1) Patient characteristics

Between January 2003 and December 2006, a total of 110 patients (84 male, 26 female) with resected stage I NSCLC including squamous cell carcinoma (SCC), adenocarcinoma (AC), and bronchioalveolar carcinoma (BAC) were enrolled in the study. All patients in the study underwent potentially curative surgery consisting of lobectomy including sleeve resection and bilobectomy (n=104), pneumonectomy (n=4), or segmentectomy (n=2) and complete mediastinal lymph node dissection. None of the patients had neoadjuvant therapy. Patients who died within one month after surgery were excluded from the study to avoid the bias of perioperative mortality. The age of the patients ranged from 41 to 79 years (mean, 62.3 years). Postsurgical pathologic tumor-node-metastasis (TNM) staging was determined according to the guidelines of the American Joint Cancer Committee (AJCC) 6th edition. There were 38 cases with stage IA (T1N0M0) and 72 cases with stage IB (T2N0M0). Follow-up data on the study population were obtained by direct contact. Follow-up oc-

curred at 3-month intervals for the initial 2 years and at 4-month intervals thereafter. Recurrences were detected by computed tomography scans or positron emission tomography and, if necessary, confirmed by pathologic examination of biopsy specimens. Patients were categorized as alive with evidence of disease or alive without disease. No patient in this series died of cancer-unrelated causes. The time from the date of the operation to the date of follow-up or death was recorded. Local recurrence was defined as tumor recurrence at the ipsilateral lung or lymph node, and distant recurrence was defined as tumor recurrence at the contralateral lung or lymph node and a distant organ such as the liver, brain, or bone.

2) Pathologic criteria

One pathologist (TIP) reviewed all the histologic slides in a blind fashion. Tumor samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Then, 4 μ L-thick sections were cut and stained with hematoxylin and eosin. Pathologic features were classified according to the histologic criteria of the World Health Organization. The degree of differentiation was divided into three groups: good, moderate, and poor. Tumor necrosis (negative, <10%; positive, \geq 10%) and visceral pleural invasion (absent vs. present) were noted. Tumor size (\leq 2 cm, 2 < & \leq 3 cm, 3 < & \leq 5 cm, 5 < & \leq 7 cm, >7 cm) was also recorded.

3) Immunohistochemical methods

Briefly, tissues were deparaffinized in xylene and rehydrated in graded alcohols and water. Endogenous peroxidase was blocked by soaking in 3% H₂O₂ at 45°C for 4 minutes. The slides were microwaved in citrate buffer (2.1 g/L, pH 6.0) at 120°C for 15 minutes to unmask the antigen and were then treated with a protein-blocking reagent before incubation at 4°C overnight with primary antibodies at a 1:50 dilution, as recommended by the supplier. After extensive washing, the sections were incubated at room temperature for 10 minutes with biotinylated anti-mouse immunoglobulin antibodies (Zymed, San Francisco, CA, USA) at a 1:20 dilution and subsequently with streptavidin-biotin peroxidase complexes at a 1:25 dilution. The reaction products were visualized by immersing the slides in 3,3'-diaminobenzidine tetrahydrochloride. Counterstaining was performed with hematoxylin. All series included

positive and negative controls. The negative controls were prepared by omitting the primary antibodies and known positive controls were included in each run.

4) Evaluation of immunohistochemical data

TTF-1 staining was assessed by the intensity relative to the strong staining intensity of type II pneumocytes as: negative (absent staining, 0), low expression (weak staining intensity, 1), medium expression (intermediate staining intensity, 2), or high expression (strong staining intensity, 3), and only nuclear staining was considered positive staining. Positivity was defined as a staining intensity of 2 and 3. EGFR expression was assessed by an intensity of staining from 0 to 3 and graded as normal (0 and 1) and overexpressed (2 and 3). E-cadherin was assessed by the percentage of positive tumor cells as follows: 0, negative; 1+, <10%; 2+, 10%–50%; 3+, >50%, and was regarded as lost (<10% of cytoplasmic staining) or preserved (\geq 10%). Survivin staining was assessed in 5 to 10 high powered fields at 400 \times magnification. Cytoplasmic immunoreactivity was evaluated semiquantitatively based on the intensity of staining. The percentage of positive tumor cells was evaluated as negative, no survivin cytoplasmic staining; 1+ (weak), <25% staining; 2+ (moderate), 25%–50% staining; and 3+ (intense), more than 50% cytoplasmic staining. Positive survivin immunoreactivity was accepted as a positive staining area of more than 25%.

