Overexpression of Cyclin B1 in Early-Stage Non-Small Cell Lung Cancer and Its Clinical Implication

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Abstract

Cyclin B1 is a key molecule for G2/M-phase transition during the cell cycle and is overexpressed in various tumor types. However, the expression status of cyclin B1 in lung cancer and its clinical significance remain unknown. We used immunohistochemistry studies to examine the expression status of cyclin B1 in 77 non-small cell lung cancer specimens from patients with histological stage I disease. All of the patients underwent curative surgical treatment. The median length of follow-up care is 8.2 years. High-level cyclin B1 expression (a cyclin B1 labeling index ≥15%) was observed in 17 of the 77 (22%) tumors. Patients whose tumors expressed a high level of cyclin B1 had a significantly shorter survival time than patients whose tumors expressed a low level of cyclin B1 (P = 0.02, log-rank test). Interestingly, overexpression of cyclin B1 was more frequently observed in tumors with squamous cell histology than in tumors with other histological cell types (P = 0.01, Fisher’s exact test). A subgroup analysis revealed that cyclin B1 overexpression seems to be an adverse prognostic factor only in patients with squamous cell carcinoma (SCC) of the lung (P = 0.02, log-rank test). Our data indicate that cyclin B1 may be dysregulated in non-small cell lung cancer, particularly in the SCC subtype, and that a high level of cyclin B1 expression may be a prognostic marker for patients with early-stage SCC of the lung.

Introduction

Lung cancer is a major cause of mortality worldwide. In the United States alone, it was estimated that 164,100 new cases of lung cancer would be diagnosed in 2000, with an estimated 156,900 deaths (1). NSCLC3 represents about 80% of all lung cancers, and its dismal survival rate has not improved significantly in the past two decades. Even patients with pathological stage I NSCLC have only a 60% survival rate at 5 years (2). To further improve the survival rate in this group of patients, their diagnostic classification based on tumor biology will be crucial. Such classification might help clinicians to make the right management decisions for each subset of patients.

Altered regulation of the cell cycle is a hallmark of human cancers (3). Cell cycle progression is governed by a series of cyclins and cdks. Individual cyclins act at different phases of the cell cycle by binding and activating corresponding cdks. Of the various cyclin/cdk complexes involved in cell cycle regulation, cyclin D1/cdk4/6 and cyclin B1/Cdc2 are of particular interest because the former directs G1/S-phase transition and the latter controls G2/M-phase checkpoint surveillance, which are in turn essential for DNA synthesis and cell proliferation. Dysregulated expression of these cyclins, cdks, or both may lead to uncontrolled cell growth and malignant transformation. Overexpression and/or amplification of cyclin B1 has been reported in a large variety of human cancers, including those of the esophagus, head and neck, lung, liver, and breast (3) and is reported to be of prognostic importance in patients with most of these tumor types (4–6). Overexpression of cyclin B1 has been reported more recently in breast, colon, prostate, oral, and esophageal carcinomas (7–11). Its prognostic value has been suggested in patients with SCC of the esophagus (11). However, little is known about cyclin B1 expression status in lung cancer and its potential clinical application in this tumor type.

To determine whether cyclin B1 expression is dysregulated in early-stage NSCLC and whether it can be used as a prognostic marker in patients with NSCLC, we examined immunohistochemically the expression pattern of cyclin B1 in 77 patients with pathological stage I NSCLC. We found that cyclin B1 was overexpressed in about 20% of the tumors analyzed (34% of SCC and 12% of non-SCC tumors) and that this overexpression is an adverse prognostic factor of survival time for patients with stage I NSCLC.

Materials and Methods

Study Population. The primary NSCLC specimens were archived tissue samples of surgically resected pathological stage I tumors from 77 patients treated at The University of Texas M. D. Anderson Cancer Center (Houston, TX). All patients were treated by surgery alone and received a median of 8.2 years of follow-up care after surgical treatment. Surgical specimens were collected between 1975 and 1990. Survival data were available for all patients and with a minimum length of follow-up care of 5 years. The study population consisted of 54 men and 23 women. The mean age of patients was 65 years. Histological subtypes included 35 SCCs, 33 adenocarcinomas, 3 bronchoalveolar carcinomas, 3 large cell carcinomas, 1 adenosquamous carcinoma, and 2 unclassified tumors (Table 1).

Immunohistochemical Staining for Cyclin B1 and Ki-67 Protein. Paraffin-embedded, 4-µm-thick tissue sections from all 77 primary tumors were stained for the cyclin B1 protein using a primary mouse monoclonal antibody (NCL-Cyclin B1; Novocastra, Newcastle, United Kingdom) and for Ki-67 (all but one case) using a primary rabbit polyclonal antibody (Dako, Carpinteria, CA). Slides were baked at 60°C for 1 h and then deparaffinized through a series of xylene baths. Rehydration was performed through graded alcohols. To retrieve the antigenicity, tissue sections were then treated with microwaves in 10 mM citrate buffer (pH 6.0) for 3 min (three times for cyclin B1, and six times for Ki-67). The sections were then immersed in methanol containing 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity and incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-cyclin B1 or anti-Ki-67 antibody at dilutions of 1:15 and 1:100, respectively. The sections were then processed using standard avidin-biotin immunohistochemistry according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Routinely processed tissue sections of normal lymph node and tonsil were used as positive staining controls and...
were also stained with the primary antibody omitted to confirm staining specificity.

