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Cyclin D1 Protein Expression in Lung Cancer

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Cyclin D1, a G1 cyclin, has been implicated in the oncogenesis of various types of malignancies via deregulation of cell cycles. Amplification of cyclin D1 as a part of 11q13 amplicon has been reported in lung cancer as well as a subset of carcinomas arising from various organs including breast, head and neck, and esophagus. In addition to its role as an oncogene, several recent studies have suggested that amplification is indicative of poor prognosis. In this study we examined the cyclin D1 protein expression in 102 consecutive cases of lung cancers using the microwave enhanced immunohistochemical staining method and correlated the data with the histologic subtype and grade, Ki-67 (MIB-1) labeling index, and survival. Nuclear positive staining was observed in 18 cases (18 %) of lung cancers. Although squamous cell carcinoma demonstrated a higher rate of expression (12 /58, 21%), three of 33 adenocarcinomas (9%) revealed overexpression and both adenocarcinoma and squamous cell carcinoma components within the adenosquamous carcinoma showed nuclear staining. There was no correlation between cyclin D1 overexpression and histologic grade, Ki-67 (MIB-1) labeling index, and survival. These observations indicate that cyclin D1 protein overexpression might be implicated in the oncogenesis of the various histologic types of non-small cell lung carcinomas but it has no usefulness as a prognostic marker.

Key Words: Cyclin D1 protein, lung cancer, immunohistochemistry

Cyclin D1 is a putative oncogene on chromosome 11q13 which was originally defined by several groups using different approaches (Matshushime *et al.* 1991; Rosenberg *et al.* 1991; Xiong *et al.* 1991). Cyclin D1 has been implicated in various types of human tumors either by translocations or amplification of the chromosome band 11q13. Overexpression of cyclin D1 by tumor type specific translocation was reported in mantle cell lymphoma (Motokura *et al.* 1991; Williams *et al.* 1993) as well as parathyroid adenoma (Rosenberg *et al.* 1991). Also amplification of the cyclin D1 as a part of 11q13 amplicon has been reported in a subset of breast, head and neck, urinary bladder, esophagus, liver and lung carcinomas (Lammie and Peters, 1991).

Despite the implication of cyclin D1 as a putative oncogene in various types of tumors by the results of molecular genetic studies (Lammie and Peters, 1991), thus far there has been little data on the overexpression of the cyclin D1 protein in these tumors due to lack of suitable immunohistochemical staining method. Recently, a highly sensitive immunohistochemical staining method using microwave antigen retrieval and newly developed cyclin D1 antibodies enable the detection of overexpressed cyclin D1 protein even in paraf-

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fin embedded tissue sections (Bartkova *et al.* 1994; Yang *et al.* 1994; Zhang *et al.* 1994; Zukerberg *et al.* 1995a). Additionally the overexpression of cyclin D1 protein has been reported in mantle cell lymphoma (Yang *et al.* 1994; Zukerberg *et al.* 1995a), breast carcinoma (Zhang *et al.* 1994; Zukerberg *et al.* 1995b), and head and neck carcinoma (Michalides *et al.* 1994).

Bcl-1 (cyclin D1) was reported to be frequently amplified in poorly differentiated squamous cell carcinomas of the lung by Southern and Northern blot analysis (Berenson *et al.* 1990). Although these methods are sensitive, immunohistochemical staining using tissue sections has several advantages over these methods and there has only been one study addressing cyclin D1 protein overexpression, the final and functional product of cyclin D1 amplification, in lung cancers (Betticher *et al.* 1996). So in this study we examined the cyclin D1 protein overexpression in 102 consecutive cases of lung carcinomas and correlated the data with the histologic subtype and grade, Ki-67 (MIB-1) labeling index, and survival to evaluate its role in lung cancers.

MATERIALS AND METHODS

Tissue samples

One hundred and two consecutive cases of lung cancers from patients operated on between 1992 and 1994 for which adequate paraffin blocks and clinical history were available were retrieved from the file of Pathology Department of Yonsei University College of Medicine. The histologic diagnosis was based on the morphologic examination of hematoxylin-eosin stained tissue sections and each case was subclassified by WHO classification.

