State of the Art

Molecular and Genetic Aspects of Lung Cancer

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INTRODUCTION

Lung cancer is the leading cause of cancer death among men and women in the United States with 170,000 deaths per year. This exceeds the sum of the next three leading causes of death due to cancer: breast, colon, and prostate. There are over one million deaths worldwide due to lung cancer, making it truly an epidemic. Fewer than 15% achieve a 5-yr survival. The vast majority (85%) present with advanced disease, although Stage I patients may have a 5-yr survival approaching 70% (1). Eighty percent of the lung cancers are non–small cell lung cancer (NSCLC; adenocarcinomas, squamous cell, bronchoalveolar and large cell carcinomas) and 20% are small cell lung cancer (SCLC). Cigarette smoking constitutes 80% of the attributable risk and asbestos, radon, other occupational and environmental exposures and genetic factors contribute to the rest. The purpose of this state-of-the-art review is to introduce the molecular genetics of lung cancer for the clinician in this rapidly progressing field. Many of the basic science concepts to follow already are being studied in clinical trials of new chemotherapeutic agents or gene therapy.

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DIAGNOSIS (CLINICAL APPROACHES)

James Alexander Miller, the first Director of the Bellevue Chest Service, reviewed primary carcinoma of the lung in 1930 (2). He presented 32 cases from Bellevue Hospital, and noted that the disease appeared to be due to urban dust and bronchial irritation but did not explicitly indict tobacco or cigarette smoking. In 1939, Ochsner and DeBakey presented a case series of seven lung cancers treated surgically by pneumonectomy and discussed the possibility that smoking caused lung cancer by irritating the bronchial mucosa (3).

Lung cancer can progress significantly before symptoms are manifest although the common symptoms of expectoration and cough increase in frequency over time in clinical cases. Dyspnea, wheeze, heaviness in the chest, chest pain, and hoarseness are not particularly helpful, but hemoptysis increases 12-fold at time of diagnosis compared with matched control subjects and loss of weight increases 3-fold (4). Among helpful clinical signs are digital clubbing which recently was observed in 29% of 111 consecutive patients with lung cancer (5). Clubbing was more common in NSCLC than SCLC, and among women than men. Paraneoplastic conditions may give rise to symptoms and signs including syndrome of inappropriate antidiuretic hormone, ectopic adrenocorticotrophic hormone, Eaton-Lambert syndrome, neurologic syndromes, hypercalcemia, deep vein thrombosis, marantic endocarditis, disseminated intravascular coagulation, and hypertrophic osteoarthropathy. The staging of lung cancer has recently been reviewed by Mountain (6). Evaluation for metastases must include a clinical and laboratory examination and, if abnormal, followed by computed tomographic (CT) scan of the head and abdomen and a radionuclide bone scan (7).

Appropriately stratified case-control studies that take cigarette smoking into account typically report that lung cancer cases have an odds ratio for having a first-order relative with a history of lung cancer of approximately 1.7 to 5.3 (8, 9). Chronic obstructive lung disease and pulmonary fibrosis are clinical risk factors for lung cancer.

Low-dose spiral CT chest scan has tremendous promise in detecting Stage I lung cancer compared with the chest X-ray. Henschke and colleagues screened 1,000 persons age 60 or over with at least 10 pack-year's smoking finding noncalcified nodules in 23% (10). Among those with positive CT, 28 were recommended for biopsy and 27 of these were malignant. Pathologic and clinical staging classified 23 of the 27 as Stage I and potentially curable. In the whole study population, malignant disease was detected four times more frequently on low-dose CT than on chest radiography.

Although sputum cytology is regarded as having too low a sensitivity to be useful in screening for lung cancer, it can be useful for detecting dysplasia. Kennedy and colleagues reported that 26% of a high risk cohort ($FEV_1 < 70\%$ predicted, $FEV_1/FVC < 70\%$ predicted, 40 pack-years of smoking) had moderate to severe dysplasia and should be a target group for research programs focusing on lung cancer prevention, early detection, and exploratory biomarker studies (11). Tockman and colleagues have used a monoclonal antibody to hnRNP (ribonucleoprotein) A2/B1 as a cancer antigen that can be detected in sputum specimens for up to 2 yr before the tumor is detectable radiographically (12). He and his colleagues reported hnRNP overexpression with a sensitivity of 91% and specificity of 88% on archived sputum of smokers who went on to develop lung cancer (13). They performed two prospective studies on sputum detection with overexpression of hnRNP A2/B1: first, 32 of 40 surgically treated primary lung cancer patients with recurrence over 12 mo were identified, and second, the test detected 69 of 94 high-risk Chinese tin miners with primary lung cancer. Computer-assisted cytometry techniques may detect early nuclear morphological changes on sputum samples (14).

Autofluorescence bronchoscopy using the laser-induced fluorescence emission system has been optimistically demonstrated to increase the dysplasia detection rate over that obtained by white light bronchoscopy from approximately 40 to 80% (15, 16). Considerable operator skill is required to detect brownish red discoloration on tertiary carinas and to distinguish these sites from the background greenish discoloration (17).

CIGARETTE SMOKING AND MOLECULAR EPIDEMIOLOGY OF LUNG CANCER

The World Health Organization estimates that 47% of men and 12% of women worldwide age 15 and over are smokers (18). Although smoking rates have decreased in industrialized countries since 1975, there has been a corresponding 50% increase in developing countries.

Case control studies reported an association between lung cancer and smoking in 1950 with a risk ratio of approximately 10; these studies were quickly followed by cohort studies in the United States and United Kingdom. The cohort studies enrolled healthy people who recorded their smoking habits and were then followed to determine the variation in mortality with the amount smoked. All showed that the mortality from lung cancer increased approximately in proportion to the amount smoked (19, 20). The American Cancer Society enrolled one million citizens prospectively in 1982 and found that the lung cancer mortality rate ratio for smokers versus nonsmokers after 9 yr follow-up was 23.9 for men and 14 for women (21). Sir Richard Doll established a cohort of 34,000 British doctors in 1951 that has been followed for over 40 yr with cigarette smoking habits recorded periodically (22). The mortality rate ratio for lung cancer in smokers versus nonsmokers was 14.9 and this dropped to 4.1 in ex-smokers. The lung cancer mortality rate ratio increased from 7.5 among current smokers smoking 1 to 14 cigarettes per day to 25.4 for those smoking 25 or more cigarettes per day. The loss of expectation of life for all cigarette smokers in the British doctor's study was 8.0 yr. It has been known since 1981 that passive smoke also increases risk for lung cancer when Hirayama and Trichopoulos and coworkers independently reported an increased risk of lung cancer in nonsmokers if their spouses smoked (23, 24). Ex-smokers have a progressive reduction in risk approaching 90% with most of the reduction occurring five or more years after quitting.

There are substantial racial differences for the incidence of lung cancer, with African-Americans having a 1.8-fold higher risk than white individuals (25), and Hispanics and Asian/Pacific Islander groups having a reduced incidence compared with white individuals. Interestingly, women are at a higher risk than men for a given level of smoking with a relative risk of 1.7. Lung cancers from women have significantly greater polycyclic aromatic DNA adducts per pack-year than men (26). As tar and nicotine per cigarette have dropped by more than two-thirds from 38 mg to 12 mg and 2.3 mg to 1.2 mg, respectively, there has been a concomitant change in the histologic type of lung cancer (27). Whereas SCLC has persisted at approximately 20% in most series, adenocarcinoma has increased to 45% with declines in squamous cell and large cell carcinoma. Thun and colleagues have suggested that these changes are the results of cigarette design, e.g., the smoke in filter tip cigarettes is inhaled more deeply than earlier, unfiltered cigarettes (more toxic), and deeper inhalation transports tobacco-specific carcinogens more distally toward the bronchoalveolar junction where adenocarcinomas often arise (28). In addition, blended reconstituted tobacco includes more stems than leaves which release higher concentrations of *N*-nitrosamines.