5) Statistical analysis

The chi-square test and Fisher's exact test were used to analyze the association between histopathologic variables, molecular variables, and recurrence. The time to relapse was defined as the period ranging from the date of surgery to the date when relapse was diagnosed. The specific time to recurrence curves were plotted using the Kaplan-Meier method, whereas the log-rank test was used to assess the statistical significance of differences between groups. Multivariate analyses were performed using the Cox proportional hazards model to identify independent prognostic factors. The criterion for significance was $p < 0.1$.

Table 1. Histopathologic findings in resected stage I non-small cell lung cancer (n=110)

Characteristics	No. of patients (%)
Cell type	
SCC	69 (62.7)
AC	37 (33.6)
BAC	4 (3.7)
T stage	
1	38 (34.5)
2	72 (65.5)
Tumor size (cm)	
\leq 3	59 (53.6)
>3	51 (46.4)
Differentiation	
Good	33 (30.0)
Moderate	60 (54.5)
Poor	17 (15.5)
Necrosis	
Absent	77 (70.0)
Present	33 (30.0)
Visceral pleural invasion	
Absent	58 (52.7)
Present	52 (47.3)

SCC=squamous cell carcinoma; AC=adenocarcinoma; BAC= bronchioalveolar carcinoma.

RESULTS

1) Histopathology (Table 1)

The histological type of NSCLC was as follows: 69 cases of SCC; 37 cases of AC; 4 cases of BAC. The average tumor size was 35.1 \pm 22.9 mm with a range of 3–130 mm and that of 51 (46.4%) patients was over 30 mm. Thirty-three tumors (30.0%) were good-, 60 (54.5%) were moderate-, and 17 (15.5%) were poor-differentiated. Visceral pleural invasion was found in 52 tumors (47.3%) and 33 tumors (30.0%) showed necrosis. Thirty-one (28.2%) patients had tumors of at least 3 cm in size as well as visceral pleural invasion tumors.

2) Immunohistochemical staining (Table 2)

TTF-1 expression was detected in 34 (30.9%) patients. EGFR overexpression was found in 54 (49.1%) of the patients. E-cadherin was lost in 10 (9.1%) cases. Overexpression of survivin was observed in 38 (34.5%) patients.

3) Association between histopathologic and molecular factors (Table 3)

TTF-1 expression was significantly high in several factors

Table 2. Immunohistochemical staining in resected stage I non-small cell lung cancer (n=110)

Characteristics	No. of patients (%)
TTF-1	
Negative	76 (69.1)
Positive	34 (30.9)
EGFR ^{a)}	
0	39 (35.5)
1	17 (15.5)
2	31 (28.2)
3	23 (20.9)
E-cadherin ^{a)}	
0	5 (5.5)
1	5 (5.5)
2	28 (30.8)
3	53 (58.2)
Survivin ^{a)}	
0	37 (33.6)
1	35 (31.8)
2	36 (32.7)
3	2 (1.8)

TTF-1=thyroid transcription factor 1; EGFR=epidermal growth factor receptor.

^{a)}According to the positive area: 0 (<25%), 1 (≥25% & <50%), 2 (≥50% & <75%), and 3 (≥75%).