Immunohistochemical staining

Immunohistochemical studies were performed on the representative tissue sections from each case by the labelled streptavidin-biotin method using a Dako LSAB Kit (Dako, Carpinteria, CA, USA). Sections for cyclin D1 and Ki-67 (MIB-1) immunostaining were pretreated in a microwave oven for 20 minutes and 10 minutes respectively in 10 mmol/L citrate buffer (pH 6.0) before immunostaining. The incubation with cyclin D1 (NCL-CYCLIN D1) (Novocastra Laboratories Ltd., Newcastle, UK) and MIB-1 (Amac, Inc., Westbrook, ME, USA) was done at 1:20 and 1:100 dilution respectively overnight. Otherwise we followed the manufacturer's protocol. Color development was performed using diaminobenzidine as chromogen with light hematoxylin counterstain.

Analysis of immunohistochemical staining

The results of cyclin D1 immunostaining were expressed as negative, + (less than 50% of the tumor cells nuclei staining), and ++ (more than 50% of the tumor nuclei staining). Ki-67 (MIB-1) labelling index was determined by light microscopy with an oil-immersion objective (magnification ×1,000) randomly counting 1,000 tumor cells and expressing the results as a percentage of positive cells.

Clinical data and statistical analysis

Clinical information on the stages at the time of surgery and follow-up data were obtained by review of hospital clinical records. Follow-up data was obtained until death of the patient or completion of the study (April 30th, 1996). Mean follow-up was 24 months (range 1-51 months). Actuarial survival curves were calculated by the Kaplan-Meier method, and the log-rank test was used to compare the survival of subgroups. We excluded the data of the patients with stage IV, large cell undifferentiated carcinoma, small cell carcinoma and carcinoid tumor for statistical analysis. The differences were considered significant when the p value was 0.05 or less.

RESULTS

Histologic subtypes of lung cancers

One hundred and two consecutive cases of lung cancers were composed of 58 cases of squamous cell carcinomas, 33 cases of adeno-

carcinomas including 4 cases of bronchioloalveolar carcinomas, 6 cases of adenosquamous cell carcinomas, 3 cases of large cell undifferentiated carcinomas, a case of small cell carcinoma and one poorly differentiated carcinoid tumor. Among 58 cases of squamous cell carcinomas, 10 cases were well-differentiated, 19 cases were moderately differentiated and 29 cases were poorly differentiated. Adenocarcinomas consisted of 12 cases of the well-differentiated type; 13 cases of the moderately differentiated type; and 4 cases of the poorly differentiated types (Table 1).

Table 1. Relationship between overexpression of cyclin D1 protein and histologic types

Histologic types	Cases showing overexpression/ Total cases(%)
Squamous cell carcinoma	12/58(21%)
Adenocarcinoma	3/33(9%)
(Bronchioloalveolar carcinoma	1/4(25%))
Adenosquamous carcinoma	2/6(33%)
Large cell undifferentiated carcinoma	1/3(33%)
Small cell carcinoma	0/1(0%)
Carcinoid tumor, poorly differentiated	0/1(0%)
Total	18/102(18%)

Immunohistochemical staining for cyclin D1 protein

Eighteen of the 102 lung cancers showed definite nuclear staining (Fig. 1) and usually the reaction tended to localize along the peripheral portion of the tumor nests (Fig. 2). Many cases also demonstrated cytoplasmic and cell membrane staining (Fig. 3) but we counted only the nuclear staining as the overexpression of cyclin D1 protein. In some cases the activated pneumocytes in the peritumoral normal lung tissue showed nuclear staining (Fig. 4).