Pershagen and colleagues demonstrated that residential exposure to radon gas increases lung cancer risk in relation to cumulative and time-weighted exposure (29). The excess relative risk of lung cancer was 3.4% per 27 pCi/L which is in the range reported for underground miners at 2 to 10% per 27 pCi/L. Hammond and colleagues assembled a cohort of 17,800 asbestos insulators in the United States and Canada in 1967 and followed them prospectively to assess lung cancer and mesothelioma risk (30). Compared with nonsmoking control subjects who had no exposure to asbestos, asbestos workers who had a history of smoking had a 53-fold increased mortality ratio from lung cancer. This was greater than the sum of the increases for lung cancer from asbestos exposure alone (5-fold) or cigarette smoking alone (11-fold). Other exposures for increased risk for lung cancer include silica, metal mining and smelting (chromium, cadmium, nickel, and arsenic), bischloromethyl ether, coke ovens (polycyclic aromatic hydrocarbons), and ionizing radiation. Diet may also influence lung cancer risk with a high-fat diet similar to that consumed in the United States enhancing risk posed by tobacco smoke carcinogens.

Tobacco smoke is complex with over 4,000 compounds identified that are suspended in an aerosol of over 10^{10} particles per milliliter of mainstream smoke. Among the more than 60 carcinogens in tobacco and cigarette smoke, the two major classes are polycyclic aromatic hydrocarbons and nitrosamines. Mainstream smoke contains 20 to 40 ng of benzo(a)pyrene per cigarette and 0.08 to 0.77 μg of the nitrosamine NNK per cigarette. The total amount of NNK required to produce lung cancer in rats is similar to the total amount of this compound to which a smoker would be exposed in a lifetime of smoking (31).

Metabolism of inhaled carcinogens was recently reviewed by Spivack and colleagues (32). Because most tobacco-derived organic carcinogens are water-insoluble, they require oxidation and conjugation for excretion in aqueous environments. The aryl hydrocarbon receptor binds incoming aromatic hydrocarbons and members of the cytochrome P450 family activate polycyclic aromatics, whereas members of the glutathione-*S*-transferase family inactivate these carcinogens. Combined phenotypes such as CYPIAI plus GSTMI null can accelerate carcinogen activation and impair inactivation leading to increased risk for lung cancer (32). DNA repair capacity as measured in a host cell reactivation assay with plasmids damaged by exposure to benzo(a)pyrene diol-epoxide (BPDE) was significantly

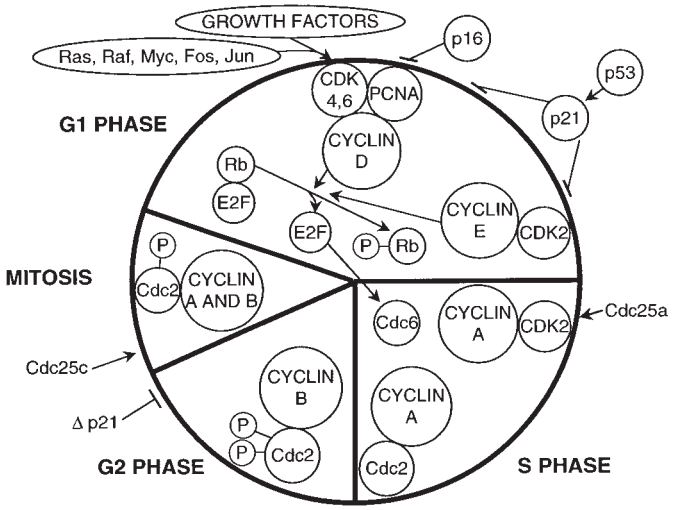

Figure 1. Cell-cycle regulators implicated in lung cancer. (Adapted from Reference 36.)

lower in lung cancer patients (3.3%) than in controls (5.1%) (33). After adjustment for age, gender, ethnicity, and smoking status, the lung cancer patients were five times more likely than control subjects to have reduced DNA repair capacity.

MOLECULAR ABNORMALITIES IN LUNG CANCER: A DISEASE OF THE CELL CYCLE

Approximately 50 tumor suppressor genes and over 100 oncogenes have now been described. Because tumor suppressor genes, telomeres, and oncogenes are intimately involved in the regulation of cell growth and division, cancer can be considered a disease of deregulation of the cell cycle. Oncogenes result from gain-of-function mutations in their normal cellular counterpart protooncogenes and act in a dominant fashion.

The classic cell-cycle model, consisting of a DNA synthesis (S) phase, a mitosis (M) phase, and two gap (G_1 and G_2) phases, has now been elucidated in molecular detail (34–36, Figure 1). Critical components of the cycle include the cyclins, cyclin-dependent kinases (Cdk), and the retinoblastoma (Rb), p53, and E2F proteins. Each Cdk is regulated by a cyclin subunit, which is required for catalytic activity and substrate specificity. A first crucial step in the cell cycle occurs late in the G_1 phase at the restriction point, when a cell commits to completing the cycle. Competence factors such as platelet-derived growth factor (PDGF) and progression factors such as insulin-like growth factor-I (IGF-I) can interact at this point to stimulate cell proliferation. Both growth factors can be made by lung tumor cells to enhance tumor growth in an autocrine fashion, usually in the late stage of tumorigenesis. Engagement of growth factors with their respective receptors leads to receptor dimerization, phosphorylation, and transmission of growth signals to the nucleus. Growth-promoting signals transduced from the cell surface to the nucleus cause a rapid and transient elevation in the D-type cyclins (early G_1). Cyclin D1 complexes with Cdk4/6 and phosphorylates the Rb protein (Figure 2) (36). Cyclin D1 overexpression is a common molecular abnormality in lung cancer (37). Hyperphosphorylation of Rb in G_1 releases the transcription factor E2F, which activates S-phase genes, including thymidine kinase, *c-myc*, dihydrofolate reductase, Cdc6, and DNA polymerase-α (38).

Two families of Cdk inhibitors are crucial in G_1 progression (Figure 3). The INK4 family on chromosome 9p21 encodes four genes (INK4a, b, c, and d) whose products bind cyclin D-Cdk4/6 dimers to inactivate the kinase function. Members of the Kip1 family (p21, p27, p57) bind the cyclin D-Cdk 4/6, cyclin E-Cdk2, and cyclin A-Cdk2 complexes (39). The cyclin E-Cdk2 complex mediates progression out of G_1, and cyclin A expression increases dramatically with the onset of S phase. Cyclin A-Cdk2 function appears to be required for DNA replication and the G_2/M transition. Loss of p53 function leads to reduced levels of p21 and hyperactivity of both cyclin D-Cdk and cyclin E-Cdk complexes, hyperphosphorylation of the Rb gene, and elevated levels of E2F (40). Inactivation of the tumor suppressor gene Rb produces the same effect resulting in increased levels of free E2F in the cell. Cooperation between the Rb and p53 pathways likely determines whether p53 induces G_1 arrest or apoptosis in response to DNA damage, with the loss of Rb tilting the balance toward apoptosis (35). Preventing p53-dependent apoptosis is a key to carcinogenicity, and lung cancers that have wild-type p53 usually have increased expression of the MDM2 gene product which binds to the p53 transactivation domain and targets p53 for ubiquitin-mediated degradation (41). Overexpression of MDM2 overcomes wild-type p53-mediated suppression of transformed cell growth (Figure 2).

