Tumor suppressor genes in human lung cancer

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Abstract : Lung cancer is the most common cause of cancer death in Japanese males, the incidence having increased markedly in recent years. Carcinogen exposure such as to tobacco-smoke and air pollution are associated with the probability of developing lung cancer. Aquired somatic mutations play an important role in the pathogenesis of environmentally induced lung cancers. Cytogenetic and molecular analysis of lung tumors has made it possible to examine this hypothesis and to search for candidate genes that may be targeted by chronic exposure to these carcinogens. Early studies implicate several distinct chromosomal loci (3p, 9p, 13q, 17p, and others) and suggest sequential genetic events occur during the initiation and progression of lung carcinogenesis. Several suppressor genes including *Rb* (13q), *P53* (17p), and *P16* (9p) have been identified and cloned at these chromosomal loci. The identification of putative tumor suppressor gene at chromosome 3p is still under work. Understanding the interaction of P53, RB, cyclins, and protein kinase inhibitors including P16 will be essential to the development of the next generation of diagnostic and therapeutic studies for lung cancer. J. Med. Invest. 44 : 15-24, 1997

Key Words : *RB, P53, P16, lung cancer, tumor suppressor gene*

INTRODUCTION

Lung cancer is the most common cause of cancer death in Japanese males, with approximately 70,000 new cases reported in 1996. There is currently no doubt that the use of tobacco products is the single most important causative factor in the development of lung cancer, although other environmental factors including air pollution may play a role. While the past 20 years has not yielded significant improvements in the diagnosis or treatment of lung cancer, this period has experienced an extraordinary expansion in the understanding of the molecular oncogenesis of lung cancer (1, 2). Investigations have strongly supported a multistep mechanism of tumor initiation and progression, induced by gene alterations (Fig.1). One search for a genetic basis of lung cancer has involved studies of alterations in the expression of certain dominant oncogenes or proto-oncogenes such as *myc* and *ras* gene family members (3-8). Activation of dominant oncogenes (by gene amplification, rearrangements, or somatic mutations) occurs in a number of bronchial epithelial cells. Another search for genetic causes of lung cancer has been the clarification of chromosomal deletions present in lung cancer cells, and the subsequent use of this information to identify cellular genes that locate at those regions and that might function in what has been referred to as tumor suppressor genes or recessive oncogenes (9-12), which is the main theme of this review article.

The concept of human tumor suppressor gene has its origins in the paradigm of familial childhood tumors such as Wilm's tumor and familial retinoblastoma. Although family studies of kindreds susceptible to these tumors demonstrated a dominant Mendelian pattern of inheritance, a recessive model of tumorigenesis was postulated by Knudson in 1971 (13, 14). In the

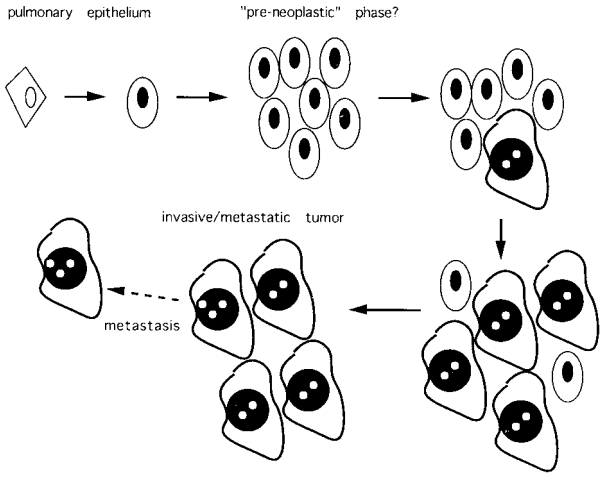

Fig.1. Multistep genetic model for lung cancer. Each step (solid arrow) progresses by somatic mutations (occurring from carcinogen exposure) or autocrine/paracrine effects of growth factors. Somatic mutations include chromosomal deletion, chromosomal rearrangement, subtle structural mutation, and gene amplification. These somatic mutations activate dominant oncogenes (*ras*, *myc*, others) or inactivate tumor suppressor genes (*Rb*, *P53*, *P16*, putative tumor suppressor gene at chromosome 3p, others).