Table 3. Association between histopathologic and molecular factors

	Cell type			Differentiation		
	SCC	AC	p-value	Good/moderate	Poor	p-value
TTF-1						
Negative	63 (91.3)	12 (33.3)	0.000	65 (69.9)	11 (64.7)	0.670
Positive	6 (8.7)	24 (66.7)	-	28 (30.1)	6 (35.3)	-
EGFR						
Normal	31 (44.9)	20 (55.6)	0.076	44 (47.3)	12 (70.6)	0.078
Overexpression	38 (55.1)	16 (44.4)	-	49 (52.7)	5 (29.4)	-
E-cadherin						
Loss	7 (12.5)	3 (9.7)	0.760	7 (9.0)	3 (23.1)	0.151
Preserved	49 (87.5)	28 (90.3)	-	71 (91.0)	10 (76.9)	-
Survivin						
Normal	44 (63.8)	25 (69.4)	0.783	63 (67.7)	9 (52.9)	0.238
Overexpression	25 (36.2)	11 (30.6)	-	30 (32.3)	8 (47.1)	-

Values are presented as number (%).

SCC=squamous cell carcinoma; AC=adenocarcinoma; TTF-1=thyroid transcription factor 1; EGFR=epidermal growth factor receptor.

such as AC (p=0.000) and visceral pleural invasion (p=0.032); however, it was not significantly associated with differentiation (p=0.670). The EGFR overexpression was more frequent in patients with SCC (p=0.076). Loss of E-cadherin was correlated with larger tumor size (p=0.061) and survivin (p=0.006, no expression).

4) Recurrence

The disease free interval was determined as the interval from the date of surgery to the date of first recurrence. Those without recurrence past 31 August 2010 were classified as censored.

Median follow-up was 55.0 (2.3–87.9) months and the 5-year disease free survival rate was 67.1%, with a mean disease free time of 65.1 months. Twenty-two patients (20.0%) had recurrent disease and 15 patients (13.6%) died from disease-related causes. Seven of the 22 patients had local recurrence and 15 patients had distant recurrence. Of the 15 patients with distant metastasis, 5 patients had recurrence in the contralateral lung, 2 patients in the bone, 1 patient in the liver, 1 patient in the brain, and 6 patients in multiple sites. Table 4 shows that histopathologic factors including differentiation (p=0.007), visceral pleural invasion (p=0.006), T stage (p=0.021), and molecular markers consisting of TTF-1 negativity (p=0.799) and survivin expression (p=0.088) were associated with recurrence. Table 5 lists the results of uni-

Table 4. Recurrence according to histologic factors and molecular markers

Characteristics	No. of patients	Recurrence	p-value
T stage			0.021
1	38	3	
2	72	19	
Tumor size (cm)			0.153
≤3	55	8	
>3	55	14	
Differentiation			0.059
Good	33	5	
Moderate	60	10	
Poor	17	7	
Visceral pleural invasion			0.006
Absent	58	6	
Present	52	16	
TTF-1			0.799
Negative	60	16	
Positive	28	6	
EGFR ^{a)}			0.924
Normal	56	11	
Overexpression	54	11	
E-cadherin ^{b)}			0.304
Loss	10	1	
Preserved	81	19	
Survivin ^{c)}			0.088
Normal	72	11	
Overexpression	38	11	

TTF-1=thyroid transcription factor 1; EGFR=epidermal growth factor receptor.

^{a)}Negative=grade 0,1; positive=grade 2,3. ^{b)}Loss=grade 0,1; preserved=grade 2,3. ^{c)}Normal=grade 0,1; overexpression=grade 2,3.

variate analysis for the prognostic significance of each histopathologic factor and molecular marker in relation to the time to recurrence. The log rank test showed that the T stage, differentiation, visceral pleural invasion, and survivin expression were prognostic factors (Fig. 1). By multivariate analysis using the Cox proportional hazards regression model, poor differentiation and survivin expression emerged as independent prognostic factors of recurrence (Table 6).