Because E2F is a transcription factor that activates S-phase genes, E2F may be critically important for replication of DNA

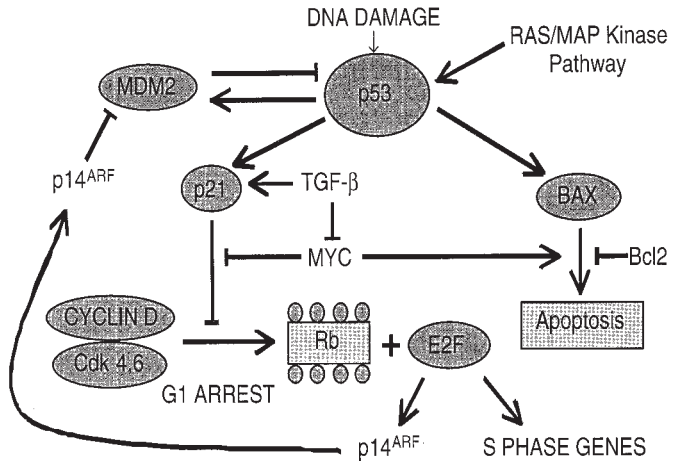

Figure 2. p53 and Rb pathways in molecular carcinogenesis.

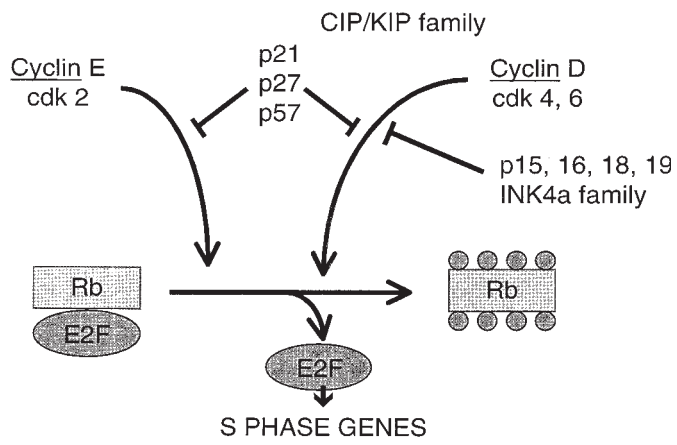

Figure 3. Sites where p21 and p16 work as checkpoint inhibitors in the cell cycle.

in the cell cycle. DNA replication occurs at multiple chromosomal sites called origins of DNA replication and is controlled, in part, by origin recognition complex (ORC) proteins (42). The ORC proteins are bound to Cdc6 which controls initiation of DNA replication (42). A prereplication complex is formed when the Cdc6/ORC interaction directs the loading of minichromosome maintenance (MCM) proteins onto chromatin; the MCM proteins are on chromatin in G_1, much less so in S, and not at all in G_2/M. Human Cdc6 messenger RNA (mRNA) and protein are not detectable in serum-deprived human diploid fibroblasts, but increase prior to the G_1/S transition as the cells are stimulated with serum (43). This transition is regulated by E2F proteins, as revealed by a functional analysis of the Cdc6 promoter showing E2F binding sites and stimulation of the Cdc6 gene by exogenous E2F (44). Immunodepletion with anti-Cdc6 antibodies prevents initiation of DNA replication (44). In lung cancer, E2F is free and may upregulate Cdc6 leading to a deregulated cell-cycle with abnormal cellular proliferation. Cdc6 may be a marker for cell-cycle deregulation and may be a target for detection or therapeutics.

ROLE OF p53 AS THE GUARDIAN OF THE GENOME AND PROTECTOR OF THE LUNG FROM ENVIRONMENTAL CARCINOGENS

The p53 tumor suppressor gene is the most commonly mutated gene in cancer (45) and is mutated in 50% (NSCLC) to 70% (SCLC) of lung cancer. Mutations in p53 commonly reflect exposures to environmental carcinogens, e.g., cigarette smoke and lung cancer or aflatoxin and liver cancer in Southeast Asia. The p53 protein has been aptly referred to as the "guardian of the genome" because the p53 gene is induced by DNA damaging agents and subsequently either delays cell-cycle progression, or steers the damaged cell headlong into programmed cell death (46). The p53 protein is a nuclear transcription factor that binds to the p21 promoter inducing its expression and inhibiting cell-cycle progression at the G_1/S cell-cycle checkpoint (39). Mutant p53 cannot activate p21, and the cell cycle proceeds unabated; thus the term "tumor suppressor." Alternatively, p53 may induce *bax*, a gene promoting apoptosis (47). Most missense mutations in the p53 gene occur in the DNA binding domain consequently inactivating its transactivation function (48). Mutations of p53 greatly enhance the half-life of the protein, allowing for frequent immunohistochemical detection of mutant p53, e.g., in the severely dysplastic bronchial epithelium or in the tumor tissue. For tumor suppressor genes, phenotypic expression requires that both alleles be lost through mutations, large deletions, or other recombinant mechanisms (49). In lung cancer cell lines Calu-1 (both p53 alleles are deleted) and A549 (containing wild-type p53), growth arrest can be induced after *in vitro* treatment with phorbol ester (50), which activates a protein kinase C signaling cascade. The induction of p21 expression by phorbol ester temporally coincides with growth arrest in G_2/M.

p53 is located on chromosome 17p and is composed of 393 amino acids. The transactivation domain is at the *N*-terminus followed by the sequence specific DNA binding domain and oligomerization domain at the *C*-terminus. p53 mutations in lung cancer are clustered in the middle of the gene at codons 157, 245, 248, and 273 (51). The apparent significance of these mutational sites became clear when the tobacco smoke carcinogen, benzo(a)pyrene, was shown to induce BPDE adducts at CpG sites in codons 157, 248, and 273 *in vitro* in bronchial epithelial cells (52). These codons contain CpG islands, and the presence of 5-methyl cytosine greatly enhances BPDE binding to guanine (53, 54). The p53 mutations seen in lung cancer are guanine to thymine transversions that occur at the CpG sites where BPDE-DNA adducts are formed *in vitro* (54). Interestingly, these mutations occur on the nontranscribed DNA strand, which is repaired relatively inefficiently. Codon 157 mutations appear to be unique to lung cancer, whereas codon 248 and 273 mutations occur at hot spots in other cancers, e.g., colon, liver, and prostate. Nonsmokers who develop lung cancer have a completely different, almost random grouping of p53 mutations.

p21 has been shown to inhibit DNA replication *in vitro* by a second mechanism dependent on proliferating cell nuclear antigen (PCNA) (55). Another molecule stimulated by p53 is the growth arrest and DNA damage gene (Gadd 45), which binds PCNA, inhibits growth, and directs DNA nucleotide excision repair (56). Inactivation of wild-type p53 function can occur through complex formation with viral oncogene products such as the large T antigen of SV40, the E1b-55 kD protein of adenovirus type 5, and the E6 gene product of the human papilloma virus types 16 and 18 (57). Mutant p53 can derepress the insulin-like growth factor-I receptor (IGF-IR) promoter allowing for high-level expression in cancer cell lines and enhancing growth-promoting signals (58). Stable expression of a dominant-negative mutant of IGF-IR in the lung cancer cell line A549 enhances sensitivity to apoptosis-inducing agents and suppresses tumor formation in nude mice by promoting glandular differentiation *in vivo* (59). Wild-type p53, when introduced into a variety of cancer cell lines, reduces colony formation in agar and carcinogenicity in animal models.

