The Expression of Cyclins D_1 and E in Predicting Short-Term Survival in Squamous Cell Carcinoma of the Lung

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Cyclins D_1 (cD_1) and E (cE) are G_1 phase cyclins believed to participate in the pathogenesis of malignancy. Overexpression of cD_1 has been reported to influence prognosis in squamous cell carcinomas (SCC) of the larynx, but was not significant in a limited study of non-small cell lung cancers (NSCLC). Altered expression of cE has been proposed as another potential prognostic marker in malignancy but its possible role in NSCLC has not been elucidated. In order to determine the prognostic value of cD_1 and cE in NSCLC, paraffin-embedded sections of 467 NSCLC were immunostained with monoclonal antibody to cD_1 (1:500, PharMingen, San Diego, CA) and 400 NSCLC with MA to cE (1:2500, PharMingen) using an enhanced sensitivity avidin-biotin complex technique. The number of tumor cells with nuclear and/or cytoplasmic immunopositivity was graded on a scale of: 0 = less than 1%, 1 = 1 to 10%, 2 = 10 to 25%, 3 = 25 to 50%, 4 = 50 to 75%, 5 = more than 75%. Results were correlated with survival by Kaplan-Meier survival plot using Stat-View software (Abacus Concepts, Berkeley, CA). Overall, 426 NSCLC with cD_1 and 360 NSCLC with cE had adequate follow-up (median, 76 mo) for survival analysis. Both cyclins independently showed significance in prognosis of SCC but not other cell types. For cD_1, absence of immunostaining was associated with worse prognosis than any immunopositivity for all stages of SCC ($P = .025$). For cE, Stage I and II SCC with less than 50% immunopositivity had a worse prognosis ($P = .029$). Of 70 Stage I and II SCC immunostained for both monoclonal antibodies, 55% of patients with tumors that demonstrated both absence of cD_1 staining and cE immunopositivity in less than 50% of cells were dead at 5 years compared to 35% of patients with tumors that demonstrated positive staining with cD_1 and cE immunopositivity in more than 50% of cells. These results strongly suggest cD_1 and cE can independently predict prognosis in early stage SCC. Worse prognosis was associated with loss of expression, consistent with mechanisms other than overexpression of these cyclins in the progression of SCC.

KEY WORDS: Cyclin D_1, Cyclin E, Immunohistochemistry, Lung, Non-small cell carcinoma, Prognostic factors.

Mod Pathol 2000;13(11):1167–1172

Lung cancer is now the leading cause of cancer deaths in both men and women in the United States (1). Histologically, lung cancer can be classified into one of four major subtypes: adenocarcinoma (AC), squamous cell carcinoma (SCC), large cell carcinoma (LC), and small cell carcinoma. Whereas small cell lung cancers have a uniformly aggressive clinical picture, non-small cell carcinomas (NSCLC) are a heterogeneous group of tumors with variable clinical courses. Therefore, the detection of prognostic markers is essential in order to specify treatment protocols and determine which patients need more aggressive surgery, adjuvant chemotherapy, or radiotherapy.

Studies have reported alterations of proto-oncogenes and tumor suppressor genes that may contribute to the progression of NSCLC and serve as potential prognostic markers. Cyclins D_1 and E are relatively recently described proteins implicated in the pathogenesis of NSCLC. Cyclins (primarily D_1, D_2, D_3, and E) and their associated cyclin-dependent kinases regulate progression of the cell cycle through the G_1 phase and into the S-phase of DNA replication (2). Overexpression of cD_1 and cE has been demonstrated to shorten the G_1 phase of the cell cycle (3, 4). Increased expression of cyclin D_1 (cD_1), as a result of amplifications and rearrangements, has been reported in parathyroid adenomas (5); some B-cell lymphomas (6); and in esophageal (7), head and neck (8, 9), hepatic (10, 11), colorectal (12), and some breast carcinomas

VOL. 13, NO. 11, P. 1167, 2000 Printed in the U.S.A.
Date of acceptance: June 2, 2000.
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(13–15). These studies have suggested that overexpression of cD_1 shortens the G_1 phase and accelerates cell growth, thus positively contributing to oncogenesis.