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case of retinoblastoma, epidemiologic data suggested there was the clinical difference between the familial and the sporadic forms of retinoblastoma. In familial retinoblastoma, retinal tumors developed earlier (occasionally present at birth) than in sporadic retinoblastoma. In addition, on average three foci of tumor were identified in patients with the familial retinoblastoma (often resulting in bilateral disease), whereas in patients with sporadic retinoblastoma, only one focus of tumor was observed. Based on these observations, Knudson proposed the existence of a retinoblastoma susceptibility gene (*Rb*), and he proposed that one allele of this gene is inactivated in the germ-line cells of patients with familial retinoblastoma (Fig.2). The germ-line inactivation could have been inherited from one of the parents or could have arisen *de novo* during embryogenesis. The result is that every cell in that case would have one inactivated allele of the *Rb* gene ; however, such cells would be otherwise phenotypically normal. Only one somatic mutation in the remaining allele of the *Rb* gene would be required to inactivate gene expression and consequently result in tumor formation in retina. In contrast, in sporadic cases, two independent somatic mutations would have to occur in the same gene and in the same cell. The probability of this event would be much less likely, thus accounting for the later onset and the finding of only one focus of retinal tumor. This hypothesis was supported several years later with the identification of cytogenetic abnormalities at chromosome 13q14 in a small percentage of patients with familial retinoblastoma (15). This was confirmed and expanded to a large proportion of retinoblastoma cases when more sensitive techniques including restriction fragment length polymorphism (RFLP) were employed to test for subtle chromosomal deletions at the molecular level (16). This indirect evidence further supported the possibility of an *Rb* gene whose tumorigenic potential would be unmasked by its inactivation through structural mutations such as a deletion. Finally, in 1986 and 1987, three groups (17-19) identified a cDNA encoding a gene at chromosome 13q14 with characteristics of a retinoblastoma susceptibility gene as initially predicted by Knudson. Subsequently, several tumor suppressor genes have been found to be mutated and inactivated in hereditary cancer-prone disorders (Table 1).

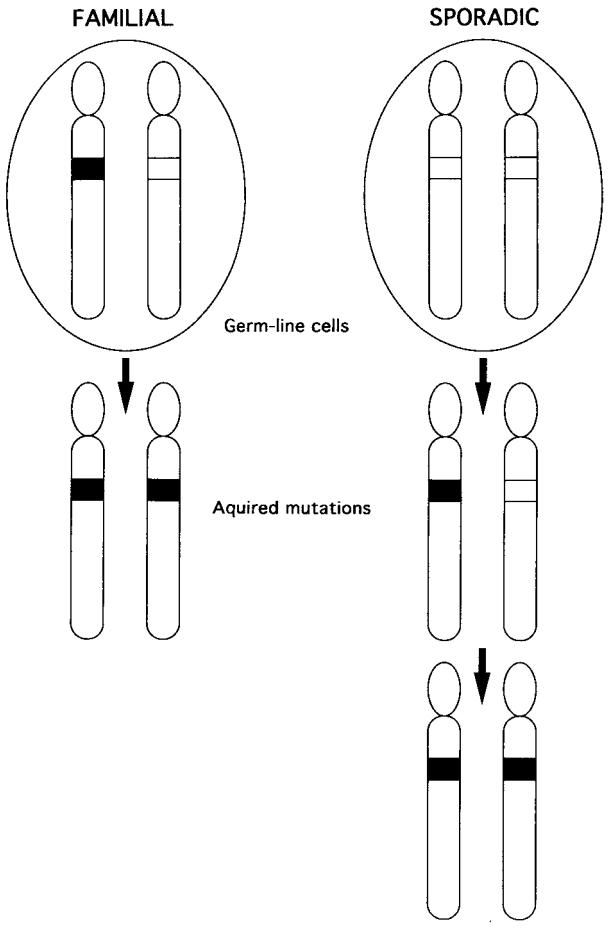

Fig. 2. Two-hit model for tumor suppressor genes. A mutation resulting in the inactivation of a tumor suppressor gene, represented by a closed square. Sporadic tumors evolve from the clonal expansion of a cell that has undergone two independent somatic (aquired) mutations, while familial tumors require only a single somatic mutation in the remaining allele.