The p16 Tumor Suppressor Pathway

The p16 protein from chromosome 9p21 binds to Cdk4 (hence *in*hibitor of *k*inase 4, or INK4) and inhibits phosphorylation of Rb (Figure 2) (60). Disruption of p16 function results in inappropriate hyperphosphorylation and, therefore, inactivation of Rb. Overexpression of the E2F transcription factor upregulates p16 expression and inhibits cyclin D–dependent kinase activity, suggesting the presence of a feedback loop. Inactivation of p16 may occur by homozygous or hemizygous deletion (61, 62), inactivation of the remaining p16 allele by point mutation (63), or by gene silencing through methylation of CpG islands surrounding the first exon of p16 (64). Methylation of CpG sequences in the p16 gene provides a way of suppressing expression of p16 in the absence of any mutation in the DNA and has been referred to as epigenetic regulation (64). p16 may be silenced by DNA methylation in early stages of NSCLC, whereas homozygous deletions and/or mutations may occur more frequently in later stages of NSCLC development. Alterations in both the p16/pRb and p53 pathways lead to enhanced proliferation of NSCLC cell lines, and correlate with significantly shorter 5-yr survival, suggesting an aggressive tumor phenotype (65). These genetic lesions can be mutually exclusive within any given tumor, consistent with the concept that they constitute equivalent steps in a single critical pathway (66). There is a reciprocal relationship between Rb mutations and p16 expression: whereas Rb is less frequently mutated in NSCLC than in SCLC, p16 expression is commonly absent (67). Functional Rb protein was absent in 90% of SCLC, and 15 to 30% of NSCLC primary lesions and tumor cell lines studied (68). Kelley and colleagues (69) found 18 of 77 (23%) of NSCLC to have p16 homozygously deleted compared with 1% of SCLC, and coincident loss of p16 and functional Rb protein was rarely observed. Immunohistochemistry showed strong p16 nuclear staining in Rb-negative NSCLC, which correlated with increased proliferative activity, especially in NSCLC with p53 mutations. Thus, there is an interesting inverse relation between p16 and Rb in lung cancer: in

SCLC, Rb is mutated and p16 is intact, whereas in NSCLC, p16 expression is disrupted and Rb is usually intact. A deregulated Rb pathway may correlate with overexpression of p53 and decreased MDM2, suggesting synergism in the deregulation of these pathways (70).

The INK4a locus at 9p21 gives rise to two RNA transcripts: each transcript has a distinct 5′ exon, E1a or E1b, which is spliced into common exons E2 and E3. p16 arises from the E1a-containing transcript whereas $p14^{ARF}$ (*a*lternate *r*eading *f*rame) contains the E1b transcript (66). The $p14^{ARF}$ protein is not a Cdk inhibitor and has no sequence homology to p15 or p16, but can induce cell cycle arrest, both in G_1 and G_2 (44). E2F and *c-myc* recently have been shown to directly activate $p14^{ARF}$ (71, 72), and $p14^{ARF}$ binds to the MDM2-p53 complex preventing p53 degradation (73, 74). $p14^{ARF}$ complexes with MDM2 and p53 which is localized in the nucleolus, and nuclear export of MDM2 and p53 is blocked (75). This provides a link of the E2F-Rb pathway to prolongation of activation of p53 and cell-cycle arrest, allowing for the repair of damaged DNA. This constitutes a further fail-safe mechanism to protect against aberrant cell growth. Loss of nuclear staining for $p14^{ARF}$ occurs in over 70% of SCLC and 25% of NSCLC (76). SCLC may have a greater propensity for cell proliferation through the loss of both the $p14^{ARF}$ fail-safe mechanism and p53.

Transforming Growth Factor-β Induces p15

Transforming growth factor-β (TGF-β) is a key cytokine-mediating inflammation in the lung, accumulation of matrix proteins in fibrosis, deactivation of macrophage immune response, and inhibition of growth of most epithelial, endothelial, myeloid, and lymphoid cells. Cancer cell lines may express integrins such as αv β1 that bind latency-associated peptide (LAP) that covalently binds inactive TGF-β; integrin binding on the surface of lung cancer cells may contribute to active TGF-β release. Because of its role in growth control, TGF-β is implicated in many cancer networks and is the strongest checkpoint inhibitor at G_1/S. TGF-β influences the cell cycle, particularly inducing p15 selectively as a checkpoint control causing cells to cease proliferation and arrest in G_1 (77). The Rb protein is a transcriptional activator of TGF-β1 and TGF-β2 (78). TGF-β treatment causes the accumulation of Rb in the underphosphorylated state, and expression of Rb-inactivating carcinogens prevents TGF-β-induced cell-cycle arrest. Withdrawal from the cell cycle may also induce differentiation, and TGF-β is a key molecule that may contribute to this process. TGF-β has also been shown to induce p21 and to repress *c-myc*, although these mechanisms have not been demonstrated in lung cancer cell lines or *in vivo* (79). TGF-β inhibition of Cdk 4/6 and Cdk2 can also occur via increased tyrosine phosphorylation in addition to inducing p15. This is mediated by the ability of TGF-β to repress the tyrosine phosphatase cdc25A (80); this has been found in cell lines deficient in p15. However, no effect on cdc25A was noted in the A549 lung adenocarcinoma cell line. The G_1/S arrest caused by TGF-β, p16, and contact inhibition is mediated by the Rb-E2F complex (81).

Role of Activated Oncogenic *ras* in the Genesis of Lung Cancer

Activation of the K-*ras* oncogene by point mutations in codon 12 occurs in 50% of lung adenocarcinomas (82), and polymerase chain reaction (PCR) techniques can identify these mutations in bronchoalveolar lavage (BAL) cells from patients suspected of having lung cancer (83). For example, in 52 patients with confirmed lung cancer, BAL cells contained K-*ras* codon-12 mutations in 14 of 25 adenocarcinomas, 1 of 3 bronchoalveolar carcinomas, 1 of 5 large cell carcinomas, and 0 of 14 squamous cell carcinomas. Tissue analysis matched the BAL-cell mutation in 35 cases, and no mutation was found in 30 patients with diagnoses other than NSCLC. K-*ras* codon-12 point mutations in lung cancer may predict significantly poorer survival and shorter duration of disease-free survival (84). An antisense K-*ras* construct in a retrovirus has been shown to inhibit ras protein expression in a lung-cancer cell line with mutant *ras*; colony formation in soft agar and carcinogenicity in nude mice were dramatically reduced in NSCLC cells expressing antisense K-*ras* (85).

The three 21-kD ras proteins (H-Ras, N-Ras, K-Ras) are members of a superfamily of proteins that in the active state bind to guanosine triphosphate (GTP) and in the inactive state bind to guanine diphosphate (GDP). Through the intrinsic ras guanosine triphosphatase (GTPase), ras returns to the quiescent state after interacting with its substrate c-Raf1 (86). The signal is subsequently transmitted by a cascade of kinases, resulting in the activation of mitogen-activated protein kinases (MAPK) (ERK1 and ERK2), which translocate to the nucleus and activate transcription factors. Most *ras* mutants are defective in GTPase activity and thus are locked into the growth stimulatory GTP-bound form. *ras* mutations usually occur by point mutations at codons 12, 13, or 61 (87) and in lung cancer most *ras* mutations occur at codon 12.

The *ras*-MAPK pathway is involved in establishing basal and induced levels of p53 (88). The mechanism of the *myc–ras* collaboration relates to activation of cyclin E-Cdk activity, loss of p27 inhibition, and induction of S phase (89). *ras* also positively regulates the synthesis of cyclin D1 (90) and stabilizes the short-lived myc protein (91). p16 can block the *ras* plus *myc*-induced transformation (92). An intact Rb protein is essential for *ras* signaling effects on the cell cycle. In Rb-deficient cells, inactivation of *ras* with a monoclonal antibody fails to cause G_1 arrest and the cells proliferate, demonstrating that multiple genetic lesions further enhance cell proliferation (90). *ras* activates the serine/threonine kinase *Raf*, which induces S-phase genes, but excess *ras/Raf* can induce p21 (93). Recently, *Rho* has been shown to suppress the expression of p21 and overcome the cell-cycle block (93). Levels of expression of *Rho* in lung adenocarcinomas would be of particular interest.