Studies examining the use of immunohistochemical staining for cD_1 or cyclin E (cE) as a potential prognostic marker are extremely rare. Overexpression of cD_1 has been reported to negatively influence prognosis in squamous cell carcinomas (SCC) of the larynx (8), but was of no prognostic value in a limited study of NSCLC (16) and mammary carcinomas (17). Increased expression of cE, another key regulatory component of cell cycle control, has been demonstrated in colorectal (18) and mammary carcinomas (19) and has been proposed as a potential and possibly better prognostic marker for breast cancer than cyclin D (19), but its possible role in NSCLC has not been elucidated. Therefore, this study was undertaken to determine the prognostic value of cD_1 and cE in NSCLC.

MATERIALS AND METHODS

cD_1 Patient and Specimen Selection

Paraffin blocks from wedge biopsies and resections (segmentectomies, lobectomies, pneumonectomies) of primary NSCLC for which patient follow-up was available were retrieved from the archives of The Methodist Hospital in Houston, Texas. A total of 467 specimens (5% wedge biopsies, 95% resections) with follow-up were available for study. The series included 270 AC, 130 SCC, and 67 LC. Pathology reports and cancer registry data were reviewed to determine tumor stage.

cD_1 Immunohistochemical Staining

After antigen retrieval by microwave irradiation in citrate buffer, sections were incubated overnight with the antibody used for the detection of cD_1 (purified mouse anti-human cD_1 mAB, clone G124–326, 1:500, San Diego, CA). Detection was by Vectastain Elite mouse IgG immunoperoxidase kit (Vector, Burlingame, CA). Diaminobenzidine was used as the chromagen, and the sections were lightly counterstained with hematoxylin. With each batch of samples, a positive control (colon) was evaluated.

cE Patient and Specimen Selection

Paraffin blocks from wedge biopsies and resections (segmentectomies, lobectomies, pneumonectomies) of primary NSCLC for which patient follow-up was available were retrieved from the archives of The Methodist Hospital in Houston, Texas. A total of 400 specimens (6% wedge biopsies, 94% resections) with follow-up were available for study. The series included 232 AC, 113 SCC, and 55 LC. Pathology reports and cancer registry data were reviewed to determine tumor stage.

cE Immunohistochemical Staining

After antigen retrieval by microwave irradiation in citrate buffer, sections were incubated overnight with the antibody used for the detection of cE (purified mouse anti-human cE mAB, clone HE12, 1:2500, San Diego, CA). Detection was by Vectastain Elite mouse IgG immunoperoxidase kit (Vector). Diaminobenzidine was used as the chromagen, and the sections were lightly counterstained with hematoxylin. With each batch of samples, a positive control (spleen) was evaluated.

Grading and Statistical Analysis

The number of tumor cells with nuclear and/or cytoplasmic immunopositivity was graded on a scale of: 0 = less than 1%, 1 = 1 to 10%, 2 = 10 to 25%, 3 = 25 to 50%, 4 = 50 to 75%, 5 = more than 75%. Histologic subtype, stage, and percent of tumor cells staining positively were correlated with survival using the Kaplan-Meier method. Significance was tested for by the log-rank (Mantel-Cox) method, and P values less than .05 were considered statistically significant. Stat-View software from Abacus Inc. was used for all statistical analyses.

RESULTS

cD_1 Staining

The patients ranged in age from 35 to 90 years at the time of diagnosis, with a median age of 64.6 years and a mean of 64.5 years. Lung cancer was known to be the primary cause of death in 88 patients. Thirty patients (7%) were nonsmokers. The male to female ratio was 263:163.

The cD_1 cytoplasmic staining results are summarized in Table 1. Overall, 48% of tumors demonstrated positive staining. The data was further subdivided by histologic subtype, stage, and percent of tumor cells staining positively (Tables 1 and 2). The immunostaining pattern was predominantly cyto-

TABLE 1. Cyclin D1 Immunohistochemical Staining Results

Dominant Cell Type	Total Number of Cases	Percent Scored Positive	Percent Scored Negative
Adenocarcinoma (all types)	270	56	44
Adenocarcinoma (not BACA)	204	56	44
BACA	66	55	45
Large cell carcinoma	67	49	51
Squamous cell carcinoma	130	30	70
Total	467	48	52

BACA, bronchioloalveolar carcinoma.

TABLE 2. Cyclin D1 Staining Results, Squamous Cell Carcinomas

Stage	Total	Score 0	Score 1	Score 2	Score 3	Score 4	Score 5
I	67	17	21	12	5	5	7
II	29	8	7	8	2	1	3
III	32	13	6	5	3	0	5
IV	2	1	1	0	0	0	0
Total	130	39	35	25	10	6	15

plasmic (Fig. 1); however, 29 tumors also demonstrated focal nuclear staining.

