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Cyclin D1 overexpression vs. retinoblastoma inactivation: Implications for growth control evasion in non-small cell and small cell lung cancer

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ABSTRACT **The cyclin-dependent kinases and their associated regulatory cyclins control cell cycle progression and cell growth. Antibodies against these proteins were used to determine their levels in several lung tumor-derived cell lines and a "normal" immortalized bronchoepithelial cell line in order to investigate their potential roles in the etiology of lung cancer. All the cell lines expressed roughly equal levels of cdk-1; cdk-2; PSTAIRE-sequence containing kinases; proliferating cell nuclear antigen; and cyclins A, B1, and E. Cyclin D1, however, was present at 4- to 100-fold higher levels in 11 of 12 non-small cell lung cancer cell lines than in the bronchoepithelial line and all but one of the small cell lung cancer lines. Furthermore, immunoblots of the retinoblastoma gene product, pRB, revealed a perfect correlation between pRB levels and tumor type with normal levels of phosphorylation-competent pRB in all of the non-small cell lung cancer lines and undetectable levels of pRB in all of the small cell lung cancer lines. These data suggest the possibility that small cell and non-small cell lung cancer may evade normal growth controls by different mechanisms: loss of the proliferation inhibitor pRB in small cell lung cancer and overexpression of the growth promoting cyclin D1 in non-small cell lung cancer.**

It has been several years since cyclins and cyclin-dependent kinases (cdk) were identified as regulators of the eukaryotic cell cycle. The discovery of cyclins and the identification of *Xenopus* maturation-promoting factor as a heterodimer of cdc2 kinase and cyclin B established the role of cyclins and cdk as regulators of mitosis (reviewed in ref. 1). Since then the story has become increasingly complicated. The identified cyclins and cdk now include cyclins A, B1–2, C, D1–3, E, F, and G and cdk1–6 (reviewed in refs. 2 and 3), and the patterns of cyclin expression cover the cell cycle in such a way that cyclin–cdk complexes could regulate every aspect of cell cycle progression. In fact the most complicated array, including the C-, D-, and E-type cyclins, now appears to be involved in progression through G_1 (3). Since this is the stage at which virtually all mammalian growth control occurs, the cyclins have recently come under scrutiny as potential oncogenes.

Much of this interest has focused on the D-type cyclins. Cyclin D1 was originally identified by three very different approaches. It was identified as a suppressor of yeast G_1 cyclin mutations (4, 5), as a gene whose expression is induced in mouse macrophages by colony-stimulating factor (6), and as PRAD1 (7, 8) and BCL1 (9, 10), genes that are overexpressed due to translocations in certain parathyroid and B-cell neoplasms, respectively. These three approaches suggest that besides being a functional cell cycle regulator, cyclin D1 may act as a growth factor response element and an oncogene. It has since been shown that human D-type cyclins are synthesized in response to growth factor stimulation and downregulated upon serum starvation (11–13), that reduction of cyclin D1 activity by antisense RNA or antibody blocks the cell cycle in G_1 (14, 15), and that cells overexpressing cyclin D1 exhibit an abbreviated G_1 phase and a reduced serum requirement (11, 15, 16). Thus, the D-type cyclins do appear to be involved in progression through G_1 and in mitogen response.

Further evidence that cyclins could be involved in loss of growth control in cancer comes from study of the retinoblastoma tumor suppressor gene (*RB*). Homozygous loss of the *RB* gene can result in malignancy, and defects in its expression are found in a number of different cancers (for example, see refs. 17–22). The *RB* gene product, pRB, undergoes a series of phosphorylation and dephosphorylation events in the course of the cell cycle (reviewed in ref. 23). During G_0 and G_1 pRB is present in hypophosphorylated or unphosphorylated forms that are able to block entry into S phase. Hyperphosphorylation by certain cyclin–cdk complexes inactivates pRB, allowing cells to proceed through the cell cycle. Although the exact mechanism is unclear, the A, D, and E cyclins are all able to overcome pRB-mediated G_1 arrest (24, 25) and, therefore, have the potential to overcome normal cellular growth controls.