Correlation of cyclin D1 staining with histologic subtypes

Squamous cell carcinoma demonstrated a

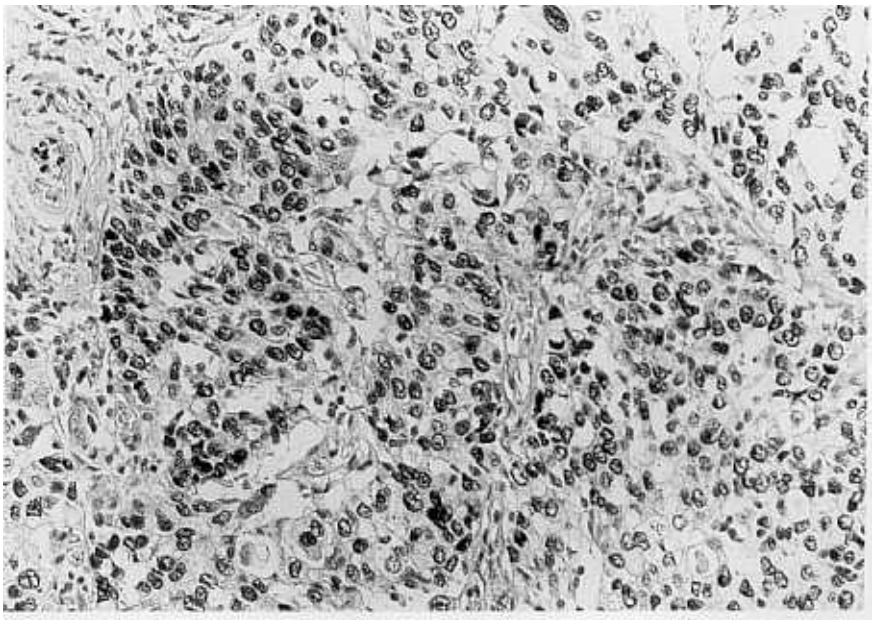

Fig. 1. *Cyclin D1 immunostaining of the poorly-differentiated squamous cell carcinoma showing intense nuclear staining. (DAB chromogen with hematoxylin counterstaining).*

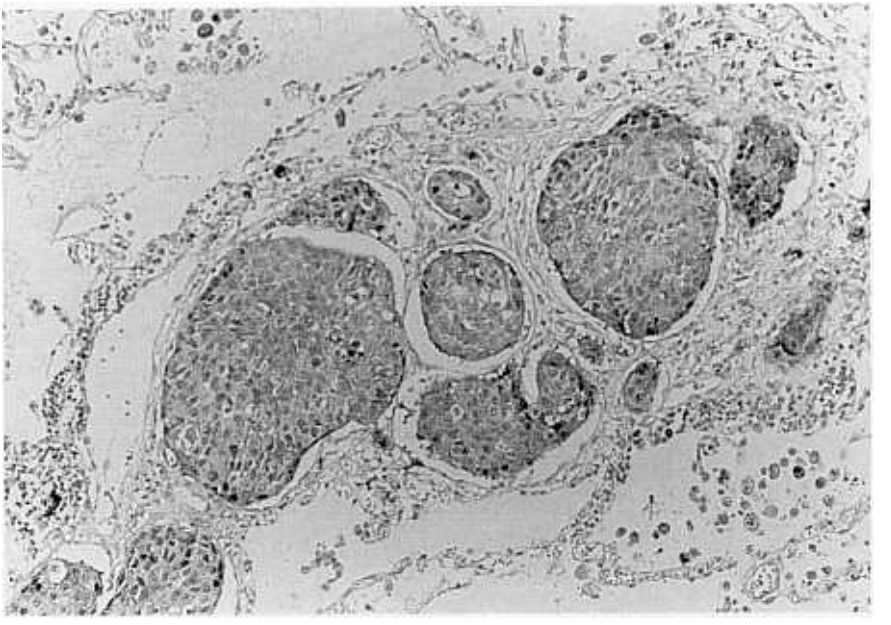

Fig. 2. Cyclin D1 immunostaining showing positive nuclear staining along the periphery of tumor nests. (DAB chromogen with hematoxylin counter staining).

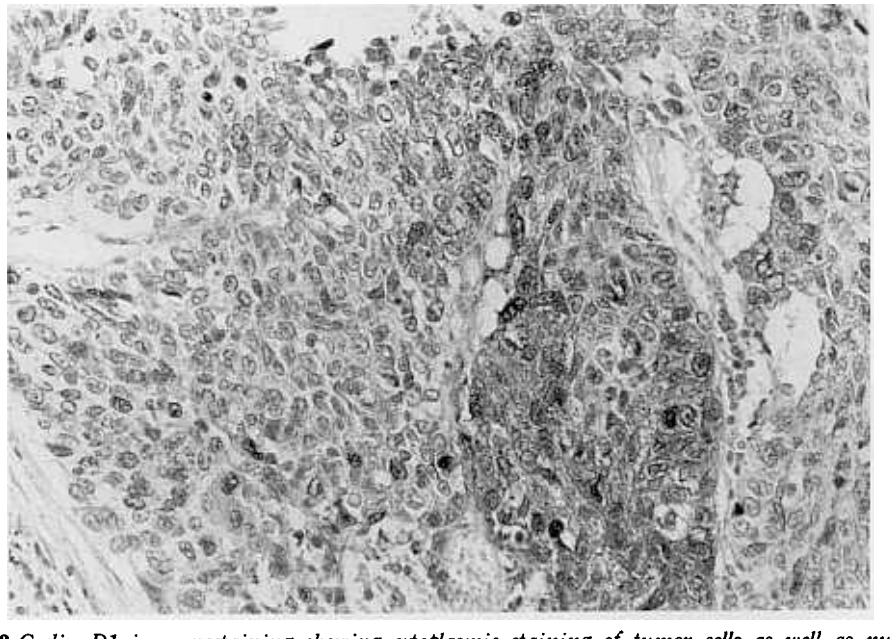

Fig. 3. Cyclin D1 immunostaining showing cytoplasmic staining of tumor cells as well as nuclear staining. (DAB chromogen with hematoxylin counter staining).

