Preface

This work, *Lung Cancer, Volume 2: Diagnostic and Therapeutic Methods and Reviews*, in the *Methods in Molecular Medicine* series presents an overview of the current status of those methods useful in the diagnosis and treatment of lung cancer—both as it exists in the clinic and as it is being revolutionized in the laboratory. The book is intended to serve as a resource for researchers wishing to increase their knowledge of current and cutting edge technologies, in order that their investigations into neoplasms of the lung may benefit from this enriched diversity of techniques and approaches.

Owing to the complex nature of the disease and the variety of methods available to analyze and attack it, no volume attempting to define diagnostic and therapeutic approaches to lung cancer can ever be complete. The sheer number of investigators involved in lung cancer research guarantees that some aspect will be inadvertently excluded. However, I hope that the range of techniques included herein will serve to open up new avenues of investigation for both the novice and experienced researcher.

As with all volumes in the *Methods in Molecular Medicine* series, the reader should find that each methods-based chapter provides clear instructions for the performance of various protocols, supplemented by additional technical notes that provide valuable insight. These notes should offer the reader a perspective on the skills and materials required for performance of both standard and novel techniques. In putting together this volume, one aim was to provide clinicians and laboratory investigators an appreciation of the current status of lung cancer diagnosis and treatment. However, *Lung Cancer, vol. 2* is also a look into the future, with descriptions of novel methods for molecular diagnosis as well as techniques for treatment based on gene therapies, new anticancer approaches, immune therapies, and chemoprevention. Chapters describing the challenges faced by those attempting to employ these new methods should also be of use to those determined to translate basic research from the laboratory to the clinic.

I would like to express my gratitude to the contributors who made this volume possible and for their patience during the period the volume was collated. I am grateful to Professor John Walker for his encouragement and guidance as series editor.

This volume is dedicated to the memory of Richard Mackenzie Brown, Warren Reardon, and Ching-Tuan T'Ang, three fathers, deeply missed.

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Molecular Alterations in Lung Cancer

Impact on Prognosis

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1. Introduction

Lung cancer is a devastating illness, and very few of the advances in chemotherapeutics have enhanced survival over the past decade. In order to make an impact on this disease, we must understand the molecular abnormalities to target better therapeutics. In this chapter, we will describe the better understanding at the molecular level that has been gained for lung cancer and its prognosis. Over the last decade, advances in molecular biology have provided important information about potentially significant determinants of prognosis in lung cancer ***(1**,**2)***. These molecular biomarkers are typically expressed in neoplastic lung tissue. Molecular abnormalities include chromosomal aberrations, telomerase expression, expression of oncogenes, and loss of tumor suppressor genes. Based on the understanding of the abnormalities at the cellular and molecular levels, newer therapies can be pursued.

2. Molecular Biological Abnormalities in Lung Cancer

2.1. Model of Lung Cancer Development

For any tumor to become cancerous, various mutations/alterations occur in the cell, which render it neoplastic. To understand the mechanisms for transformation, we will briefly describe several pathways that can be activated or suppressed in the pathogenesis of lung cancer. In terms of development of a lung cancer cell, the cell is surrounded by other cells and extracellular matrix (ECM). The ECM and various molecules in the circulation can communicate

From: *Methods in Molecular Medicine, vol. 75: Lung Cancer, Vol. 2: Diagnostic and Therapeutic Methods and Reviews*
Edited by: B. Driscoll © Humana Press Inc., Totowa, NJ

with the cell through receptors such as growth factor receptors, adhesion molecules (such as cadherins), and integrins. Once a transducing signal is achieved in the cell, different pathways may be activated, which can lead to dramatic changes that could affect the cytoskeleton, morphology, or migration of the cell. Eventually, the signal is transmitted to the nucleus, and certain genes can be activated or suppressed, which results in malignant transformation.

When a cell becomes neoplastic, many changes occur inside and outside the cellular environment. The cell not only changes its internal homeostatic mechanisms, but also changes the extracellular environment. Eventually, the cancerous population multiplies and, thereafter, may metastasize and colonize in different sites. For a carcinoma to metastasize, many events occur such as: invasion with changes in tumor cell adhesion, proteinase production, and locomotion; intra-vasation and extra-vasation from the circulatory system; colonization in the distant site; and angiogenesis in the new site of implant. For the purposes of this review, we will restrict ourselves to chromosomal abnormalities, activation of oncogenes, and loss of tumor-suppressor genes in lung cancer. We will also emphasize the importance of prognostication with the various genes that become abnormal in lung cancer.