CHARACTERISTICS OF SMALL CELL LUNG CANCER (SCLC) AND NON-SMALL CELL LUNG CANCER (NSCLC)

Lung cancer has been conveniently separated into two major categories (designated as small cell or non-small cell lung cancer) on the basis of clinical behavior and histologic appearance under light microscopy (Table 2) (19-23). Small cell lung cancer (SCLC) constitutes about 20% of all cases of lung cancer and is an aggressive lung tumor with a high propensity for disseminated spread throughout the body. As a result, surgical resections of SCLC are rare, limiting the ability of the investigator to obtain primary tumor sample. Although SCLCs are initially sensitive to anticancer chemotherapy and/or radiotherapy, with a tumor reduction seen in almost all treated cases, the disease usually recurs, at which time it is usually resistant to any further treatments. The appearance of SCLC as a small cell with pyknotic nuclei (oat cell or lymphocytic appearance) is likely a crush artifact from the bronchoscopic biopsy, while well-preserved tissue sections show larger epithelial cells with a fine nuclear chromatin pattern. Nonetheless, the histologic appearance of SCLC is characteristic and remains the only finding to establish the diagnosis.

A major advance in the study of the biology of lung cancers was the ability to generate many continuous cell lines derived from biopsies of patients with lung cancer. Cell lines obtained from SCLC specimens characteristically grow as tight clusters of 20 to several hundred cells that nonadherently float in liquid culture medium, making it difficult to obtain viable single cell suspensions (Fig.3) (23). Early biochemical investigations confirmed the observation that SCLC is a neuroendocrine tumor characterized by neurosecretory granules and the production of several neuron-specific enzymes and peptides, including dopa-decarboxylase

Table 1. Hereditary tumors and affected genes

	gene	chromosomal locus
Retinoblastoma	*RB1*	13q
Wilm's tumor	*WT1*	11q
Familial polyposis coli	*APC*	5q
Li-Fraumeni syndrome	*P53*	17p
von Recklinghausen neurofibromatosis	*NF1*	17q

Table 2. Characteristics of SCLC and NSCLC

	SCLC	NSCLC
Clinical		
Method of diagnosis	Light microscopy	Light microscopy
Distribution of cases	20%	80%
Primary treatment	Chemo (Radio) therapy	Surgery
Dissemination at diagnosis	Frequent	Less frequent
Paraneoplastic syndromes (Ectopic peptide secretion)	Common	Uncommon
Growth in tissue culture	Nonadherent clusters	Adherent monolayer
Biochemical		
Neuroendocrine markers (L-DCC, NSE, Chromogranin CK-BB, Neurosecretory granules)	Common	Rare
Peptide secretion (ADH, ANP, GRP)	Present	Absent
Extracellular matrix production (Laminin)	Common	Rare

SCLC, small cell lung cancer ; NSCLC, non-small cell lung cancer ; L-DCC, L-dopa decarboxylase ; NSE, neuron-specific enolase ; CK-BB, brain isotype of creatine kinase ; ADH, antidiuretic hormone ; ANP, atrial natriuretic peptide ; GRP, gastrin releasing peptide

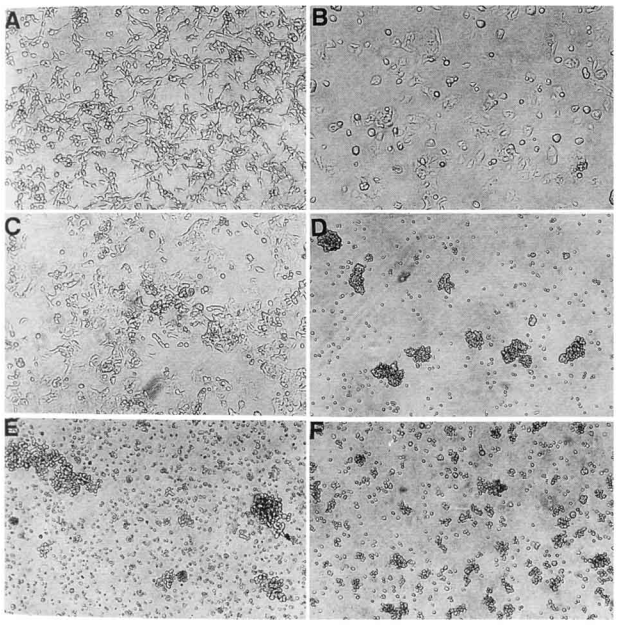

Fig. 3. Appearance of several lung cancer cell lines in liquid culture by inverted microscopy : Non-small cell lung cancer (NSCLC) lines (A : Ma-44, B : Ma-31, C : Ma-29) and small cell lung cancer (SCLC) lines (D : N417, E : Ms-10, F : H69)

(DDC), neuron-specific enolase (NSE), the brain isoenzyme of creatine kinase (CK-BB), chromogranin A, bombesin-like immunoreactivity or gastrin releasing peptide (GRP), adrenocorticotropic hormone (ACTH), anti-diuretic hormone (ADH), atrial natriuretic peptide (ANP), and others (Table 2) (20-24). The biologic significance of the production of functionally active enzymes such as enolase and creatine kinase is unknown. However, the expression of other products, such as GRP, has been speculated to play a mitogenic role in an autocrine mechanism. In summary, these immortalized cell lines have continued to serve as a valuable resource for biochemical and cytogenetic studies that have ushered in the first discovery of genetic alterations in lung cancer.