Four hundred twenty-six patients had adequate follow-up for examination by Kaplan-Meier survival analysis. For analysis purposes, follow-up was truncated at 4 years. Of the 426 patients, 96% were followed for at least 4 years. At 4 years, 214 patients were dead, 197 were alive, and 15 had been lost to follow-up. The only subset that generated a statistically significant *P* value was the group of all 130 SCC (all stages). This group showed a trend for better survival in cD_1-positive *versus* negative tumors ($P = .0902$). When follow-up was truncated at 5 years, this value dropped to $P = .0555$; when follow-up was further censored to 4 years, this became statistically significant ($P = .0246$, Fig. 2). The same trend was observed regardless of stage of SCC. No correlation between cytoplasmic staining score and grade or stage was observed. In fact, it was relatively consistent across such categories. Analysis of the 29 tumors that showed focal nuclear staining did not demonstrate a clear relationship to cell type, stage, grade, cytoplasmic staining, smoking history, or prognosis.

cE Staining

The patients ranged in age from 39 to 90 years at the time of diagnosis, with both the median and mean age being 64.2 years. Lung cancer was known to be the primary cause of death in 88 patients. Twenty patients (5.5%) were nonsmokers. The male to female ratio was 230:130.

The overall cE staining results are summarized in Table 3. Overall, 32% of tumors demonstrated positive staining in more than half of the cells. The data was further subdivided by histologic subtype, stage, and percent of tumor cells staining positively. Table 4 outlines the cE immunostaining results for SCC. The cE immunostain showed intense nuclear staining; no cytoplasmic staining was demonstrated (Fig. 3).

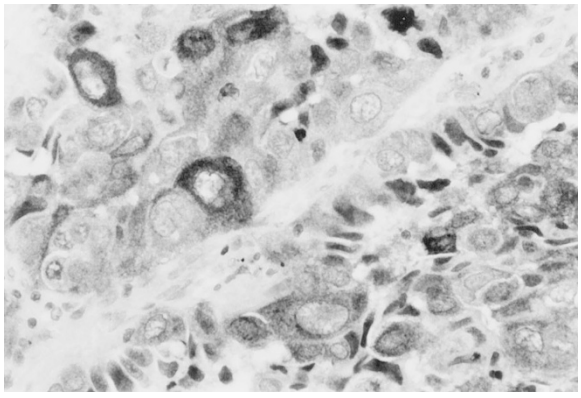

FIGURE 1. A squamous cell carcinoma demonstrating cytoplasmic staining for cyclin D_1 (original magnification, 40×).

Three hundred sixty cases had adequate follow-up for examination by Kaplan-Meier survival analysis. Follow-up was not truncated for this study set. Two hundred thirty-nine patients were followed until their death; the remaining 121 patients have been tracked to the present or have been lost to contact. The mean follow-up for these 121 patients was 82.6 mo, with a median of 76 mo. The only subset that generated a statistically significant *P* value was the group of all 70 stage I and II SCC. This group demonstrated significantly better survival in cases showing immunopositivity in greater than 50% of cells ($P = .029$, Fig. 4).

A seemingly contradictory trend was noted for cE positivity in 184 stage I and II AC (including bronchioloalveolar carcinomas). Comparing survival analysis for patients with negatively *versus* positively staining tumors, absence of staining showed a trend toward better survival ($P = .0647$). This *P* value is very close to statistical significance.

Conclusion

The results of our study strongly suggest that cD_1 and cE expression is associated with better prognosis in a human cancer, specifically NSCLC. The expression of cD_1 in our 130 pulmonary SCC demonstrates a trend toward increased patient survival outcome, which was statistically significant only at a relatively short (4 years) follow-up period. Long-term follow-up may achieve statistical significance when applied to a larger series of SCC cases. In early stage SCC, better survival was demonstrated in cases showing immunopositivity for cE in more than 50% of tumor cells. These results were statistically significant. The cD_1 and cE expression provided prognostic significance in SCC, but not tumors of other cell types.