While these results suggest that cyclins could act as oncogenes, other recent results have directly implicated cyclin overexpression in oncogenesis. Overexpression or amplification of cyclin D1 has been demonstrated in a variety of cancers including B-cell leukemias; centrocytic lymphomas; and esophageal, squamous cell, colorectal, hepatocellular, and breast carcinomas (7, 26–30). Cyclin D2 overexpression due to proviral insertion is common in murine leukemia virus-induced T-cell leukemia (31). Cyclin A is a target of hepatitis B virus integration in hepatocellular carcinoma (32), and cyclin E expression is elevated or altered in some breast and colorectal carcinoma cell lines (29, 33).

Little is known about the role of cyclins and cdk in lung oncogenesis. We have used antibodies against a variety of cell cycle proteins to determine their levels in an immortalized normal bronchoepithelial cell line and several lung tumor-derived cell lines. The primary division of lung cancer into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) is based on morphological differences, but the two types also differ clinically in terms of prognosis and chemosensitivity and biochemically in terms of the markers they express (34, 35). Our results suggest that a further difference may exist in the mechanism by which these tumors evade normal growth controls and that cyclin D1 overexpression may play a role in the etiology of NSCLC.

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Abbreviations: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; PCNA, proliferating cell nuclear antigen.

MATERIALS AND METHODS

Cell Lines and Reagents. Cell lines were obtained from the University of Colorado Cancer Center Tissue Culture Core Facility. The NCI lines were originally established at the National Cancer Institute, Bethesda, MD (36); NU6, NE18 (37), and UCLC11 were established at the University of Colorado Health Sciences Center, Denver; and UMC19 was established at the State University of New York Health Science Center (38). BEAS-2B (39) was a gift from Karen Kelly (University of Colorado Health Sciences Center).

Antibodies were obtained from the following sources: anti-PSTAIRE kinase, cdk1, cdk2, cyclin A, cyclin B1, and cyclin D1 were from Upstate Biotechnology, Lake Placid, NY; anti-cyclin E was from Pharmingen; anti-pRB was a gift from Wen-Hwa Lee (University of Texas Health Science Center, San Antonio); anti-proliferating cell nuclear antigen (PCNA) was a gift from Wilbur Franklin (University of Colorado Health Sciences Center); and horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad.

Cell Extracts. Unless otherwise indicated cell lines were cultured in 175-cm^2 flasks in RPMI 1640 medium with 5–10% fetal calf serum (FCS). Some lines (H324, H372, H720, H748, and H1048) were grown in RPMI 1640 medium with 10 nM hydrocortisone/5.0 μg of insulin per ml/100 μg of transferrin per ml/10 nM 17β-estradiol/30 nM sodium selenite and 2–5% FCS. Cells were harvested at 50–80% confluence and washed in phosphate-buffered saline (PBS). Aliquots were removed for determination of protein concentration and for fluorescence-activated cell sorter (FACS) analysis by the University of Colorado Cancer Center Flow Cytometry Core to determine cell cycle distribution. The remaining cells were resuspended in Laemmli sample buffer (40) at 1–4 $\times$ 10^7 cells per ml, immediately boiled for 5 min, sheared through a syringe needle to reduce viscosity, aliquoted, and stored at −80°C.

Western Analysis. Volumes of cell extracts corresponding to ≈50 μg of total protein were subjected to SDS/PAGE (40). Proteins were transferred according to the manufacturer's instructions to Immobilon P (Amersham) transfer membranes for 45 min at 0.5 A on a Genie Electroblotter (Idea Scientific, Minneapolis) and immunoblotted using the Amersham enhanced chemiluminescence kit. Film exposures ranged from 2 sec to 1 hr depending on the primary antibody. Bands were quantitated on a Molecular Dynamics computing densitometer using IMAGEQUANT software.

RESULTS

Cyclins and cdk in Lung Tumor Cell Lines. The lung cancer cell lines and tumor types from which they were derived are listed in Table 1. Cultures were harvested at 50–80% confluence to ensure that the extracts reflect a growing population of cells. FACS analysis was performed (data not shown) to verify this and to allow differences in cell cycle protein levels resulting from differences in cell cycle distribution to be recognized. Cell cycle distributions did vary, but the percentage of cells in S phase usually fell between 20% and 40%.