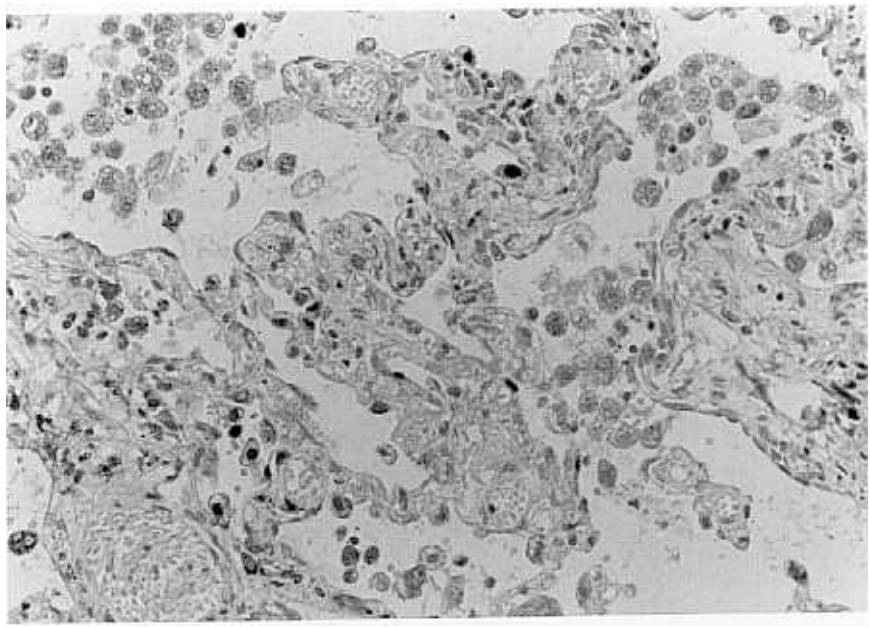

Fig. 4. *Cyclin D1 immunostaining showing positive nuclear staining of activated pneumocytes. (DAB chromogen with hematoxylin counter staining).*

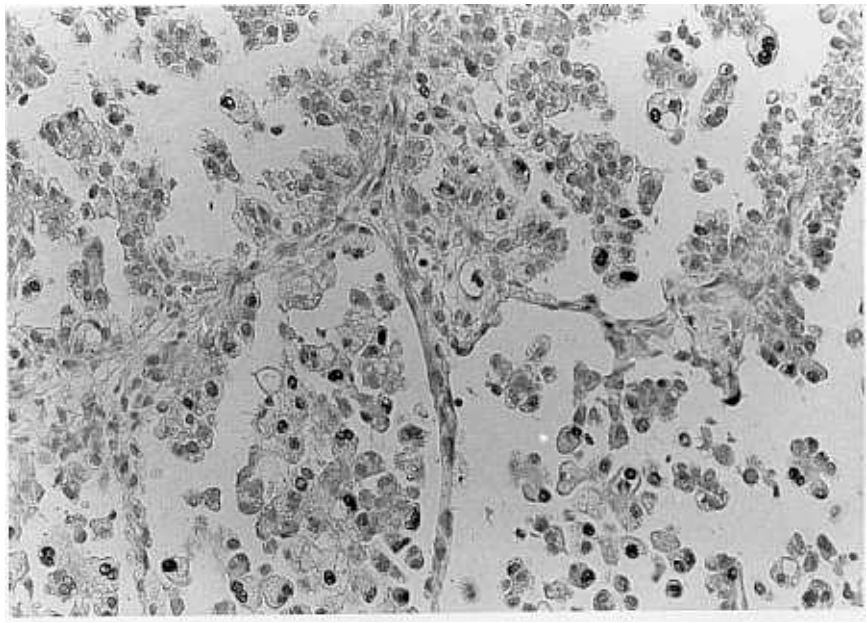

Fig. 5. *Cyclin D1 immunostaining of a case of bronchioloalveolar carcinoma showing positive nuclear staining. (DAB chromogen with hematoxylin counter staining).*

higher rate of expression (12/58, 21%) compared to adenocarcinoma (3/33, 9.1%). Among 4 cases of bronchioloalveolar carcinomas included in the adenocarcinomas, one case expressed cycin D1 protein (Fig. 5). Two cases in the 6 cases of adenosquamous cell carcinomas revealed nuclear positivity and both adenocarcinoma and squamous cell carcinoma areas showed nuclear positivity in either case. One in three cases of large cell undifferentiated carcinomas expressed nuclear cyclin D1 protein and a case of small cell carcinoma and poorly differentiated carcinoid tumor did not show positive reaction.