Most of the previous studies involving cD_1 and cE have found overexpression of these cyclins in human tumors from various sites and suggested a link between overexpression and oncogenesis. Knowledge of the cell cycle provides a reasonable expla-

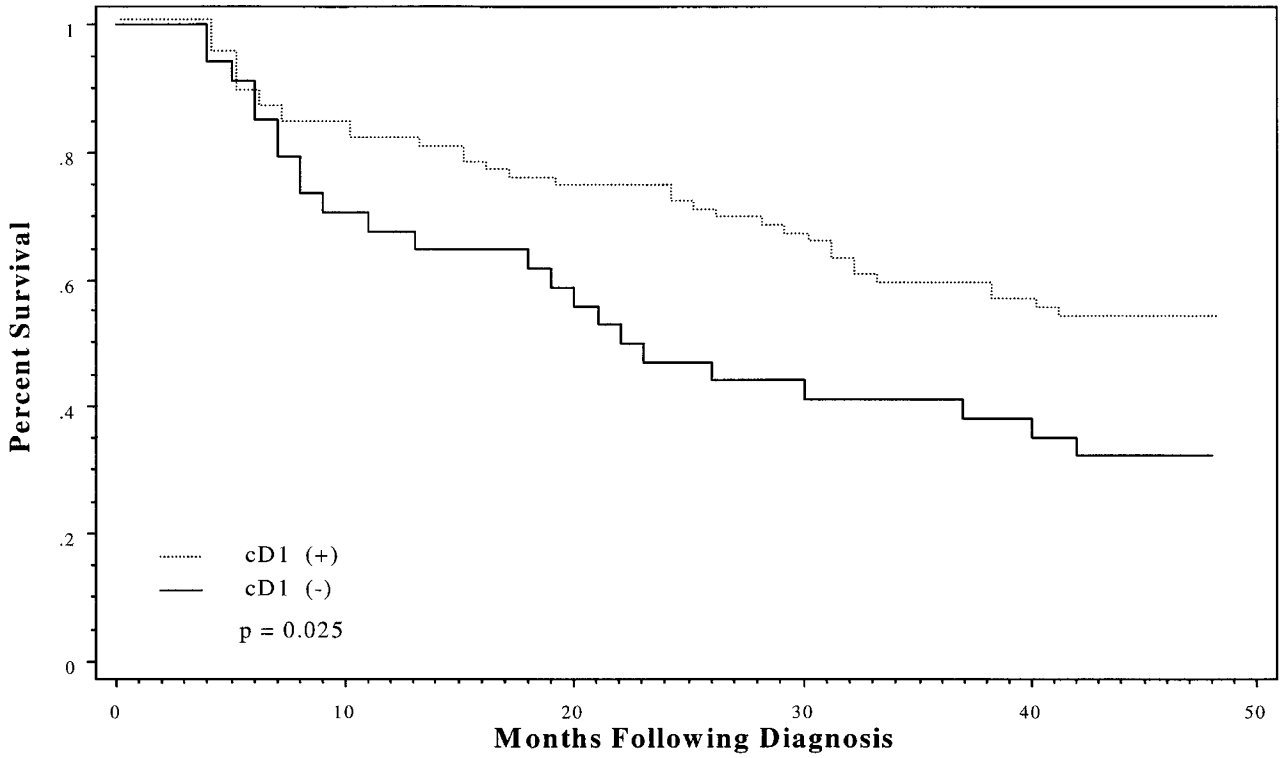

FIGURE 2. Graph of 130 squamous cell carcinomas (all stages) showing better survival in cD_1-positive *versus* negative tumors ($P = .0246$).

TABLE 3. Cyclin E Immunohistochemical Staining Results

Dominant Cell Type	Total Number of Cases	Percent Scored 0–3	Percent Scored 4–5
Adenocarcinoma (all types)	232	77	23
Adenocarcinoma (not BACA)	179	74	26
BACA	53	87	13
Large cell carcinoma	55	51	49
Squamous cell carcinoma	113	58	42
Total	400	68	32

BACA, bronchioloalveolar carcinoma.