2.2. Chromosomal Abnormalities, Telomerase Activation, and Implications

Using both actual tumor specimens and cell lines, various chromosomal and oncogene abnormalities have been identified in lung cancer. There have been a number of reports on the various chromosomal abnormalities (including loss of complete chromosomes or portions thereof) that can occur in lung cancer. For example, in non-small cell lung cancer (NSCLC), chromosomal aberrations have been described on 3p, 8p, 9p, 11p, 15p, and 17p with deletions of chromosomes 7, 11, 13, or 19. Also, in small cell lung cancer (SCLC), chromosomal abnormalities have been described on 1p, 3p, 5q, 6q, 8q, 13q, or 17p ***(3)***.

One of the most consistent chromosomal abnormalities in lung cancer has been the loss of the short arm of chromosome 3 (3p[14-25]) ***(4)***. The loss of alleles at 3p is observed in >90% of SCLC tumors and approx 50% of NSCLC tumors ***(5)***. Various groups are trying to clone out the tumor-suppressor genes, which may be involved in the loss of 3p regions. As an example, the FHIT gene (for fragile histidine triad) has been localized to 3p14.2 and about 80% of SCLC tumors shown abnormalities of this gene ***(6)***. The protein product of the FHIT gene is involved in the metabolism of diadenosine tetraphosphate into ATP and AMP. Loss of FHIT gene results in the accumulation of diadenosine tetraphosphate and could lead to the stimulation of DNA synthesis and proliferation. The FHIT gene may represent one of several potential tumor

suppressor genes located on chromosome 3p involved in the pathogenesis of SCLC.

Other genetic losses have, although not consistently, been identified in lung cancer. In NSCLC, these include genetic loss at chromosome 8p(21.3-22) and may affect in 50% of multiple samples ***(7)***. Genetic loss at 9p(21-22) could potentially involve the p16 (MTS1/$p16^{INK4A}$) and p15 (MTS2/$p15^{INK4B}$) tumor suppressor genes, which are involved in cell-cycle regulation at the G1 checkpoint by inhibiting cyclin-dependent kinase CDK4 and may be affected in 67% of tumor samples ***(8,9)***. Genetic loss at 11p (p13 and p15) may involve the Wilms' tumor suppressor gene at region p13 and can be affected in 20% to 46% of tumor samples ***(10)***. SCLC exhibits infrequent loss of 9p, but more losses than NSCLC of 3p, 5q, 13q, and 17p ***(5)***.

Telomeres, which are genetic elements at the ends of linear eukaryotic chromosomes from degradation, illegitimate recombination, or cellular senescence. Long telomeres are present in germ cells and most cancer cells via the telomerase enzyme, and this probably maintains the ability of the cells to divide indefinitely ***(11,12)***. Telomerase activity has been directly correlated with malignant and metastatic phenotype of a wide array of solid tumors. In one study, 80% of tumor tissue from lung cancer had telomerase activity ***(13)***. Telomerase activity was measured in bronchial washings with 18 of 22 patients with lung cancer being positive, whereas only 1 of 19 without cancer ***(14)***. Because telomerase activation is essential for long-term growth of many malignancies, inhibition of this enzyme would be an attractive target for therapy ***(11)***.

Compared to the chronology of colon cancer development, it is quite difficult to arrive at the chronology of events for a normal cell to develop from a preneoplastic lesion to a frank neoplasia in lung cancer. It is possible that multiple synchronous molecular abnormalities occur in response to toxins such as cigarette smoke, which transform the normal lung cell into a cancerous cell.

2.3. Oncogenes and Tumor-Suppressor Genes

Various oncogene expressions have been investigated in NSCLC and SCLC. There are two forms of oncogenes: dominant oncogenes, and tumor-suppressor genes. Dominant oncogenes, such as RAS, MYC, HER-2/NEU, and BCL-2, exert their effect by overtaking the normal cellular growth function; tumor-suppressor genes that exert their effect in controlling cellular growth. Once suppressor genes such as p53, RB, $p16^{INK4A}$, $p15^{INK4B}$, and genes on chromosome 3p are deleted or mutated, normal control mechanisms are not available. None of the genes have been implicated in the etiology of lung cancer 100% of the time ***(1)***.

2.3.1. Oncogenes

2.3.1.1. RAS Genes

The RAS-dominant oncogenes play an important role in signal transduction and cellular proliferation. The RAS proteins have a molecular weight of 21 kDa and consist of K-RAS, H-RAS, and N-RAS. The RAS proteins are active when bound to guanosine triphophate (GTP) and are inactivated by GTPase-activating protein (GAP) by hydrolyzing GTP to guanosine diphosphate (GDP). These proteins acquire transforming potential secondary to a point mutation at codon 12, 13, or 61 in the encoding gene. Mutations at or near the GTP-binding domain of RAS protein prevents the inactivation of GTP, thereby resulting in continuous RAS activity.