DISCUSSION

The prognosis for patients with NSCLC was highly variable and a better understanding of the biology of tumors

should provide useful information for predicting clinical outcomes and for individualizing treatment. Because lung cancer is a heterogeneous disease resulting from the acquisition of multiple somatic mutations, the prognostic role of histopathologic and molecular markers is difficult to determine [12]. As a significant fraction of the patients at stage I experience disease recurrence and die after a curative resection, the differentiation of patients with a high risk of early recurrence and the identification of patients who must receive adjuvant therapy are topics of great interest in the study of early stage NSCLC. However, stratification of patient prognoses using only the TNM staging system does not allow one to differentiate between patients with stage I lung cancer that are and are not at risk of tumor recurrence. It has been demonstrated that adjuvant therapy is not effective in all patients with stage I disease; it is only effective in specific subsets of patients. It is important to select a subgroup of patients with stage I disease that might benefit from adjuvant therapy and for this reason, investigators have attempted to pinpoint factors that predict poor prognosis through analysis of clinical and histopathologic factors [13-16]. Therefore, the goals of this study were to identify these prognostic factors and to evaluate the association between histopathologic and molecular markers. A multitude of histopathologic factors associated with survival have been described in the literature through retrospective series studies. Harpole et al. [3] identified vascular invasion, visceral pleural invasion, a high mitotic index, and a tumor size greater than 3 cm as risk factors associated with recurrence in 289 resected stage I lung cancer patients. In this study, histological factors including tumor size, differentiation, and T stage were identified as significant factors. The degree of differentiation of the primary tumor is known to be a significant prognostic factor and poor differentiated tumors are defined as negative prognostic factors in patients with stage I NSCLC [17]. Tumor size was rearranged in the 7th edition of the guidelines of the AJCC and this detailed size rearrangement was more effective in predicting recurrence than the 6th edition guidelines (data not shown). Although observation data based on clinical and histopathologic description aids in dividing patients into risk groups, much variation exists in the interpretation of these qualitative variables. Therefore, many molecular biologic markers have recently been studied in lung

Table 5. Association between histopathologic and molecular factors

Factors	Variables	Univariate analysis
Age (yr)	< 65, ≥ 65	0.497
Sex	Male/female	0.332
Location	Central/periphery/mid	0.136
Differentiation	Good/moderate/poor	0.026
Histology	SCC/AC/BAC	0.402
Visceral pleural invasion	Absent/present	0.000
Necrosis	Absent/present	0.284
Tumor size (cm)	≤2, 2 < & ≤3, 3 < & ≤5, 5 < & ≤7, >7	0.210
T stage	1/2	0.023
TTF-1	Negative/positive	0.304
EGFR	Normal/overexpressed	0.924
E-cadherin	Loss/preserved	0.332
Survivin	Normal/overexpressed	0.049

SCC=squamous cell carcinoma; AC=adenocarcinoma; BAC=bronchioalveolar carcinoma; TTF-1=thyroid transcription factor 1; EGFR=epidermal growth factor receptor.