Volumes of extracts corresponding to roughly equal amounts of protein were electrophoresed and immunoblotted with anti-cell cycle protein antibodies (Fig. 1*A*). Immunoblots revealed essentially constant levels of 33- to 34-kDa cdk containing the trademark PSTAIRE sequence (at least cdk1, -2, and -3). Specific antibodies gave the same result for cdk1 (also known as cdc2) and cdk2. Also at constant levels were PCNA (41, 42) and cyclins A and B1, cyclins specific to S phase and M phase, respectively. With the exception of one overexpressing line, H345, the levels of the G_1/S-specific cyclin E were also fairly constant. Cyclin E exists during the cell cycle as at least two distinct species—a 51-kDa G_1/S form and a 55-kDa G_2/M form (43). Differences in the distribution of these species among cell lines are apparent and do not correlate with differences in cell cycle distribution (data not shown). While these differences may be intrinsic to the cell lines, the lack of reproducibility in multiple extracts from the same cell lines (data not shown) argues against this possibility. We have also looked at cyclins D2 and D3 and find no detectable D2 and very low levels of D3 in all of the lines tested (data not shown). These results are consistent with the current literature regarding tissue specificity of D-type cyclin expression (11, 13).

Table 1. Cyclin D1 and pRB expression in SCLC- and NSCLC-derived cell lines

Tumor type	Cell line	Cyclin D1	pRB
Small cell	NCI-H69 (2)	−/±	−
	NCI-H345 (3)	−	−
	NCI-H372 (2)	−/±	−
	NCI-H446 (1)	−	−
	NCI-H524 (2)	+	−
	NCI-H526 (2)	±	−
	NCI-H748 (1)	−	−
	UMC19 (1)	−	−
	NCI-H1048 (1)	−	−
Non-small cell			
Adenocarcinoma	NCI-H322 (1)	+	+
	NCI-H324 (1)	+	+
	NCI-H441 (3)	++	+
	NCI-H2122 (2)	++	+
Large cell	NCI-H460 (4)	±/+	+
	NCI-H661 (1)	±	+
	UCLC11 (1)	++	+
Mesothelioma	NCI-H28 (1)	+	+
	NCI-H290 (1)	++	+
Squamous	NCI-H157 (2)	++	+
	NU6 (2)	+	+
	NE18 (2)	+	+
Pulmonary carcinoid			
Atypical	NCI-H720 (1)	−	−
Typical	NCI-H727 (1)	+	+

Parentheses indicate the number of extracts of each cell line tested.

Clearly the levels of the proteins described above are not completely constant. Some lines were low (e.g., H1048) or high in all proteins tested, reflecting differences in protein loading or in the fraction of proliferating cells. Other differences such as the relatively high level of cyclin A in H661 and the low level of cyclin A in H460 may reflect real differences between lines but show no tumor type pattern and include apparent under- and overexpression. We therefore conclude that cyclins A, B1, and E; PCNA; and the typical cdk are generally expressed at constant levels in the lines tested and did not play a significant role in the progression of the tumors from which these lines, with the possible exception of H345, are derived.

Overexpression of Cyclin D1 in NSCLC Lines. In contrast to the above proteins, cyclin D1 levels varied dramatically in a reproducible, tumor-type-specific manner. As shown in Fig. 1*A* and summarized in Table 2, high levels of cyclin D1 were detected in 11 of the 12 NSCLC lines, including lines from 4 subtypes of NSCLC. In contrast only 1 of 9 SCLC lines showed a high level of cyclin D1, while 5 of 9 and an extrapulmonary small cell line contained no detectable cyclin D1. Two other lines, H720 and H727, were derived from pulmonary carcinoid tumors, a poorly defined and variable class of neuroendocrine tumors that can resemble SCLC or the large cell subtype of NSCLC (44). H720, an atypical carcinoid line with the small cell morphology and floating aggregate growth pattern typical of a SCLC line, contained no detectable cyclin D1, while H727, a typical carcinoid with