The cyclin B1 labeling index was defined as the percentage of tumor cells displaying cytoplasmic or nuclear immunoreactivity and calculated by counting the number of cyclin B1-stained tumor cells among at least 1000 tumor cells for each section. Similarly, the Ki-67 proliferative index was defined as the percentage of nuclear-stained cells among 1000 or more tumor cells. Representative areas of each tissue section were selected, and cells were counted in at least four fields (at ×400) in these areas. All slides were scored concomitantly by a pathologist (S. J. J.) and another investigator (J-C. S.).

**Statistical Analysis.** Survival curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. Fisher's exact test and the χ² test were used to analyze the association between two categorical variables. P < 0.05 was considered to be statistically significant. Immunohistochemical analysis was performed in a blinded manner with respect to the clinical information of the subjects. The Pearson correlation coefficient was used to test the strength of association between continuous variables (i.e., cyclin B1 and Ki-67).

**Results**

In normal ciliated bronchial epithelial cells, peribronchial gland cells, or alveolar pneumocytes, cyclin B1 expression was not detectable. Intestinal or lymphoid cells of tumor areas were occasionally immunostained. In tumor cells that stained positively for cyclin B1, the immunostaining was detected predominantly in the cytoplasm (Fig. 1, a–c), although in a few cases, nuclear staining was also observed. In the positive control tissues (tonsil), the normal stratified squamous epithelium displayed cyclin B1-positive cells in the basal and parabasal layers. In the adjacent lymph nodes, cyclin B1-positive cells were localized predominantly in the germinal centers. Positive Ki-67 immunostaining was restricted to the cell nuclei (Fig. 1, d–f). For Ki-67, as well as for cyclin B1, the immunohistochemical staining showed a wide heterogeneity from rare scattered cells to a homogeneous pattern for the vast majority of cells examined, suggesting that phenotypic heterogeneity is a major feature in NSCLC (Fig. 1). If ≥15% of the cells were positive for cyclin B1, the case was considered to have cyclin B1 overexpression. For Ki-67 staining, cases with ≥25% positive cells were considered to have high expression of Ki-67 based on previous reports (12–14). As shown in Fig. 2, a weak but statistically significant correlation (correlation coefficient = 0.30; P = 0.009) was found between cyclin B1 index and Ki-67 score.

**Cyclin B1 overexpression was observed in 17 of the 77 (22%) stage I NSCLC specimens. Low cyclin B1 expression was observed in 60 tumors. Table 1 shows the relationships between expression of cyclin B1 and clinicopathological factors. The frequency of cyclin B1 overexpression did not differ significantly in different age groups or by sex. Interestingly, overexpression of cyclin B1 was observed more frequently in the SCC subtype than it was in other histological subtypes. Twelve of the 35 (34%) SCCs exhibited high levels of cyclin B1 expression, whereas only 5 of 42 (12%) patients with tumors with non-SCC subtypes (mainly adenocarcinoma) showed overexpression of cyclin B1 (P = 0.01, Fisher’s exact test).

We subsequently analyzed the relationship between cyclin B1 expression and length of survival. Fig. 3A shows a comparison of the Kaplan-Meier survival curves between patients whose tumors expressed a high level of cyclin B1 and those whose tumors had low cyclin B1 expression. Patients with tumors that overexpressed cyclin B1 had significantly shorter survival times than patients with tumors that displayed low levels of cyclin B1 (P = 0.02, log-rank test). About 60% of the patients whose tumors had a low cyclin B1 expression were alive at 5 years compared with only 30% of the patients whose tumors had high cyclin B1 expression (Table 1). The prognostic significance of cyclin B1 expression was further explored in terms of the major histological subtypes. Cyclin B1 overexpression was a significant adverse prognostic factor among patients with tumors of the SCC subtype (P = 0.02, log-rank test; Fig. 3B) as opposed to patients with adenocarcinoma (Fig. 3C). Because Ki-67 and cyclin B1 expression had a weak correlation, the prognostic significance of Ki-67 was also explored, but no statistically significant effect was observed (Fig. 3D).

**Discussion**

Cyclin B1 is an important mitotic cyclin in the G₂ and M phases of the cell cycle. Its association with the active form of cdc2 initiates chromosome condensation, destruction of the nuclear membrane, and assembly of the mitotic spindle. An increasing body of data suggests that altered expression of cyclin B1 is a frequent event in tumor cells. Overexpression of cyclin B1 has been demonstrated in colorectal, prostate, breast, esophagus, and head and neck cancers as well as Hodgkin and MALT lymphomas (7–11, 15, 16). Significant differences in the labeling index of cyclin B1 between benign/premalignant lesions and breast carcinomas suggest a consequential role of cyclin B1 overexpression in the malignant transformation of breast epithelial cells (7). Furthermore, cyclin B1 overexpression was statistically associated with depth of tumor invasion and the presence of venous invasion in esophageal SCC, which was thought to affect length of survival as established in a multivariate analysis (11).