nation to support these findings. The *retinoblastoma-1* (*Rb*) tumor suppressor gene produces a protein (pRB) thought to be the major repressor of G_1 phase progression (20). pRB is active in early G_1 in its hypophosphorylated form; in mid/late G_1, the protein becomes inactivated by phosphorylation. Cyclins D_1 and E, in association with their catalytic partners, the cyclin-dependent kinases (cdk), are responsible for this phosphorylation of pRB and regulate progression of the cell cycle from G_1 into S-phase (2). The protein product of *Rb* appears to inhibit cD_1 and cE promotor activity by binding E2F transcription factors. In mid-to-late G1, when pRB is initially phosphorylated, perhaps by cdk-cyclin D complexes, E2F transcription factors are released, which promote transcription of the cD_1 and cE genes (21, 22). Increased cD_1 and cE expression continues pRB phosphorylation, releasing additional E2Fs that continue cD_1 and cE transcription. Therefore, it is plausible that overexpression of cD_1 or cE heightens phosphorylation of pRB and promotes cellular proliferation by suppressing pRB activity. This concept of cD_1 and cE as positive regulators of cell proliferation is supported by studies that have demonstrated that microinjection of anti-cyclin D_1 and E antibodies prevent S-phase entry (3, 23, 24). The studies that have found overexpression of cD_1 or cE in tumors have postulated that cD_1 and cE are positively involved in tumorigenesis because increased levels of cD_1 and cE have been shown to promote cell growth by shortening the G_1 phase (3, 4). It is possible then that overexpression of these cyclins may contribute not only to tumorigenesis but also to clinical aggressiveness of a tumor.

However, our study has shown that cD_1 and cE immunopositivity confers a better prognosis in human lung cancers, and recent studies have raised the possibility that cD_1 actually functions as a negative regulator of cellular proliferation (3, 4, 25). We have already established the feedback mechanism between cD_1, cE, and pRB. Several studies have reported a lack of cD_1 expression in pRB-deficient tumors (21, 26). The absence of cD_1 in the higher stage tumors in our study may be related to mutations in the *Rb-1* gene. It is likely that mutations in *Rb-1* gene are more significant than those of the cyclins and further study involving cD_1 and *Rb-1* expression in concert is necessary to support this intriguing possibility. Additionally, this concept is supported by Lukas *et al.* (27), who reported that

TABLE 4. Cyclin E Staining Results, Squamous Cell Carcinomas

Stage	Total	Score 0	Score 1	Score 2	Score 3	Score 4	Score 5
I	58	2	8	12	16	9	11
II	25	1	2	3	7	7	5
III	28	2	3	6	3	7	7
IV	2	0	0	0	1	1	0
Total	113	5	13	21	27	24	25

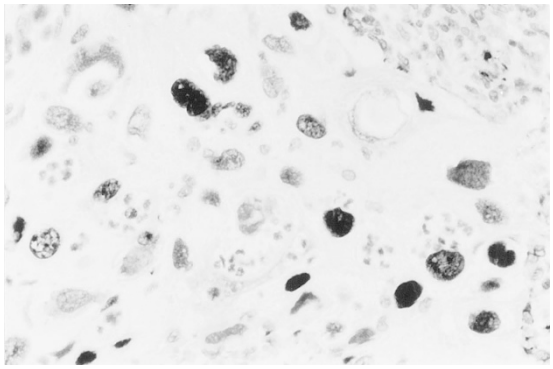

FIGURE 3. The cyclin E immunostain showing strong nuclear staining (original magnification, 20×).

microinjection of anti-cyclin D_1 antibodies accelerates S-phase entry in pRB-deficient mouse embryonic fibroblasts. The results of this enticing study are in accord with our findings that suggest lack of cD_1 expression promotes cell proliferation.

Another possible explanation for our findings concerns the amount of cD_1 and the timing at which these levels occur. It has been demonstrated that high levels of cD_1 occurring at the G_1/S phase junction arrest S-phase entry (3, 23, 28), suggesting that the amount and timing of cD_1 can exert different effects on cellular proliferation. For unknown reasons, it appears that cD_1 can be either a positive or negative regulator of cellular proliferation in different situations. Additionally, cD_1 has been shown to accumulate in senescent, differentiated cells, which may explain why cD_1 immunopositivity was present in the better-differentiated tumors in our study (29, 30). In summary, the potential oncogenic properties of cD_1 *in vivo* are unclear.

Interestingly, our study reveals a seemingly contradictory trend for early stage AC. Tumors showing absence of staining for cE demonstrated a trend toward better survival. This is in agreement with other studies