The discovery of $p14^{ARF}$ has provided further insights into how the oncogenes *c-myc* and *ras* promote carcinogenesis. $p14^{ARF}$ is essential for the p53-dependent arrest provoked by *ras* (94), and a loss of either of $p14^{ARF}$ or p53 would contribute to *ras* transformation. $p14^{ARF}$ is also upregulated by *c-myc* (72). For *c-myc* overexpression to succeed in cell transformation and proliferation, p53-induced apoptosis must by blocked. Analogous to *ras*, loss of $p14^{ARF}$ or p53, which are common genetic lesions in lung cancer, would enable an amplified *c-myc* unfettered opportunity for cell proliferation and transformation. $p14^{ARF}$ appears to bridge a gap between oncogenic signals and p53 whereby $p14^{ARF}$-induced activation would be critical to move the compromised cell toward apoptosis. Mice with targeted deletions of $p14^{ARF}$ are prone to develop cancers at an early age and methylation of INK4a or mutations or deletions of exon 2 which disrupt $p16^{INK4a}$ and $p14^{ARF}$ are common in human lung cancer (81, 95).

Oncogenic Pathways: *c-myc* in Lung Cancer

The *c-myc* proto-oncogene belongs to a family of related genes (*c-myc*, N-*myc*, L-*myc*) that are amplified in a subset of SCLC and, less commonly, in NSCLC. The product of *c-Myc* is a transcription factor that forms a heterodimer with Max that activates genes involved in growth control and apoptosis. myc-Max dimers activate the promoter of cdc25A, which acti-

vates Cdk2 and Cdk4, growth-factor-responsive stimulators of G_1/S progression (96). Cdc25A and cdc25B can cooperate with activated *ras* to transform primary rodent fibroblasts (97). The Mad family of proteins bind Max and antagonize the *c-myc* transactivation function (98). The Mad proteins contain a Sin 3 interaction domain that complexes with histone deacetylase which exerts transcriptional repression.

A novel growth-enhancing effect of *c-myc* is to repress growth arrest genes, e.g., *gas1*, which activates a transactivation-independent p53-mediated growth arrest function (99), gadd 45 (100), and p21 (T. C. Lee, unpublished observation). The growth arrest gene, *gas1*, is activated in G_0 growth-arrested cells, and its expression keeps cells in G_0 arrest (101). The activity of *gas1* in G_0 arrest is dependent on the presence of wild-type p53 (101).

c-myc is a positive regulator of G1-specific cyclin-dependent kinases, particularly of cyclin E/CDK2 complexes. We have observed that *c-myc* protein is overexpressed in tumor samples compared with non-neoplastic lung tissue, and that the *c-myc* antagonist Mxi1 is abundantly expressed in nonmalignant lung samples in contrast to barely detectable expression in tumors (T. C. Lee and W. N. Rom, unpublished observations). These results are consistent with active cell cycling in lung cancer tissue. *c-myc* upregulates and prevents inhibition of cyclin E/CDK2 activity by causing inactivation of the CDK inhibitor p27 and probably also of p21 and p57 by transcriptional and/or post-translational mechanisms. The cell cycle deregulation seen in NSCLC may be explained, at least in part, by *c-myc* overexpression which leads to enhanced cyclin E/CDK2 activity and Rb phosphorylation/inactivation, and entry into S phase. The most common abnormality involving *c-myc* and its other family members in lung cancer is gene amplification or gene overexpression without amplification. Overexpression of a *c-myc* family gene, with or without amplification, occurs in 80 to 90% of SCLCs (102). Only one *c-myc* gene family member is amplified in any one given tumor. In contrast to SCLC, amplification of the *c-myc* gene occurs only in approximately 10% of NSCLCs. However, *c-myc* overexpression in the absence of gene amplification occurs in over 50% of NSCLC specimens (103, 104).

Chromosomal Abnormalities: Preneoplastic Changes in Bronchial Epithelial Cells

Field cancerization is a concept that applies to lung cancer to describe the frequent occurrence of multiple primary tumors (105) or metachronous second primary lung cancer. Auerbach dissected airways of cigarette smokers and observed widespread and dispersed metaplasia (106). He and Saccomanno (107) suggested a progressive pathway to bronchial carcinogenesis in smoking uranium miners whereby dysplasia progressed to *carcinoma in situ* (CIS) over a period of 10 to 15 yr. Dysplastic lesions followed progressively have a risk for developing into invasive cancer; approximately 25% progress over 36 mo for lung, and similar incidences occur for bladder, breast, and cervical carcinomas (108).

Franklin and colleagues (105) recently observed widely dispersed p53 mutations in dysplastic respiratory epithelium dissected from a lifelong smoker who had died suddenly from coronary artery disease. Seven of 10 microdissected dysplastic lesions from both lungs had an identical G→T transversion of codon 245 in exon 7 which is a cancer "hot spot". Widely dispersed loss of heterozygosity (LOH) has also been reported in the respiratory epithelium for chromosome 3p (109). It is likely that multiple clones with varying genetic mutations develop concurrently.

The short arm of chromosome 3p (3p14.2) contains a tumor suppressor gene associated with smoking and lung cancer (110). Known as the fragile histidine triad (*FHIT*) gene, it is frequently deleted or mutated in lung cancer, and is the earliest deletion in bronchial preneoplastic lesions (111–113). Burke and colleagues found allelic deletion in 30% of 73 NSCLC when five microsatellites were evaluated encompassing *FHIT* (114). *FHIT* transfection in cancer cell lines reduces their tumorigenicity in athymic nude mice (115).

Bronchial epithelium has been evaluated for mutant p53 in areas unaffected by tumor in lung specimens resected for lung cancer. In 34 patients reported by Bennett and colleagues, there were 0/22 (mutant p53/total) in normal mucosa, 1/15 in squamous metaplasia, 3/11 in mild dysplasia, 5/17 in moderate dysplasia, 11/18 in severe dysplasia, 15/25 in CIS, and 9/11 in invasive cancer (116). Thus, mutant p53 has been identified by laser capture microdissection of early preneoplastic lesions of the lung and may have a distinct role in enhancing the proliferative behavior of the bronchial epithelium (Figure 4) (117). Human bronchial epithelial cells transfected with mutant p53 are tumorigenic when inoculated into athymic nude mice (118).

Two recent reports highlighted LOH by PCR on multiple bronchial biopsies from almost 100 long-term smokers without lung cancer (119, 120). LOH was detected in 75%, 57%, and 18% of informative subjects at chromosomal sites containing

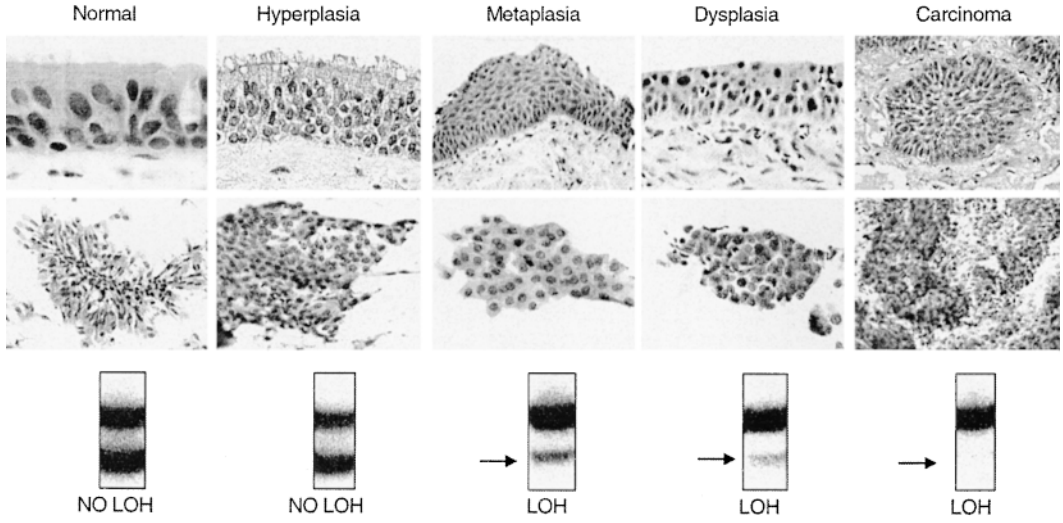

Figure 4. Loss of heterozygosity of the *p53* gene in multistage carcinogenesis. Normal, hyperplastic, metaplastic, dysplastic, and neoplastic epithelial biopsies (*top panel*); epithelial cell aggregates obtained via laser capture dissection microscope (*middle panel*); and LOH analysis by PCR (*lower panel*). Both parental alleles are retained in normal and hyperplastic epithelium, whereas metaplastic, dysplastic, and tumor samples demonstrate partial or complete loss of the lower allele. (Reprinted with permission from Maitra and coworkers [117].)