Non-small cell lung cancer (NSCLC) includes all other types of lung cancer and, as a result, is a collection of at least four histologic types : squamous cell carcinoma, bronchioloalveolar and adenocarcinoma, large cell carcinoma, and undifferentiated lung carcinoma (24). Although NSCLCs are less likely than SCLCs to undergo early dissemination, they are characterized by aggressive local spread and are less responsive to anticancer chemotherapy and/or radiotherapy (24). Namely, NSCLC patients are more likely to undergo surgical treatment, allowing the collection of primary tumor specimens for laboratory investigations. In addition, tumorigenic cell lines can also be derived from these samples and such cultures typically grow as an adherent monolayer with fibroblast-like morphology (Fig.3) (23). Although this classification of SCLC vs. NSCLC has been useful for both pathologists and clinicians, it incorrectly implies an understanding of the cell of origin of these cancers. Generally, it is believed that SCLCs arise from rare cells with neural APUD (amine precursor uptake and decarboxylation) features that are buried in the bronchial mucosa, while NSCLCs arise from other precommitted bronchial epithelial cells. For many years, however, pathologist have observed lung cancers characterized by admixtures of both SCLC and NSCLC histology. Furthermore, about 10-15% of NSCLCs produce neuroendocrine enzymes and peptides (24). These observations have raised the hypothesis (unitarian theory of lung cancer) of a single progenitor cell that gives rise to different types of lung cancers. However, the molecular genetic analysis of SCLC and NSCLC tumors has revealed as many similarities as differences between the two histologic types, and at present medical oncologists will continue to use the same pathology-based classification system.

THE *RB* GENE AND LUNG CANCER

Reviews of cytogenetic data in lung cancer samples revealed numerous examples of deletions or unbalanced translocations involving the chromosome band 13q14 present in karyotypes of SCLC cell lines (Table 3) (9, 10, 25-28). These had been previously overlooked due to the extensive aneuploidy found in SCLC samples, and this suggests that additional marker chromosome abnormalities may yet be found. A subsequent review of RFLP data from primary lung cancer samples demonstrated frequent loss of heterozygosity, not only on chromosome 3p, but also on 13q and 17p in SCLC (11,12). Although SCLC had a completely different clinical presentation than retinoblastoma, both grow nonadherently in liquid culture medium as tight clusters, and both frequently amplify *myc* family genes (4, 29). Therefore, the *Rb* gene, located at chromosome 13q14, was a potential target for somatic mutations in SCLC.

Early DNA and RNA studies found evidence of structural mutations in 20% of SCLC samples tested and absent mRNA in approximately 40% of SCLC cell lines (30). Furthermore, in each case where a matched primary cancer sample was available, the abnormality detected was identical to that observed from the derived SCLC cell line (30). Further studies of RB protein revealed that the majority of SCLC samples that expressed apparently normal mRNA had no detectable RB protein (31, 32). In addition, several examples of mutant RB proteins were found as a result of subtle mutations, generally single point mutations. To date these have included aberrant deletion of nucleotide sequences from exons 16, 21, or 22, yielding stable, but truncated, proteins with intact amino- and carboxyl-terminal residues (32, 33). Some of these same alterations have been observed in other tumor types, such as a deletion of exon 21, which was reported in both a bladder cancer and a prostate cancer, while an example of a deletion of exon 22 has also been seen in a case of NSCLC. Another interesting mutation identified in SCLC was a single nucleotide change in exon 21 that led to a missense mutation (cys^{706} to phe^{706}) (34). Each of these *in vivo* RB protein mutants share the same phenotype : they are defective in viral oncoprotein binding (and, therefore, also defective in their ability to bind to the corresponding cellular RB binding proteins such as E2F) and in phosphorylation (Fig.4) (35). The mutations do not directly affect a cluster of serine and threonine residues that are the presumed sites of phosphorylation, these mutations, but instead, appear to block a specific conformation required for protein binding and phosphorylation. The *in vivo* RB mutants, therefore, are presumed to be inactive (loss of function mutants), and this is consistent with their inability to transform primary rat embryo cells by themselves or in cooperation with an activated *ras* gene (36).