In the present study, we demonstrated that cyclin B1 is overexpressed in a significant fraction of NSCLCs. Overall, 17 of the 77 (22%) tumors expressed cyclin B1 in ≥15% of tumor cells. Interestingly, cyclin B1 expression was different when SCC tumors were compared with non-SCC samples. High cyclin B1 expression was observed in 34% of SCC tumors but in only 12% of tumors with other histological subtypes (Table 1). Furthermore, our data show that high cyclin B1 expression is a significant unfavorable prognostic factor in patients with stage I NSCLC. The fact that all of the patients in the study were treated at a single institute and received lengthy follow-up care after surgery makes survival analysis reliable. In the histological subgroup analysis, we found that the prognostic value of cyclin B1 seems to be limited to the squamous cell subtype and has no predictive value among patients with adenocarcinoma (Fig. 3, B and C). This particular observation, together with the fact that cyclin B1 expression itself differs among histological subtypes, highlights the biological differences among different subtypes of NSCLC. Different abnormalities in oncogenes and tumor suppressor genes among histological subtypes of NSCLC are well-known. Indeed, K-ras mutations are much more common in adenocarcinomas than in SCCs, whereas the p53 mutant immunophenotype is more frequent in squamous carcinomas than in adenocarcinoma (17, 18).

To determine whether overexpression of cyclin B1 in NSCLC simply reflects the increased cell proliferation, we analyzed Ki-67, a well-established marker of cell proliferation that is not phase specific in the cell cycle, in contiguous sections of the tumors. Consistent with previous reports, a weak correlation (0.30; P = 0.009) was found

![Table 1](cancersres.aacrjournals.org)
between the cyclin B1 index and Ki-67 score (9, 10). Although some of the previous studies have suggested that Ki-67 is a possible indicator of length of survival (12, 19, 20), we failed to show any significant prognostic value for Ki-67 in our study population. The absence of prognostic value for Ki-67 remained even when survival analysis was performed using different cutoff levels published previously [i.e., 5%, 10%, or 20%; data not shown (9, 20, 21)]. Nevertheless, the prognostic significance of Ki-67 in lung cancer is not yet firmly established because some studies suggest that a high Ki-67 expression level is predictive of poor survival (12, 19, 20), whereas others did not demonstrate any effect (13, 14, 21). Collectively, these observations suggest that high cyclin B1 expression in NSCLCs is not a mere consequence of cell proliferation, but rather an indicator of aberrant cell cycle progression at the G2-M-phase transition in cancer cells. Cyclin B1 also seems to play a pivotal role in the biological behavior of NSCLCs, particularly in SCC, and, in doing so, represents a potential new prognostic marker as well as a therapeutic target in NSCLC.

Fig. 1. Immunohistochemical staining patterns of cyclin B1 and Ki-67 in NSCLC. a, an adenocarcinoma with most cancer cells expressing cyclin B1 in the cytoplasm. b, a squamous cell carcinoma with most carcinoma cells positive for cyclin B1. c, an adenocarcinoma with rare scattered cells expressing cyclin B1 (original magnification, ×400). d, an adenocarcinoma showing a strong positive reaction in the nuclei of carcinoma cells. e, a squamous cell carcinoma with most carcinoma cells positive for Ki-67. f, a bronchoalveolar form with few cells positive for Ki-67 (original magnification, ×400).

Fig. 2. Distribution of the cyclin B1 labeling index and Ki-67 proliferative index for the study population.

corr. coef. = 0.30
p-value = 0.009

Fig. 2. Distribution of the cyclin B1 labeling index and Ki-67 proliferative index for the study population.
How overexpression of cyclin B1 participates in tumor progression remains to be established. Many authors suggest that the oncogenic role of cyclins is probably related to their unscheduled expression, namely, their expression throughout the cell cycle (22, 23). The unscheduled expression of cyclins in tumor cells could be the result of either impaired degradation or continued synthesis during the cell cycle. Regardless of the cause, the continuous presence of cyclins may cause activation of their respective partner kinases (cdks), which remain essentially invariable throughout the cell cycle. This, in turn, might lead to unscheduled phosphorylation of a variety of proteins, thus driving the cell through the cycle and bypassing the respective checkpoints. Recent findings that p53 controls a G2 checkpoint through down-regulation of cyclin B1 and that constitutive activation of cyclin B1 and associated cdc2 kinase can override this p53-mediated G2-M-phase arrest support this notion (24, 25).

In conclusion, this study has shown, using a homogeneous population of 77 patients with stage I NSCLC, that there is overexpression of cyclin B1 in lung tumors, particularly in SCC, and that such overexpression was associated with patients’ prognosis. More comprehensive studies involving greater numbers of patients are necessary to confirm these findings.

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Fig. 3. A, survival curves of patients with stage I NSCLC according to cyclin B1 expression; B, survival curves of patients with stage I squamous cell carcinoma according to cyclin B1; C, survival curves of patients with stage I adenocarcinoma according to cyclin B1; D, survival curves of patients with stage I NSCLC according to Ki-67.

References

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