putative tumor suppressor genes: 3p14, 9p21 (p16), and 17p13 (p53), respectively (119). There was an association between increasing metaplasia and LOH. Only one of five nonsmokers had LOH at 3p14, and none had it at 9p21. Multiple LOH lesions were found more frequently in CIS lesions, and ex-smokers had similar results as smokers, suggesting that these changes were persistent. In another report (121), five of seven tumors had LOH at 9p, and LOH was detected in 38% of hyperplastic foci, 80% of dysplastic foci, and 100% in CIS lesions adjacent to the tumor. The Rb gene has been reported to be normal in bronchial dysplasia but overexpression of cyclin D1 or loss of p16 expression in 30 to 42% of the lesions disrupts the Rb pathway (122, 123). Mutations of the K-*ras* gene also occur early in the development of lung cancer. K-*ras* gene mutations were present in 18 of 57 (32%) adenocarcinomas resected from patients who had discontinued smoking 1 to 10 or more years before their resection (124), similar to adenocarcinomas in current smokers. Ten patients who had K-*ras* mutations in their lung tumors had detectable K-*ras* mutations in their sputum before the clinical detection of lung cancer, suggesting that K-*ras* activation occurs early in lung tumorigenesis (125). Wistuba (126) and colleagues microdissected 94 foci from 12 surgical resections that included uninvolved lung to study the entire 3p region as well as 9p21, 13q14 (Rb), and 17p13 (p53). They found that a third of normal epithelium had genetic changes but 42% of mildly abnormal bronchial epithelium had allelic loss at one or more regions. Other findings included: increasing LOH frequency with increasing severity of dysplasia; earliest allelic loss occurred at 3p21 followed by 9p21; p53 allelic loss was present in 80% of advanced dysplasia or CIS; and allele-specific mutations occurred in 90% of dysplasias and tumors despite determining that the dysplasias were clonally independent. Taken together, loss of the p16 and p53 loci results in the loss of tumor suppressor pathways that are critical steps in the neoplastic evolution of lung cancer. The addition of a constitutively active mitogenic pathway (*ras*-MAPK) by oncogenic activation of *ras* (or activation of receptor tyrosine kinases such as EGF-R and c-ERB-B2), whether early or late, provides a further growth program for sustained cell proliferation.

Chromosomal Abnormalities: Telomeres and Telomerases in Lung Cancer

The telomere–telomerase hypothesis states that continued shortening of telomere length which occurs in normal cells eventually results in the induction of cellular senescence, and that activation of telomerase results in unlimited replicative potential. This hypothesis is based on observations that most normal human somatic cells do not have detectable telomerase activity, whereas most human tumors have shortened telomeres and demonstrate telomerase activity.

Telomeres are repetitive noncoding DNA (TTAGGG)n nucleoprotein structures that protect the ends of linear chromosomes. Maintenance of telomere length and function depends on a specialized reverse transcriptase known as telomerase, which consists of two components—the telomerase reverse transcriptase (TERT) component, and the telomerase RNA (TR) component (127).

Telomerase activity is very low or undetectable in most human somatic tissues and primary cells. Telomeres shorten with each cell division *in vivo* and *in vitro*. A critical telomeric length, known as the Hayflick limit (128), is reached in human primary cells which limits replicative capacity and induces cellular senescence. This telomeric length checkpoint response is mediated by the Rb and p53 tumor suppressor pathways. Primary cells deficient in Rb or p53 demonstrate continued growth beyond the Hayflick limit, and suffer from marked telomere shortening, genetic instability, and massive cell death—a phenomenon known as crisis. Telomere dysfunction activates a p53-dependent checkpoint (129). The loss of telomere function and p53 deficiency as seen in mice doubly null for mTR and p53 cooperate to initiate the process of cellular transformation (129). Thus, potential cancer cells must overcome two telomeric tumor suppression mechanisms, replicative senescence and crisis. Ectopic expression of human TERT in normal human primary cells results in maintainence of telomeric length and unlimited growth (130). Telomere shortening in the absence of telomerase activity, therefore, is a critical signal for entry into senescence, and that activation of telomerase blocks this process. Immortalization of some epithelial cells, however, requires not only TERT expression but also a defective RB/p16 pathway (131). In mice doubly null for the telomerase RNA (mTR) and the INK4a tumor suppressor genes, the loss of telomere function and the inability to activate telomerase reduced the cancer incidence by greater than 50% *in vivo* (132). Reintroduction of mTR into cells significantly restored the oncogenic potential, demonstrating that telomerase activation is a cooperating event in the malignant transformation of cells containing very shortened telomeres (132).

Telomerase is expressed in most human cancers, including lung cancers. Telomerase activity in 136 primary lung cancer resection specimens and 68 adjacent nonmalignant tissues was evaluated using a PCR-based telomeric repeat amplification protocol (TRAP assay) (133). Telomerase activity was detected in 80% (109 of 136) of primary lung cancer samples versus 4% (3 of 68) normal adjacent tissue samples. Eleven of the 136 surgically resected specimens (from 11 patients) were primary SCLCs which demonstrated very high levels of telomerase activity whereas the other 125 specimens (primary NSCLCs from 125 patients) had a wider range of telomerase activity. A high telomerase activity in primary NSCLC was found to be associated with increased cell proliferation rates and advanced pathologic stage (134).

Telomerase activity was also detected in lung cancer cells obtained from bronchial washings from 82% (18 of 22) of lung cancer patients (135), whereas cytologic examination detected malignant cells in only 41% (9 of 22). Telomerase activity was detectable regardless of the tumor's location (central versus peripheral). In a similar study of 37 primary lung cancer patients diagnosed histologically, there were 24 positive cytologies and 29 positive for telomerase activity (136). A positive diagnostic outcome increased to 32 when both cytology and telomerase activity were considered. Thus, assaying for telomerase activity with the TRAP assay in addition to cytologic examination increases the sensitivity of cytology alone in making the diagnosis of lung cancer in bronchial washings.

Reactivation of telomerase expression is necessary for the continuous proliferation of cancerous cells to reach immortality and its deregulation may occur in preneoplastic bronchial epithelial dysplasias. Fresh and archival tissue samples from 40 patients (34 invasive lung cancers, 5 CIS without invasion, and 1 without lung carcinoma), were studied using the TRAP assay and *in situ* hybridization for hTR (137). Telomerase positivity was present in basal epithelial cells of normal bronchial epithelium (7 of 27, 26%) and in peripheral lung samples (14 of 60, 23%; epithelium of small bronchi and bronchioles) (137). Telomerase activity was detected in a much higher proportion of abnormal bronchial epithelial samples: hyperplasia (20 of 28, 71%), metaplasia (4 of 5, 80%), dysplasia (9 of 11, 82%), and CIS (11 of 11, 100%). Whereas normal cells demonstrate shortening of telomere length with each cell division, tumor cells show no net loss of telomere length, suggesting that

telomere stability may be a requirement for bronchial epithelial cells to escape replicative senescence.