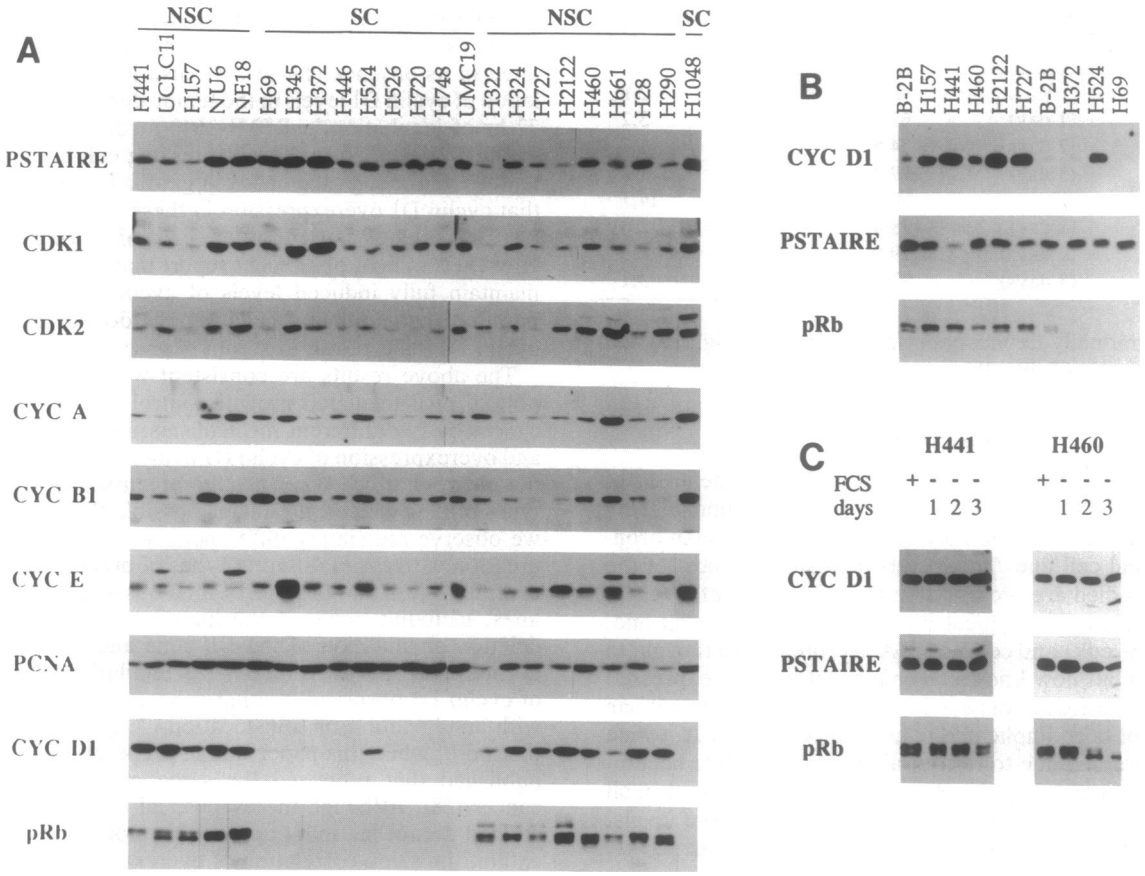

FIG. 1. Immunoblot analysis of cdk, cyclin, and pRB levels in lung tumor-derived cell lines and a normal immortalized bronchoepithelial cell line. Extracts were prepared from the indicated cell lines and subjected to SDS/PAGE. Proteins were then transferred to membranes and blotted with the indicated antibodies. (*A* and *B*) The indicated cell lines were grown in the medium specified in *Materials and Methods*. (*C*) Cells were incubated in RPMI 1640 medium with or without 10% FCS as indicated.

the morphological and growth properties of a NSCLC line, contained high levels of cyclin D1.

In light of evidence that D-type cyclins may regulate pRB (24, 25) and that perturbations in pRB expression are common in SCLC (17–20, 22), we were also interested in pRB levels. Anti-pRB immunoblots revealed a perfect correlation between tumor type and pRB levels (Fig. 1*A*). All the NSCLC lines and the NSCLC-like carcinoid line had equal levels of pRB of normal molecular weight that migrated as a doublet of hypo- and hyperphosphorylated species. In contrast, none of the SCLC lines nor the atypical carcinoid line had detectable levels of pRB. Thus, with the apparent exception of H661, all cell lines with normal levels of pRB also express high levels of cyclin D1 (Table 1).