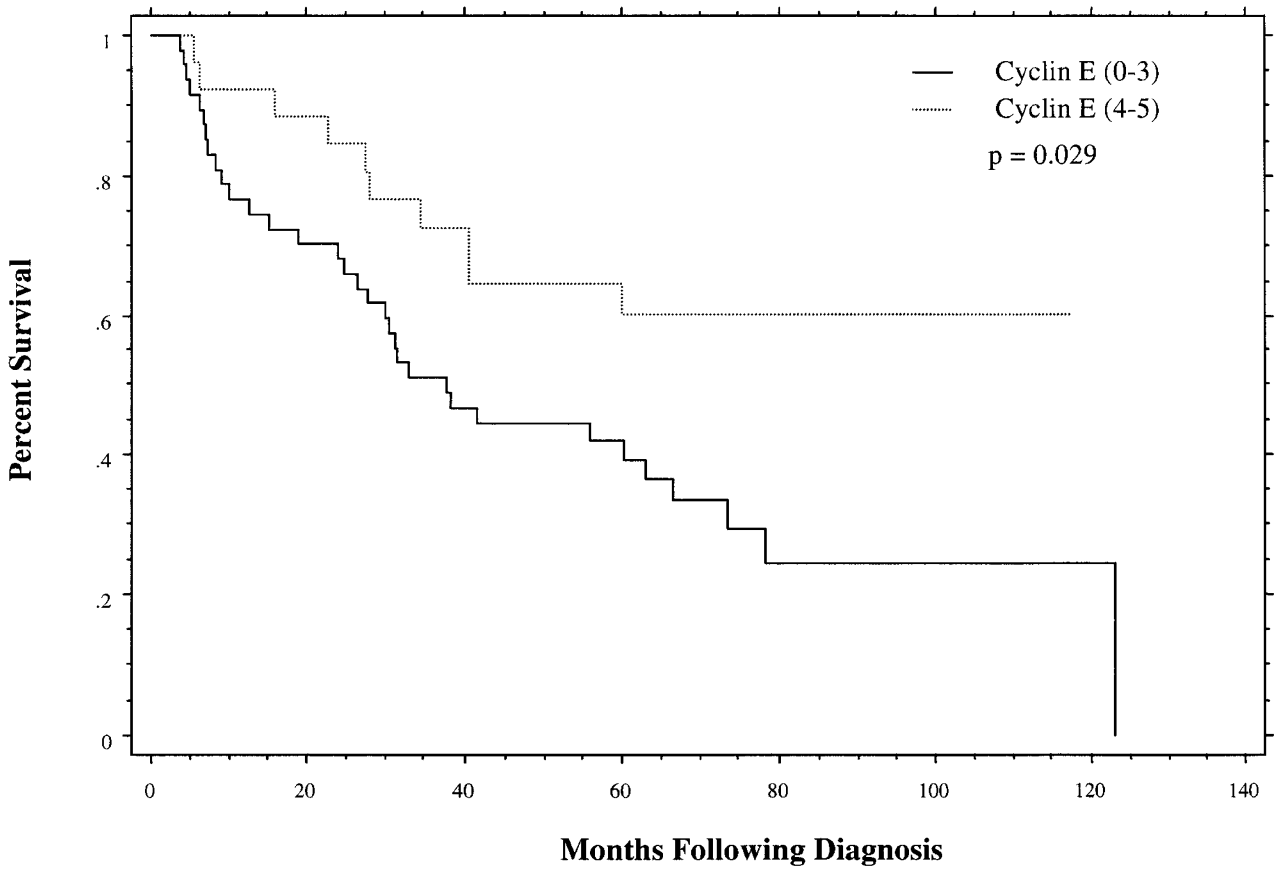

FIGURE 4. Graph of all 70 stage I and II squamous cell carcinomas demonstrating better survival for cases in which more than 50% of the tumor cells showed immunopositivity (P = .029).

that have demonstrated increased cE expression in increasing grade and stage of AC of the breast (19).

The cyclins and pRB are only two components of a complex regulatory pathway. p16 is a member of a family of regulatory proteins that compete with cD_1 for the binding of cdk 4 and 6 (20). Cellular growth factors determine the amount of cdk 4 or 6 associated with cD_1 or p16, thereby determining phosphorylation of pRB and cell proliferation. Additionally, cD_1 appears to be regulated by another proto-oncogene, *c-myc*. Studies have shown *Myc* can induce cD_1 transcription and that ectopic *Myc* expression suppresses cD_1 expression in cells that lack functional pRB (25, 31).

It has been difficult to identify individual cell cycle regulatory proteins to serve as reliable markers for prognosis in human lung cancer because of inconsistencies in studies in the literature. Our findings underscore the need to evaluate more than one protein that may affect the same pathway in order to predict prognosis. Because of these complex interactions, a molecular profile consisting of a battery of potential aberrant proteins for an individual lung cancer may be much more dependable in predicting long-term prognosis.

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