MODELING LUNG CANCER GENETIC LESIONS IN TRANSGENIC MICE

There are few animal models of human lung cancer that portray its molecular pathogenesis. Existing lung cancer animal models include urethane, asbestos, or benzo(a)pyrene-induced pulmonary adenocarcinomas in A/J mice, Fisher 344 rats exposed to tobacco-specific nitrosamine NNK, or the SV40 large T antigen driven by the Clara cell 10 kD (CC10) protein or surfactant protein C (SP-C) gene promoter (138, 139).

Using transgenic and knockout strategies, mice have been engineered to contain inactivated critical tumor suppressor genes (p53, Rb, and INK4a) or express oncogenic *ras*. p53 null mice ($p53^{-/-}$) develop malignant lymphomas by 6 mo (140), but only 2 of 96 heterozygous $p53^{+/-}$ mice developed tumors. Harvey and colleagues (141) investigated both p53 and Rb pathways by crossing Rb-mutant heterozygous mice with p53-mutant heterozygous and homozygous mice. All of the $p53^{+/-}$ $Rb^{+/-}$ mice developed tumors by 66 wk of age, and they occurred significantly sooner than either of the singly heterozygous mice, suggesting cooperation in tumorigenesis by these two tumor suppressor genes. $p53^{-/-}$ $Rb^{+/-}$ mice resulted in reduced survival, with a heavy tumor burden, but 40% of these mice had bronchial hyperplasia characterized by foci of pulmonary endocrine cells located basally in bronchial and bronchiolar epithelium (142).

More specific to the lung, the SV40 large T antigen which binds the tumor suppressor gene products p53 and Rb, has been used in conjunction with lung-specific promoters SP-C or CC10 to produce transgenic mice with a high risk of lung cancer (138, 139, 143). Adenocarcinomas were found in the lung as early as 9 wk and most animals developed large tumors by 30 wk. We have shown that transgenic mice with a lung-specific CC10 promoter-driven dominant negative p53 gene lacking the DNA binding and transactivation domains are predisposed to lung cancer (144). Mice with targeted deletion of the INK4a locus (deleting p16 and $p14^{ARF}$) or $p14^{ARF}$ knockout mice are highly susceptible to tumors including pulmonary adenocarcinomas (95, 145).

Benzo(a)pyrene (BaP) is the most important carcinogen among the polycyclic aromatic hydrocarbons, and mainstream smoke contains 20 to 40 ng BaP per cigarette. The carcinogenicity of BaP-containing substances was recognized in chimney sweeps by Sir Percival Pott in 1775, in mulespinners in the cotton-spinning industry, among topside coke-oven workers, and finally, among smokers by Sir Richard Doll in the Report of the U.S. Surgeon General in 1964. The strain A/J mouse lung tumor bioassay has been frequently used for testing of a number of compounds for carcinogenic activity. This mouse strain has a high incidence of spontaneous lung tumors and is highly susceptible to lung tumor induction by chemical carcinogens, including BaP. Hecht and coworkers (146) used the A/J mouse and treated by weekly exposure to BaP in amounts typical for a smoker and produced lung adenomas in 70 to 100% of exposed mice compared with 7% for vehicle alone. A combined regimen of the nitrosamine NNK and BaP produced 100% lung adenomas at 16 wk. Mass and colleagues (147) correlated BPDE adduct formation in A/J mouse lungs after a single BaP intraperitoneal injection with the occurrence of G→T transversions in K-*ras* in 25 to 56% of the tumors that subsequently developed. There was a dose-dependent increase in the number of tumors per mouse lung. *ras* mutations in BaP-induced tumors occur with 93% frequency in codon 12.

GENE THERAPY FOR LUNG CANCER

Gene therapy is an exciting prospect for treatment and potential cure of lung cancer because genes can be transduced into cancer cells to correct genetic lesions such as replacing defective tumor suppressor genes or inactivating oncogenes. Experimental studies have shown that it is not necessary to correct all the genetic lesions manifest in a tumor; for instance, replacement of p53 function alone is often sufficient to induce the apoptotic death of cancer cells. Genetic approaches can also be used to express toxic genes in the tumor, and the immune response can also be modified by genetic means.

Gene Delivery Systems

A significant challenge for gene therapy is targeting, i.e., getting the gene of interest into the cancer cell. Transfection following the infection of naked DNA into most organs is not efficient. Despite this, the level of gene expression after injection of DNA into muscle or dermis may be sufficient to induce an immune response to enable a genetic vaccine strategy, as demonstrated by Conry and colleagues in a primate model using a plasmid containing the carcinoembryonic antigen (CEA) cDNA (148). There is also some efficacy after direct infection of plasmid DNA expressing p53 directly injected into liver tumors, and clinical trials using CEA-expressing plasmids for NSCLC are in progress. Plasmids attached to gold particles can be delivered by a gene gun that uses helium pressure to propel the particle through the skin approximately 70 cells deep into the dermis (149).

Liposomes, complexes of polycationic lipids with DNA, have greater transfection efficiency than naked DNA and have minimal toxicity. A Phase I study of transfected cancer cells expressing interleukin-2 in limited-stage SCLC is in progress (150), and the genes for HLA-B7/β_2-microglobulin have been transferred to patients with NSCLC with objective clinical response. Liposomes containing wild-type p53 can be introduced into the bronchial epithelium of p53 knockout mice, and this can significantly inhibit tumor formation after intratracheal tumor inoculation in athymic nude mice (151).

The most efficient means of delivering genes to tumor cells are via the viruses. The retrovirus has the advantage of integration of the genetic material into the infected cell's chromosome, thereby conferring stable expression. The retrovirus can only be produced at low titer and thus large injection volumes are required. The retrovirus is also not stable in the circulation as a consequence of complement-mediated inactivation and is therefore not suitable for intravenous administration. However, adenovirus can be produced at high titer and is now most commonly used. Other viruses including adeno-associated virus, vaccinia virus, and Sindbis virus are being investigated in experimental systems.

One strategy to target gene delivery to specific cells can be achieved by using specific receptors that may be expressed on tumor cells. The vector can then be targeted to the receptors either by the use of specific antibodies or by modification of viral structural proteins. Examples include the use of antibodies directed against neural cell adhesion molecule (NCAM) to target vectors to SCLC since 90% of SCLC express NCAM. Other examples include the modification of structural viral proteins to express a specific ligand, for instance, the gastrin releasing peptide ligand (152) or insertion of RGD sequences to target cell surface integrins (153, 154). Another strategy is to place the therapeutic gene under transcriptional control, for example, placing the promoter for the surfactant protein A (SP-A) gene in front of a toxic gene (155). The toxic gene will thus only be expressed in cells that normally express SP-A. Although not tumor-specific, this strategy does enable a level of

selectivity if the tumor expresses SP-A as 30 to 50% of NSCLCs do.