Cyclin D1 Levels in NSCLC Lines Are High and Unregulated. The high levels of cyclin D1 in NSCLC lines relative to SCLC lines could be interpreted as elevated levels in the former or as reduced levels in the latter. To address this question directly we obtained a "normal" cell line, BEAS-2B, derived from normal human bronchoepithelial cells but immortalized by infection with a hybrid adenovirus–simian virus 40. It is partially transformed but noninvasive, essentially nontumorigenic, and able to repopulate normally a deepithelialized rat trachea (39). Comparison of BEAS-2B extracts to fresh extracts of some of the tumor lines on immunoblots reproduced the earlier results for the tumor lines and revealed normal pRB and a detectable but low level of cyclin D1 in BEAS-2B cells (Fig. 1*B*). The levels of all other proteins tested in Fig. 1*A* were the same in BEAS-2B as in the tumor lines (Fig. 1*B*; data not shown). Quantitation by densitometry and normalization to PSTAIRE kinase levels indicated that the NSCLC lines contained from 4-fold (H460) to >100-fold (H441) higher levels of cyclin D1 than BEAS-2B. By comparing relative levels of cyclin D1 in Fig. 1*A*, we conclude that all of the NSCLC cell lines with the possible exception of line H661 overexpress cyclin D1 relative to the normal line. However, it also appears that the cyclin D1 levels in the SCLC lines with no detectable cyclin D1 are reduced relative to the normal cell line and that both of the above interpretations may apply.

Table 2. Summary of cyclin D1 expression in SCLC- and NSCLC-derived cell lines

Tumor type	Negative	Low	M–H
Small cell	5/9	3/9	1/9
Non-small cell			
Adenocarcinoma	0/4	0/4	4/4
Large cell	0/3	1/3	2/3
Mesothelioma	0/2	0/2	2/2
Squamous	0/3	0/3	3/3
Total	0/12	1/12	11/12
Pulmonary carcinoid			
Atypical	1/1	0/1	0/1
Typical	0/1	0/1	1/1

M–H, moderate to high.

As described earlier, D-type cyclins in normal cells are regulated by growth factors and their levels drop rapidly upon serum starvation. When extracts were prepared from several cell lines grown in the presence and absence of serum, immunoblots revealed that the high levels of cyclin D1 in four NSCLC lines and in the one overexpressing SCLC line were not significantly affected by 1–3 days of incubation in the absence of serum (Fig. 1*C*; data not shown), although the

Table 3. Cell cycle distributions for populations in Fig. 1*C*

Cell line	Serum	% G_0/G_1	% S	% G_2/M
H441	+	45.2	51.0	2.8
H441	− (1 day)	67.6	29.1	3.2
H441	− (2 days)	74.5	23.7	1.7
H441	− (3 days)	79.1	19.5	1.2
H460	+	50.3	35.0	14.7
H460	− (1 day)	48.0	40.0	12.0
H460	− (2 days)	79.9	12.6	7.5
H460	− (3 days)	92.5	4.4	3.1

cells did gradually dephosphorylate their pRB (Fig. 1*C*) and arrest in G_0/G_1 (Table 3).

DISCUSSION

In this study immunoblots were used to look at the levels of various cell cycle proteins in a collection of human lung cancer cell lines and one normal immortalized human bronchoepithelial cell line. The results indicate that most of the proteins studied are present at essentially constant levels in all lines tested. The typical PSTAIRE-containing cdk and, specifically, cdk1 and cdk2; PCNA, a protein once thought to be a cyclin but now known to be a constitutively expressed DNA polymerase factor; cyclin B1, a mitotic cyclin and one that has not been implicated in any cancer; cyclin A, which is overexpressed due to retroviral insertion in some hepatocellular carcinomas (32); and cyclin D3 were constant in all cell lines. Cyclin D2 was not present at detectable levels. Finally, cyclin E was overexpressed in one SCLC line, H345, but constant in all others. Cyclin E overexpression has been implicated in other types of cancer (29, 33), but the fact that H345 was an isolated example in our study argues against a general role for cyclin E overexpression in lung cancer progression. Overall we conclude that perturbations of the levels of PCNA; typical cdk; and cyclins A, B1, D3, and E are not generally involved in the etiology of lung cancer.

Cyclin D1, however, provided a sharp contrast to the above results, varying dramatically in different cell lines in a tumor type-specific manner. At least 11 of the 12 NSCLC-derived lines as well as one NSCLC-like carcinoid line overexpressed cyclin D1 relative to an immortalized, but nontumorigenic human bronchoepithelial line and all but one of the SCLC-derived lines. Levels in the NSCLC lines were 4- to 100-fold higher than normal, while levels in the SCLC lines were depressed relative to the normal line.