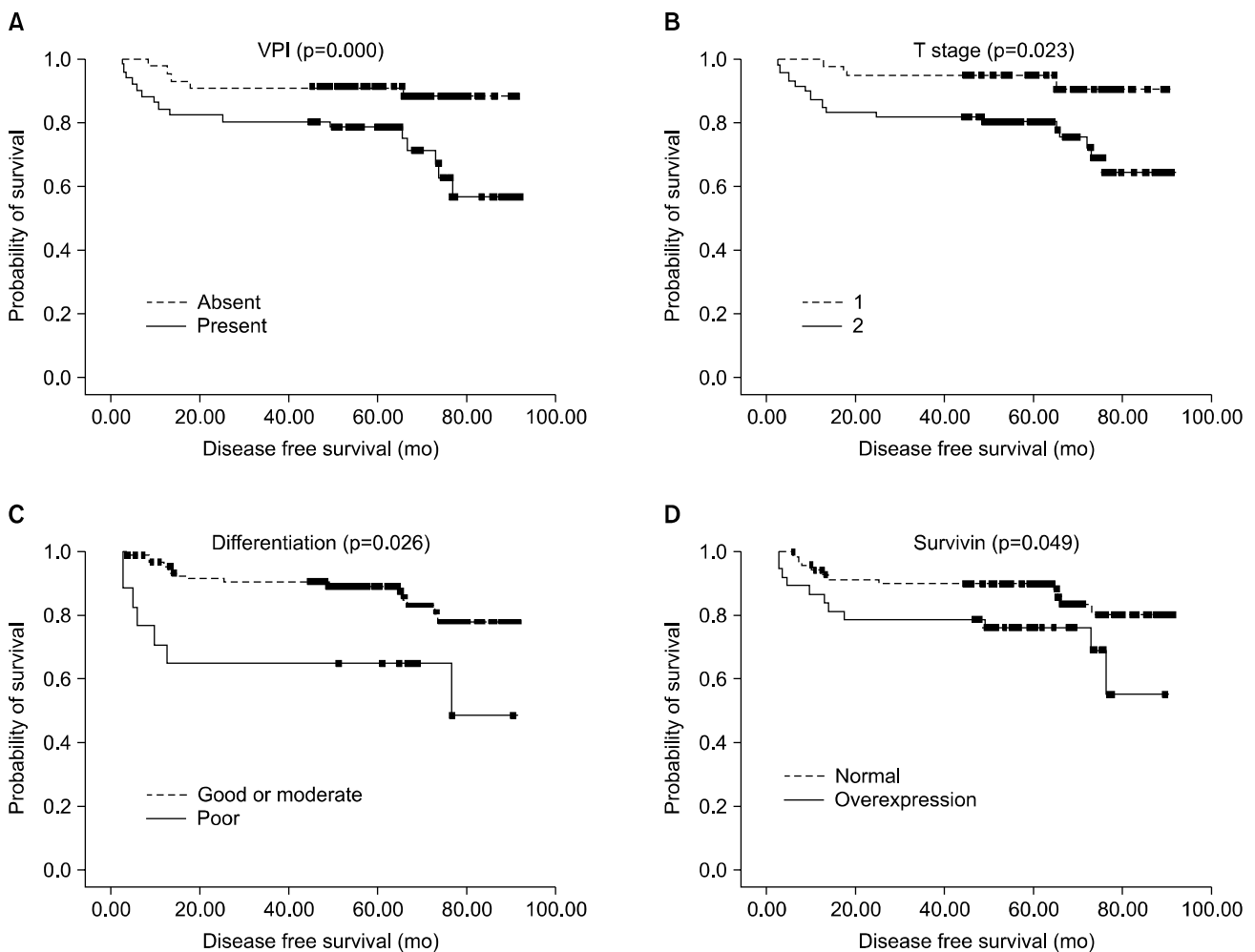


Fig. 1. Kaplan-Meier analysis of disease free survival for (A) visceral pleural invasion (VPI) (B) T-factor (C) differentiation, (D) survivin.

Table 6. Multivariate analysis of disease-free survival by Cox proportional hazards regression model

Variables	HR	95% CI	p-value
T stage	1.729	0.365–8.199	0.490
Visceral pleural invasion	2.532	0.750–8.549	0.135
TTF-1 negative	0.440	0.157–1.232	0.118
Survivin overexpression	2.212	1.014–5.652	0.045
Poor differentiation	3.147	1.296–7.639	0.011

HR=hazard ratio; CI=confidence interval; TTF-1=thyroid transcription factor 1.

cancer to determine their prognostic importance. Gene copy number and mutation analyses are now being explored in prospective clinical studies in enriched populations. These assays are very promising, but they are costly and are not widely available. In contrast, protein expression studies by immunohistochemistry are rapid, reproducible, and routinely performed in many hospitals. During the last decade, the aggressive phenotypes of lung cancer have been characterized by a number of molecular abnormalities, so multiple algorithms were created based on relative protein expression profiles using up to 15 factors with possible significance.