In summary, almost all SCLC samples (>90%) have absent or aberrant RB protein expression (31, 32, 37). In contrast, only about 15% of NSCLC samples have inactivated RB function (31, 32, 37). RB protein status in lung cancer, extrapulmonary small cell carcinoma, and mesothelioma from American and Japanese patients is shown in Table 4 (37, 38). This lower frequency of *Rb* inactivation is seen in a wide range of adult tumors, such as bladder, prostate, breast, and hematologic tumors. The biologic significance of the *Rb* gene as a high-frequency target in SCLC, and a lower frequency target in other common tumors, is still unclear.

Reintroduction of the *Rb* gene in a variety of $Rb^{-/-}$ cell lines, such as retinoblastoma, osteosarcoma, prostate, and bladder cancer derived cell lines, has resulted in complete or partial suppression of tumorigenicity when measured as the ability to form subcutaneous tumors in nude mice (24). Two studies have demonstrated similar findings in both NSCLC and SCLC cell lines (39, 40). Similar to the findings reported in prostate and bladder cancer,

Table 3. Cytogenetic abnormalities in SCLC and NSCLC

SCLC	NSCLC
3p14-3p24	3p21-3p25
13q14	3p14
17p	i (3q)
11p	11p
1p	17p
5q	1q1-1q3
	9p
	17q
	19q13
	5q1
	7q

SCLC, small cell lung cancer ; NSCLC, non-small cell lung cancer.

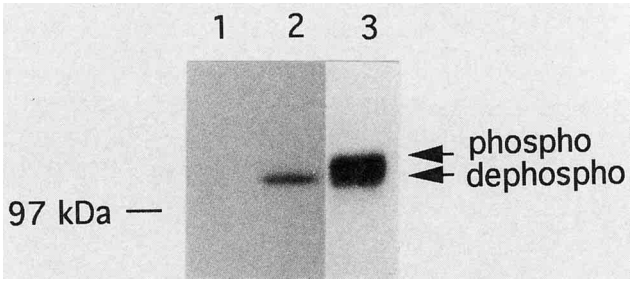

Fig. 4. RB protein expression of lung cancer cells by western blotting. Lanes : 1, N417 SCLC ; 2, H209 SCLC ; 3, Ma-31 NSCLC. RB protein from N417 cells (lane 1) was not detected. RB protein derived from H209 cells (lane 2) was a dephosphorylated type that migrated as a single band at 105 kDa, indicating that the protein was mutant. The protein that was defective in phosphorylation and in oncoprotein binding such as adenoviral E1A and SV40 large T antigen, was identified as having a point mutation within exon 21 resulting in a single cysteine to phenylalanine substitution at codon 706. RB protein derived from Ma-31 cells (lane 3) exhibited differently phosphorylated species that migrated at 105～110 kDa on 7.5% SDS/PAGE, indicating that the protein was the wildtype.

Table 4. Altered RB protein expression (absent or mutant) in SCLC, NSCLC, extrapulmonary small cell carcinoma (EPSC), pulmonary carcinoid, and mesothelioma cell lines derived from American and Japanese patients

	SCLC	EPSC	carcinoid	NSCLC	mesothelioma
USA	67/76	4/5	1/6	12/81	0/4
Japan	10/13			1/15	

however, the suppression was not complete ; some mice developed tumors following injection with cells reexpressing *Rb* gene, although these were up to 10 times smaller than the corresponding tumors in the same mice following injection with the same parent cell line injected with plasmid alone. In lung cancer cells, the reexpression of a functional *Rb* gene can result in tumor suppression *in vivo*, but the effect appears to be variable. This probably reflects the multiple genetic abnormalities accumulated in lung cancer cells. This accumulation of genetic defects contrasts to familial retinoblastoma, which results from the disruption of a single locus, the *Rb* gene. Interestingly, the *Rb*-mediated tumor-suppressive effect can be totally reversed in lung cancer cells by co-injection with an extract of an extracellular matrix (40). The capacity for *Rb*-mediated tumor suppression to be overcome by tissue-specific factors may also explain the inability of Rb reexpression to exert a tumor-suppressive effect on retinoblastoma cells following intraocular injection in athymic mice (41).