Approximately 30 to 50% of NSCLC express the epidermal growth factor receptor (EGFR) which is the product of the c-erb B1 gene and is structurally similar but distinct from the c-erb B-2 or HER2/neu oncogene protein (156). The oncogenic potential of c-erb B-1 is mediated through three potential mechanisms: overexpression, mutation in the extracellular domain (EGFR III) resulting in constitutive activation of the receptor, and heterodimerization with HER-2/neu. Tumor cells that secrete EGF and express EGFR form an autocrine loop that enhances the growth of the tumor. Expression of c-erb B-2 correlates with shortened survival time (157). Specific targeting of malignant cells has been accomplished with anti-erb B-2 monoclonal antibodies, and these can be conjugated to toxins, radioactive species, or enzyme prodrugs. Deshane (158) and Grim and colleagues (159) reported that intracellular expression of the anti-erb B-2 single-chain antibody delivered in an adenovirus was cytotoxic to c-erb B-2 overexpressing lung cancer cells and prolonged survival in animals (158, 159). Sindbis virus has been retargeted with removal of the E2 receptor binding domain followed by insertion of the IgG binding domain of protein A, a protein derived from *Staphylococcus aureus* that has a strong affinity for the Fc region of IgG. Modified Sindbis virus can then bind a monoclonal antibody directed to c-erb-B-1 or -2 and deliver a toxin to the targeted cancer cell (160).

Recently the concept of targeted replication has evolved. The adenovirus expresses a protein to inhibit p53 called the E1b-55kD protein that enables viral replication in normal cells. This protein may be redundant in cells that do not express p53, and thus deletion of the viral protein may target viral replication to cells with p53 mutations (161).

Gene therapy by introducing wild-type p53 is a successful strategy in animal models of lung cancer and has been extended to human clinical studies. Roth and colleagues administered a retroviral vector expressing the wild-type p53 gene to nine patients with NSCLC in whom conventional treatments had failed (162). The virus was administered by direct intratumoral injection by either bronchoscopic or transthoracic approaches. The treatment was safe with no clinically significant vector-related toxic effects noted up to 5 mo after treatment. Vector-specific p53 sequences were detected by *in situ* hybridization and DNA PCR in posttreatment biopsies. Tumor regression was noted in three patients, and tumor growth stabilized in three other patients. Apoptosis was more frequent in posttreatment biopsies than in pretreatment biopsies. Schuler and colleagues conducted a Phase I dose escalation study of a single intratumoral injection of a replication-defective adenoviral vector expressing wild-type p53 in 15 patients with NSCLC at doses up to 7.5×10^{12} particles (163). The vector was administered by intratumoral injection by bronchoscopy or by CT-guided percutaneous injection. No clinically significant toxicity was observed. Vector-specific wild-type p53 mRNA sequences could be demonstrated in posttreatment biopsies of six patients who received higher vector doses. Transient local disease control was observed in four of those six successfully transduced patients. There was no evidence of clinical responses at untreated tumor sites (164). Swisher and colleagues administered *Ad.p53* to 28 patients with advanced NSCLC whose cancers had progressed on conventional treatments (165). The virus was administered by intratumoral injection at monthly intervals with a maximum of six administrations. Twenty-three patients received *Ad.p53* by CT-guided percutaneous fine-needle injection and five patients by bronchoscopy at doses up to 10^{11} plaque-forming units (PFU). Adenoviral vector DNA was detected by PCR in 18 of 21 patients with evaluable posttreatment biopsy specimens. Vector-specific p53 mRNA was detected by means of reverse transcriptase/polymerase chain reaction (RT-PCR) analysis in 12 of 26 patients. Apoptosis was demonstrated by TUNEL (*in situ* DNA nick end labeling) assay in posttreatment biopsy specimens from 11 patients. Vector-related toxicity was minimal and therapeutic activity in 25 evaluable patients included two partial responses and disease stabilization in 16 patients.

Monitoring, Therapeutics, and Delivering Toxins via Gene Therapy

Marker genes can be used to assess the efficacy of gene transfer. Tursz and colleagues (166) evaluated six patients with inoperable lung cancer with an endobronchial lesion accessible by bronchoscopy that was injected with a single dose of a recombinant, replication-deficient adenovirus (rAd.RSV.beta-gal). Beta-galactosidase was expressed in tumor biopsy specimens of three patients, and after therapy, endoscopic and clinically objective antitumor responses were seen in four patients, including one patient who showed a complete response by pathologic evaluation (166). Gahery-Segard and colleagues (167) were interested to see whether a recombinant adenovirus could induce long lasting immunity against an expressed transgene despite an anticipated immune response directed against the virus. They administered a recombinant adenoviral vector that expressed the β-galactosidase protein to four patients with lung cancer by a single intratumoral injection. The β-galactosidase protein was detected in biopsies at Day 8 from all patients. Recombinant viral DNA was also detected by PCR in tumor biopsies at Day 60 in three patients. All patients developed potent $CD4^+$ T helper cell, type 1 (Th1) responses to adenoviral particles, and cytotoxic T-lymphocyte responses to adenovirus were boosted. Thus, despite antiadenovirus responses, transgene protein expression was sufficient to induce strong and prolonged immunity suitable for immunizing patients against the transgene protein.

Sterman and colleagues have reported (168) that intrapleural administration of an adenoviral vector containing the *Herpes simplex virus thymidine kinase* (*HSV.tk*) gene is well tolerated and results in detectable gene transfer when delivered intrapleurally at high doses in patients with mesothelioma. The *HSV.tk* gene product can phosphorylate ganciclovir to a triphosphate form that is toxic to the cancer cell containing the *HSV.tk* gene. In addition, the triphosphate form of ganciclovir diffuses to adjacent cells through intercellular junctions killing adjacent cells known as the bystander effect. Twenty-one previously untreated mesothelioma patients received doses up to 1×10^{12} PFU of a replication-incompetent recombinant adenoviral vector containing the *HSV.tk* gene under control of the Rous sarcoma virus promoter. The *Ad.HSV.tk* was introduced directly into the pleural cavity of patients followed by 2 wk of systemic therapy with ganciclovir at a dose of 5 mg/kg twice a day. Patients underwent thoracoscopic pleural biopsy 3 d after vector delivery. Dose-limiting toxicity was not reached, and side effects were minimal and included fever, anemia, transient liver enzyme elevations, and bullous skin eruptions. Intrapleural and intratumoral immune responses were generated. *HSV.tk* gene transfer was documented in 11 of 20 evaluable patients in a dose-related fashion using RT-PCR, *in situ* hybridization, immunohistochemistry, and immunoblotting. A similar adenovirus expressing the *HSV.tk* gene is being administered in a Phase I trial to patients with NSCLC using bronchoscopic or transthoracic injection (W. N. Rom and S. Woo, unpublished results).

Important to the progression from a colony of cells to a growing tumor are induction of genes that stimulate endothelial cell incursion to form capillaries, and nearby stromal cell

activation to release metalloproteinases with the capability to digest matrix proteins and allowing for tumor cell invasion. Central to these concepts is an internal, central hypoxia in the tumor mass which leads to induction of transcription factors, e.g., hypoxia-inducing factor (HIF-1) to activate genes such as vascular endothelial growth factor (VEGF) necessary for capillary formation (169). At this juncture, the orchestration of the cancer phenotype is well underway, albeit clinically undetectable. Inhibiting VEGF with antiangiogenesis genes would be another gene therapy.

SUMMARY: CELL-CYCLE NETWORKING

Insights into cell-cycle networking have grown exponentially in the past several years leading to the concept that lung cancer is a disorder of the cell cycle. Although many of these findings are applicable to the lung, lung cancer may be unusual in that the progenitor cells give rise to squamous cell carcinoma, adenocarcinoma, small cell carcinoma, or other cell types. The lung is also the target organ for many environmental toxicants, consequently extrapolating from *in vitro* studies to the lung requires studies of various lung cells directly. It is clear that mutations of cell-cycle genes occur in a sequential manner in the lung eventually leading to clonal cell expansion. After 8 to 12 mutations, a malignant clone proliferates into a CIS lesion where the apoptotic pathway to destroy wayward cells has been sabotaged.

Treatment strategies to cure lung cancer will have to focus on these early genetic lesions to enhance their repair, or to trigger the apoptotic pathway to eliminate wayward cells. The lung would be an excellent target for a strategy that involves inhalation of such a chemopreventive or protective agent.

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