Correlation of cyclin D1 staining with histologic grade

There was no correlation between cyclin D1 protein overexpression and histologic grade (Table 2). There was a even distribution of well and poorly differentiated subtypes in six cases showing the intense nuclear staining over half of the tumor cells. Although the poorly differentiated subtype of adenocarcinoma and adenosquamous carcinoma showed a higher rate of overexpression, the well differentiated squamous cell carcinoma revealed a higher rate of overexpression compared to the poorly differentiated subtype.

Table 2. Relationship between overexpression of cyclin D1 protein and histologic grade

Histoligic type	Histologic grade (% of overexpression)	Cyclin D1 protein expression		
		Negative	+	++
Squamous cell carcinoma				
	WD(20%)	8	1	1
	MD(32%)	13	6	0
	PD(14%)	25	2	2
Adenocarcinoma				
	WD(15%)	11	0	2
	MD(0%)	13	0	0
	PD(25%)	3	1	0
Bronchioloalveolar	(25%)	3	1	0
Adenosquamous carcinoma				
	WD(0%)	1	0	0
	MD(25%)	3	1	0
	PD(100%)	0	0	1

WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated

Table 3. Relationship between overexpression of cyclin D1 protein and survival

Stage	Cyclin D1 overexpression	3 years survival(%)	Mean survival ±SE(months)	Median survival ±SE(months)	p value
I	N(n=30)	47.0	37±3	36	0.79
	P(n=3)	66.7	39±7		
II	N(n=13)	36.9	26±5	25±10	0.40
	P(n=6)	62.5	38±8		
IIIA	N(n=33)	37.3	29±3	25± 7	0.31
	P(n=8)	37.5	15±3	13± 4	

N: negative, P: positive, SE: standard error

Correlation of cyclin D1 staining with Ki-67 (MIB-1) labelling index

No correlation was found between Ki-67 (MIB-1) labeling index and cyclin D1 protein overexpression. The mean MIB labeling index of the cases showing cyclin D1 protein overexpression was 39.5% and was lower than that of the cases showing no overexpression (mean, 43.9%). Cases showing strong expression of cyclin D1 protein (++) had a similar mean value (36.4%).

Correlation of cyclin D1 staining with stages and survival

The differences in survival by stages were assessed by the log-rank test across all four stages and were found to be significant ($p = 0.01$). Among the 17 cases showing cyclin D1 overexpression, 3 cases were Stage I, 6 cases were stage II and 8 cases were stage IIIA at the time of surgery. No significant difference in survival could be detected according to the presence of overexpression of the cyclin D1 protein in each pathologic stage (Table 3).

DISCUSSION

Due to involvement in the regulation of cell cycles, cyclins and associated cell regulatory molecules, such as cyclin dependent kinase and p21, have recently been considered as attractive candidates for oncogenes. Among several types of human cyclin gene family members, only cyclin D1 has been strongly implicated in the oncogenesis of several types of malignancy (Lammie and Peters, 1991) and more direct evidences supporting cyclin D1 as an oncogene were provided by several recent studies using gene transfer techniques (Bodrug *et al.* 1994; Hinds *et al.* 1994; Wang *et al.* 1994). Most of the studies so far on the cyclin D1 among various cancers were about the amplification using blot techniques, and there have been few studies detecting the cyclin D1 protein in tissue sections which have many advantages compared to blot techniques. Recently several antibodies specific to cyclin D1 protein have been developed and some of them worked even on formalin-fixed and paraffin-embedded tissue sections. In this study, we detected cyclin D1 protein overexpression in 18 of the 102 cases of lung cancer using the newly developed commercialized antibody (NCL-CYCLIN D1, Novocastra Laboratories Ltd., Newcastle, UK) combined with highly sensitive microwave-enhanced immunohistochemical staining method as previously used for cyclin D1 protein immunostaining (Bartkova *et al.* 1994; Yang *et al.* 1994; Zukerberg *et al.* 1995a). We counted only nuclear staining as positive but in many cases cytoplasmic and cell membrane staining were noted with or without nuclear staining. Most of the previous works using various polyclonal and monoclonal antibodies to cyclin D1 protein don't mention this cytoplasmic staining pattern except for one group of authors on lung cancer (Betticher *et al.* 1995; Betticher *et al.* 1996). However some of our unpublished observations have included cytoplasmic staining even in lymph nodes as well as breast and head and neck cancers and this may be due to nonspecific cross-reaction, cell-cycle dependent solubility change of protein or a novel cyclin D1 transcript by mutation (Betticher *et al.* 1995; Lukas *et al.* 1995; Betticher *et al.* 1996). We have also observed definite nuclear staining of the reactive pneumocytes. This is not unusual due to some proliferative zone of normal tissue may express this protein (Bartkova *et al.* 1994). Additionally this pattern of expression can be observed in cases of squamous cell carcinoma showing limited positivity, in that most of the positive nuclei are localized along the peripheral portion of the tumor nests.