The presence of a disruption of the *Rb* locus in almost all SCLC cell lines and a significant number of NSCLC cell lines and tumor samples clearly indicates a central role for the RB protein in both cell growth regulation and molecular carcinogenesis. Although alterations of *Rb* gene expression have been implicated as a negative prognostic factor in both sarcomas and bladder carcinomas, similar implications in lung cancer are not established (Table 5) (37, 42-45). However, there have been recent reports of decreased survival in early-stage NSCLC with *Rb* gene abnormalities and a higher incidence of *Rb* gene abnormalities in stage Ⅲ and Ⅳ NSCLC tumor specimens. When RB protein status is combined with analysis of *P53* status, there is an increased survival advantage of 34 months (12 months vs. 46 months, $P<0.001$) in stage Ⅰ and Ⅱ NSCLC (46). The current lack of effective chemotherapeutic agents in NSCLC and chemoresistant SCLC makes clinical application of survival data in lung cancer difficult (47-52). However, with improved methods (adenoviral vector) of gene transfer for pulmonary disease such as cystic fibrosis and pulmonary emphysema, there exists a possible future role for the use of the *Rb* gene in directed gene therapy in lung cancers.

Table 5. Prognostic significance of RB alteration

Tumor	Sample	Method	Altered RB on survival
Sarcoma	56 fresh tumors	IHC, IB	Worse
Bladder	48 fresh tumors	IHC	Worse
Bladder	43 fresh tumors	IHC	Worse
NSCLC	194 fresh tumors	IHC, NB, SB	NS
NSCLC	68 cell lines	IB	NS
SCLC	62 cell lines	IB	NS

IHC ; immunohistochemistry, IB ; immunoblot, NB ; Northern blot, SB ; Southern blot, NS ; not significant.

THE *P53* GENE AND LUNG CANCER

The *P53* gene encodes a 53 kDa nuclear protein that localizes to chromosome 17p13. It was originally identified as a host cellular protein that bound to the large T viral antigen of animal cells infected with simian virus 40 (SV 40) (53, 54), and those early reports speculated that the large T antigen exerts its transforming effect by binding to and, therefore modulating, the activity of the cellular *P53* gene (53). Several years later, however, it was classified as a dominant oncogene on the basis of elevated protein levels in many tumors and the ability of its cDNA to transform primary rat embryo fibroblasts when transfected in cooperation with an activated *ras* gene (55). In 1988, however, it was recognized that all transforming clones of *P53* had undergone activating somatic mutations, while, in contrast, wild-type *P53* appeared to exert a phenotype of growth suppression when transfected into immortalized cell lines (56). Furthermore, the elevated levels of P53 protein observed in many tumors were the result of mutations that markdly increased the protein half-life, and the detection of increased protein levels on western blotting is one of the methods used to screen samples for the presence of activating *P53* mutations.

Earlier RFLP analyses had implicated tumor-specific loss of heterozygosity on chromosome 17p in both lung cancer and colon cancer (12). Shortly afterward it was demonstrated that the gene on 17p targeted for allele loss in colon cancer was the *P53* gene. Similar investigations of lung cancer revealed that essentially all (70-100%) SCLCs have aquired mutations, while approximately 50% of NSCLCs show evidence of *P53* mutations (57). The *P53* mutations are distributed throughout the open-reading frame. The majority of mutations are substitutions of one amino acid residue for another at a single codon, but frame shifts, insertions, deletions, and splicing mutations have been also reported (58). *P53* is composed of 11 exons, and the majority of mutations are found in the central exons, exons 5 through 8. As in *Rb* mutations observed in lung cancer, G-to-T transitions characteristic of tobacco-induced mutations have been seen in *P53* mutations characterized from lung cancer specimens or cell lines (59). These findings suggest the need for an objective assay to identify exogenous and endogenous carcinogens acting on individual humans.