In this study, we have evaluated factors such as TTF-1 and surviving, which have not been extensively studied. TTF-1 is mainly expressed in the thyroid and lung during embryogenesis, and it plays a physiological role in their development and morphogenesis. Positive TTF-1 staining by immunohistochemistry has been described in 25% to 80% of primary adenocarcinoma; however, the vast majority of squamous cell carcinoma of the lung lacks TTF-1 expression [7]. In our study, TTF-1 positivity was 8.7% in squamous cell carcinoma and 66.7% in adenocarcinoma. TTF-1 positive adenocarcinoma had significant relationships with the prevalence of female, nonsmoker, negative staining of p53, less frequent retinoblastoma protein loss, and preserved expression of p27 [18]. Yatabe et al. [19] demonstrated that TTF-1 was consistently expressed during development and this pattern was preserved in transformed cells and metastatic lymph node. Expression was not affected by the differentiation state. However, Anagnostou et al. [7] showed that patients with stage I adenocarcinoma with TTF-1 expression had a longer median overall survival than those without expression and re-

vealed TTF1-expression as an independent lower risk factor for patients with stage I adenocarcinoma.

EGFR is a member of the receptor tyrosine kinase (TK) family, and TKs regulate signaling pathways that control critical cellular activities [20]. The clinical importance of EGFR has increased with the development of EGFR TK inhibitors (TKI). EGFR mutations were observed more frequently in women, never-smokers, and in adenocarcinoma, and EGFR TKIs were more effective in patients with EGFR mutations. Suzuki et al. [21] compared mutation with expression and reported that EGFR overexpression was significantly higher in cases where EGFR mutation was positive rather than negative. In this study, EGFR overexpression was more common in squamous cell carcinoma, men, and ever-smokers, which is in contrast with the findings of EGFR mutation. Therefore, the exact mechanism remains unclear whether EGFR mutation is the major contributor to EGFR overexpression.

E-cadherin and catenins are components of the adherens junction protein, with crucial roles in maintaining intercellular junctions in epithelial cells; reduced E-cadherin expression could potentially affect tumor differentiation, metastasis, and prognosis [10,11]. Although the number of patients who have demonstrated the loss of E-cadherin is small, it still represents a significant prognostic factor.

Survivin is a recently identified protein that functions as an inhibitor of apoptosis, suppressing programmed cell death and regulating cell division. The expression of survivin is undetectable or found at very low levels in normal tissues but at high levels in various malignancies and also embryonic and fetal tissues [22]. The overexpression of survivin may overcome apoptotic checkpoints and promote aberrant progression of the transformed cell through mitosis. A high level of survivin expression in malignancies is considered to be an important indicator of poor prognosis and associated with high tumor grade, tumor progression, and chemoresistance [23]. Survivin staining was detected in both the nucleus and the cytoplasm of NSCLC. However, it was shown that nuclear survivin, rather than cytoplasmic staining, was predictive of poor survival in patients with NSCLC and esophageal cancer. Shinohara et al. [8] showed patients who had nuclear staining for survivin had a significantly increased risk of disease recurrence and concluded that the nuclear presence of

survivin may be an independent biomarker for disease recurrence and overall survival in patients with resected stage I and II NSCLC. Atikcan et al. [24] evaluated the prognostic significance of both nuclear and cytoplasmic survivin expression in NSCLC and showed that cytoplasmic staining was found to be significantly increased in squamous cell carcinoma and that nuclear survivin expression might predict prognosis in NSCLC, whereas cytoplasmic survivin has no prognostic significance.

This study had limitations. First, immunohistochemical scoring is at best a semi-quantitative exercise for which no standard criteria have yet been proposed or adopted. The scoring methods that have been used include estimating the grade of staining intensity, the percentage of tumor cells stained, the cellular localization of the antigen, and systems that combined these parameters. Second, a more important confusion is the method of determining cut-offs for dichotomizing scores in log-rank tests. Cut-offs are often arbitrary and are sometimes selected to obtain the desired effect using the minimum p-value approach. Therefore, different antibodies and grading systems should also be taken into account; the counting of positive tumor cells and grading of staining intensity may be subject to interobserver variability, which should be evaluated.

CONCLUSION

In resected stage I non-small cell lung cancer, poor differentiation and survivin overexpression have been identified as independent predictors of poor disease-free survival.

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