The expression rate of cyclin D1 protein in our study is much higher than the data from the previous study on the amplification of the cyclin D1 oncogene among lung cancers (Berenson *et al.* 1990). Actually this discrepancy could be anticipated from the previous reports that overexpression of cyclin D1 protein or mRNA could occur in the absence gene amplification (Buckley *et al.* 1993; Bodrug *et al.* 1994; Zukerberg *et al.* 1995a). It suggests that mechanisms other than gene amplification, such as

abnormally increased protein stability and clonal rearrangements, operate in lung cancers as in other organs. Compared to the data on cyclin D1 protein overexpression in breast (Zhang *et al.* 1994; Zukerberg *et al.* 1995b) and head and neck cancers (Michalides *et al.* 1995), this study showed a lower rate of overexpression of cyclin D1 protein. But there are certain limitations in comparing the data on the overexpression rate of cyclin D1 protein in various types of cancers using different antibodies due to the great variation of the sensitivity of the antibodies (Yang *et al.* 1994). Cyclin D1 overexpression was observed even in the cases of adenocarcinoma which is significantly different from the previous data showing no gene amplification among 51 cases of adenocarcinoma (Berensen *et al.* 1990). But recent studies using the Western blot analysis (Schauer *et al.* 1994) and the immunohistochemical staining method (Betticher *et al.* 1996) as in this study revealing cyclin D1 protein overexpression in adenocarcinoma cell lines and resected tumor tissues support our results.

Because cyclin D1 overexpression can possibly disturb the cell cycles by shortening the G1 phase, we examined if there is a correlation between the Ki-67 (MIB-1) labeling index and the cyclin D1 protein overexpression but found none. These results differ from the study showing a high correlation between cyclin D1 amplification and proliferative activity measured by flow cytometry in head and neck squamous cell carcinoma (Callender *et al.* 1994); but they are in accordance with the results of the study on breast cancer showing no correlation between cyclin D1 protein overexpression and proliferative activity measured by bromodeoxyuridine labeling (Zukerberg *et al.* 1995b). Although we believe there are correlations between proliferative indices measured by various methods, the above mentioned discrepancies may result from different methods for measuring cyclin D1 overexpression and proliferation index as well as small sample size.

We could find no correlation between cyclin D1 protein overexpression and histologic grade and survial in contrast to the results of recent studies suggesting association with poor prognosis (Schuuring *et al.* 1992; Callender *et al.* 1994; Muller *et al.* 1994; McIntosh *et al.* 1995; Michalides *et al.* 1995). However, the study on the breast carcinoma using similar methods as in this study (Zukerberg *et al.* 1995b) demonstrated no correlation between cyclin D1 overexpression and any histologic and clinical parameters except ER and PR status and the recent study on a small series of lung cancers even suggested cyclin D1 overexpression as a good prognostic marker (Betticher *et al.* 1996). Therefore the results of this study justify larger scale immunohistochemical study to confirm the prognostic meaning of cyclin D1 protein overexpression in various types of cancer.

In conclusion, we have shown that cyclin D1 protein overexpression might be implicated in the oncogenesis of the various histologic types of non-small cell lung carcinomas by demonstrating cyclin D1 protein overexpression in 18 % of lung cancers using the newly developed antibody and a highly sensitive, microwaved enhanced, immunohistochemical method. However in contrast to most of the previous studies, we found no correlation between overexpression and histologic grade, proliferation index and survival.

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