THE *P16* GENE AND LUNG CANCER

The *P16* (*INK4A, MTS1*) has been identified as a candidate for the familial melanoma gene at chromosome 9p21, and germ line mutations in the *MTS1* have been identified in several kindreds with familial melanoma (60). In addition, aquired mutations have been identified in a variety of transformed cell lines and several primary tumors (60). Inactivation of the RB protein by phosphorylation is mediated by a family of cyclins and cyclin-dependent kinases (CDKs), and inhibited by CDK inhibitors. P16 has kinase

Table 6. Summary of P16 and RB status in 88 lung cancer cell lines

		P16 (+)	P16 (−)	total
SCLC	RB(+)	1 (14%)	6 (86%)	7
	RB(−) or (mt)	48 (100%)	0 (0%)	48
NSCLC	RB(+)	4 (15%)	22 (85%)	26
	RB(+) or (mt)	6 (86%)	1 (14%)	7

+ wild-type ; - absent ; mt mutant.

inhibitor activity that is specific for CDK4 and is a strong inhibitor *in vitro* for RB protein phosphorylation (61). An interesting observation is that lung cancer cell lines that are RB-positive without inactivating *Rb* mutations are almost invariably P16-negative when examined by immunoblotting (Table 6) (62, 63). In summary, in only 10% of SCLC samples is P16 protein expression absent, while 70% of NSCLC samples have loss of P16.

OTHER TUMOR SUPPRESSOR GENES

The accuracy with which cytogenetics and RFLP analyses predicted the presence of tumor suppressor genes at the 13q (*Rb*), 17p (*P53*), and 9p (*P16*) regions suggests that evidence for the presence of nonrandom, tumor-specific allele loss is an important clue for the identification of additional tumor suppressor genes. Many other chromosomal loci have been identified as frequent sites for gross structural mutations in lung cancer (Table 3) (9, 10, 25-28), suggesting that many of these sites also harbor novel genes playing a role in normal growth and development for pulmonary tissues. Although all karyotypes demonstrated multiple alterations, the most consistent abnormality was a deletional event on the short arm of chromosome 3, and more precise cytogenetics allowed the smallest consensus deletion to be mapped to the region of 3p14-3p24. This finding was tested by RFLP analyses of both primary SCLCs and NSCLCs (12). These investigators verified that there was evidence at the molecular level for DNA loss on one allele of 3p in more than 90% of SCLCs, while a similar allelic loss was observed in approximately 50% of NSCLC samples. The consistent finding of tumor-specific chromosomal deletions in this region led to the hypothesis that a lung cancer susceptibility gene located at this area. One approach to identify a putative 3p tumor suppressor gene has been to examine interesting genes that have previously been characterized and mapped to this region. Several candidate genes have been studied, including the thyroid hormone receptor (c-erbA-β), a retinoic acid receptor (rarβ), and tyrosine phosphatase (PTP-λ) (24). Although each of these genes undergo allelic loss in most tumor samples from patients with SCLC, evidence for homozygous mutations and/or loss of wild-type protein function has not been reported for these cases. Attempts to identify the putative tumor suppressor gene on 3p have not been successful to date.

TUMOR SUPPRESSOR GENE PRODUCTS AND CELL CYCLE REGULATION

The *Rb* gene encodes an 105-kDa nuclear phosphoprotein. The gene product is a substrate for the cyclin-dependent kinases (CDKs) and is phosphorylated in a cell-cycle-dependent manner (64-67). To fully understand the importance of the *Rb* gene product in the regulation of the cell cycle, proteins involved in cell cycle regulation are necessary (68). The schema of the role of tumor suppressor proteins in cell cycle regulation is shown in Fig.5. The resting or nondividing cell (G0) is characterized by the presence of hypophosphorylated RB protein. The signals that maintain the cell in the resting phase are complex but include a variety of exogenous growth factors. The mitogenic stimuli result in a cellular commitment in late G1 to undergo DNA replication, or enter S phase (69-71). This check-point is the equivalent of the G1 decision point in budding yeast known as START. After the restriction to enter S-phase has passed, the cell is committed to DNA replication under the influence of the cyclins and their associated CDKs, which are further subject to regulation