Because of the evidence for regulation of pRB by cyclin D1 and the many reports of pRB inactivation in SCLC, we also looked at the levels of pRB. The result was a perfect correlation between pRB level and tumor type. All of the NSCLC lines expressed a constant and normal level of phosphorylation-competent pRB, while none of the SCLC lines contained detectable pRB. Thus, all of the lung tumor-derived cell lines that express normal levels of pRB are NSCLC-derived lines and also overexpress cyclin D1. Conversely, all of the lines containing reduced levels of cyclin D1 are SCLC lines and are lacking in pRB. NSCLC lines and tumors that lack pRB, as well as SCLC lines with apparently normal pRB, are in the minority but have been reported (17–19, 22, 45). We hope to look at cyclin D1 levels in these lines in the future. We note also that we have not directly demonstrated that the pRB detected in our NSCLC lines is active. However, since all of the characterized RB structural mutations in the literature that affect pRB activity result in loss of either pRB protein or its phosphorylation competence (for examples and discussion, see refs. 19 and 46), we assume that these NSCLC lines do contain active pRB.

Since cyclin D1 has been shown in hematopoietic and fibroblast cell lines to be induced by serum and downregulated in its absence (11–13), we also looked at serum regulation of cyclin D1 levels in the tumor-derived cell lines. After 72 hr of incubation in RPMI 1640 medium without serum, cyclin D1 levels in the overexpressing cell lines were unaffected even though the cells did gradually arrest, suggesting that cyclin D1 overexpression in these lines is accompanied by, or perhaps caused by, a loss of normal regulation. However, in light of a recent report that resting hepatocytes maintain fully induced levels of cyclin D1 (47), it is also possible that epithelial cells simply do not downregulate cyclin D1 in response to serum deprivation.

The above results are consistent with a model in which normal pRB-mediated growth control is overcome in lung cancer by two different mechanisms: loss of pRB in SCLC and overexpression of cyclin D1 in most NSCLCs. Since it is not clear what cell types are the precursors for the various types of lung cancer, it remains possible that the differences we observe are simply differences in the characteristic expression patterns of different types of precursor cells. However, it is clear that the lack of pRB observed in many SCLC lines, including some of the lines in this study, is due to deletion or mutation of the *RB* gene and not simply a low normal level of expression (17–19). Similarly, overexpression of cyclin D1 observed in other cancers is clearly associated with amplification or translocation (7, 26, 27, 29, 30). Furthermore, while this paper was in preparation, a report was published that proposed the same model for esophageal cancer (28). Although the esophageal tumors and cell lines studied do not fall into apparent histological types, they do exhibit the same correlation of overexpression of cyclin D1 with normal pRB in some tumors and of low cyclin D1 with a lack of pRB in others. Thus, the proposed model may be a general one for loss of normal growth control in cancers of many types.

The mechanistic details of cyclin D1 overexpression and the overriding of pRB-mediated growth inhibition remain unclear. We have used the term overexpression to refer to an elevated steady state level of cyclin D1 protein. Since cyclin D1 is part of a complex, highly regulated growth control pathway, it seems likely that altered expression could be achieved by a variety of mechanisms. In fact, the overexpression of cyclin D1 in breast cancer tumors mentioned earlier was found to result in different tumors from different mechanisms including DNA amplification and apparent transcriptional and posttranscriptional mechanisms (26). The question of how cyclin D1 overcomes pRB growth inhibition is a controversial one, though it is clearly capable of doing so (24). Possible mechanisms include pRB inactivation by direct or indirect phosphorylation or by direct binding of cyclin D1 to pRB. A further possibility that has been proposed is that cyclin D1 has its own growth-promoting activity independent of pRB and may itself be regulated by pRB (25).

It is perhaps important to note that so far we have looked only at tumor-derived cell lines and not at primary tumors. It remains a formal possibility that the differences we see are artifacts of establishing a cell line. It seems unlikely, however, that such a strict tumor type correlation would be seen for events occurring in the cell lines and not in the original tumor progression. Furthermore, the RB data are supported by other work with primary SCLC tumors (17, 19, 20), and the similar observations of cyclin D1 overexpression vs. loss of pRB in esophageal cancer were made primarily with tumor tissue (28).

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