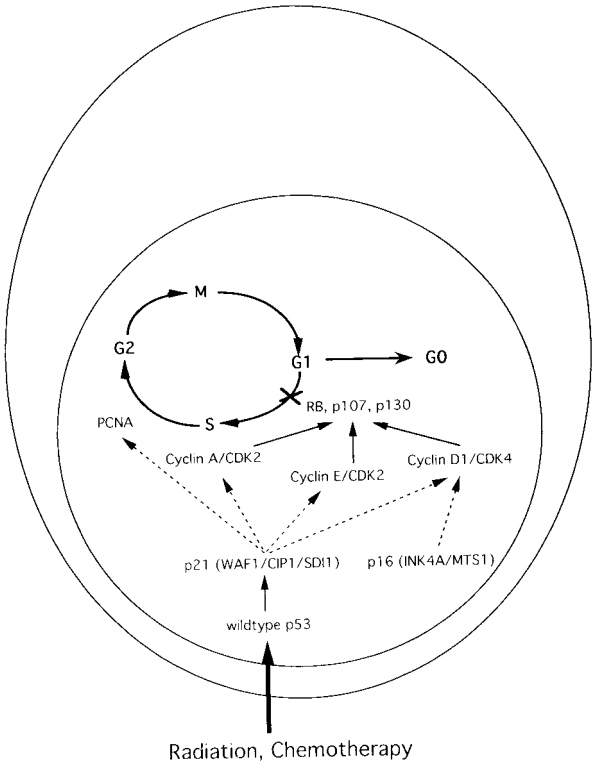

Fig.5. The cell cycle and tumor suppressor proteins. RB protein and other RB family members (p107, p130) remain hypophosphorylated in the quiescent (G0/G1) cell. As cyclin D levels rise in response to mitogenic stimuli, the cyclin D-associated CDKs (CDK2, CDK4) target RB for hyper-phosphorylation. The cell commits to DNA replication (S-phase) following a rise in cyclin E levels, and also interacts with the CDK family members to target RB for further phosphorylation. RB remains phosphorylated until late G2, when phosphatase activity leads to specific RB dephosphorylation. The CDK inhibitors (p16, p21) act to repress kinase activity of the CDK family members. DNA damage, induced by radiation/chemotherapy, transcriptionally activates p21 via wild-type p53.

by a group of CDK inhibitors (72). Following mitogenic stimulation, eukaryotic cells in late G1 demonstrate a rapid increase in the levels of the D family cyclins (cyclins D1, D2, D3) (69-71). The levels of the cyclin D family members appear to be highly sensitive to the presence of growth factor stimuli. The D family cyclins associate with and regulate several CDKs, most specifically CDK2 and CDK4, positively modulating their kinase activity, resulting in the phosphorylation of target proteins possessing canonical kinase sequences. The 105-kDa RB protein has at least nine phosphorylation target motifs in its primary amino acid sequence and is a cellular substrate for the CDK system. Although cyclin D-mediated RB hyper-phosphorylation is an important early response to mitogenic stimuli, the rate limiting factor in determining the G1/S-phase transition appears to be cyclin E protein levels, which begin to rise in late G 1. Cyclin E associates with CDK2 and probably plays a role in mediating additional RB hyperphosphorylation. Overexpression of members of the E, D, and A family of cyclins has been demonstrated to overcome RB-mediated growth suppression (73), demonstrating a possible role for these cyclins in regulating *in vivo* RB function.

As the cells enter S-phase, there is progressive phosphorylation at a series of serine and threonine residues identified as target residues of the CDK system (68). During G2 phase, the RB protein remains in a hyperphosphorylated state until specific phosphatase activity dephosphorylates the protein as it reenters G1/G0. The capacity to serve as a target for phosphorylation by the CDKs is one of several critical functional properties that is lost in mutant forms of the RB protein identified from transformed cell lines. This loss of the wild-type RB phenotype can easily be identified by immunoblotting. Conventionally, wild-type RB protein isolated from asynchronously growing cells migrates as a series of bands varying in size form 105 to 110 kDa. The slower migrating bands represent the hyperphosphorylated forms of RB protein. In contrast, mutant forms of the RB protein migrate as a single band, representing exclusively the hypophosphorylated RB moiety (Fig.4). As the cell progresses through the cell cycle, these mutant forms of the RB protein remain hypophosphorylated, in contrast to normal wild-type cell cycle fluctuations (32-35).

The molecular significance of the CDK system and its role in RB phosphorylation are further emphasized by an important class of identified and cloned CDK inhibitors, P16, P21 (WAF1, SDI1, CIP1), and P27 (KIP1) (68). These factors play a role in regulating the activity of the various CDK members, in essence functioning as a check on the proliferative signal that is the result of cyclin-mediated CDK activation. As negative controlling elements of the cell cycle, these inhibitors are themselves strong candidates of tumor suppressor genes. P21 is transcriptionally activated by P53 (72). P21 binds to cyclins A, D1, E, and CDK2 and is a potent inhibitor of the CDKs and RB phosphorylation. Expression of this gene thus directly inhibits cell cycle progression, and loss of expression via inactivation of the transcriptional activity of P53 would directly cause loss of cell cycle regulation. P21 provides a link between the functional roles of P53 and RB. P21 thus appears to be a direct mediator of growth-suppressive properties of P53.

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