Activation of the K-RAS oncogene is an adverse prognostic factor in resectable adenocarcinoma of the lung. As an example, a point mutation in the K-RAS oncogene was noted in 29% of 69 resected specimens in one series ***(15)***. Tumors associated with the mutation tended to be smaller but more poorly differentiated. Death during a median follow-up of 3 yr was more common in patients with the K-RAS mutation than in those without mutation (63% vs. 32%). There were no significant associations between K-RAS mutations and tumor size, stage, nodal status, or tumor differentiation. Several other reports also demonstrated a decrease in survival associated with K-RAS mutations in patients with respectable NSCLC ***(16,17)***, but such findings have not been universal ***(18,19)***.

2.3.1.2. MYC Genes

The MYC-dominant oncogenes, c-MYC and N-MYC, and L-MYC, encode for nuclear DNA binding proteins, which are involved in transcriptional regulation. The general mechanism of activation of MYC genes in lung cancer is gene amplification with resulting overexpression ***(20,21)***. The frequency of abnormal expression of MYC genes is low in NSCLC (10%) and variable in SCLC (10–40%). Studies have shown that amplification of c-MYC genes adversely affect survival in SCLC ***(22–24)***. In cell lines established after progression from cytotoxic chemotherapy, 44% of the cell lines had MYC amplification ***(25)***. A total of 80–90% of SCLC also show overexpression of MYC RNA as compared with normal lung tissue ***(26)***. c-MYC amplification is rarely seen in NSCLC ***(27)***. In a study of Japanese patients with NSCLC ***(28)***, the restriction fragment-length polymorphism (RFLP) of the L-MYC gene may be a marker for metastatic potential. However, there may be geographical differences, since no RFLP changes of the L-MYC gene were detected in NSCLC tumor samples from Australian, Norwegian, and North American patients ***(2,10)***.

2.3.1.3. HER2/NEU Gene

c-erbB-1 proto-oncogene encodes the epidermal growth factor receptor (EGFR) and has been a classic model for signal-transduction events in normal and transformed cells. A related proto-oncogene, c-erbB-2 (also known as HER2/NEU), encodes for a protein product of molecular weight 185 kDa ($p185^{neu}$), and is a growth-factor receptor. The frequency of normal expression of c-erbB-2 in NSCLC is approx 25%, and it has not been reported to be abnormal in SCLC. Overexpression of the c-erbB-2 has been shown to be associated with an adverse prognosis in adenocarcinoma of the lung ***(29)***. An antibody against the $p185^{neu}$ has been shown to inhibit proliferation of NSCLC cell lines ***(30)***.

2.3.1.4. BCL-2 Gene

The BCL-2 proto-oncogene product inhibits programmed cell death, termed apoptosis. BCL-2 overexpressing cells have expansion of cell populations secondary to lack of apoptosis. The expression of BCL-2 in NSCLC has been evaluated ***(31)***. Of the various tumors evaluated, BCL-2 protein was abnormally expressed (where the pattern of expression in lung cancer cells differs from that in adjacent normal tissue) in 20 of 80 squamous cell carcinomas and in 5 of 42 adenocarcinomas. Basal cells in adjacent normal epithelium were positively stained for BCL-2; however, the more differentiated columnar cells were negative. In a group of patients with squamous cell carcinomas, 5-yr survival was better for patients with BCL-2- positive tumors (78% vs 48%, $p < .05$) evaluated ***(31)***. The explanation for better prognosis for BCL-2-positive tumors has not yet been determined; however, there is a possibility that the increased expression of BCL-2 involved in the pathogenesis of some cases of lung cancer confers a survival advantage through its anti-apoptotic effects ***(32)***.

2.3.2. Tumor-Suppressor Genes

2.3.2.1. p53 Gene

The p53 family of genes includes p53, p73, and p63. The original p53 gene, located at the chromosome 17p13.1 encodes a nuclear protein that acts as a transcription factor and blocks the progression of cells through the cell-cycle late in the G_1 phase. The most common genetic changes associated with cancer involve mutations of the p53 gene. p53 gene mutations cause a loss of tumor-suppression function, promoting cellular proliferation. Some p53 mutant proteins also have transforming properties, and can bind and inactivate available wild-type (normal) p53. The Li-Fraumeni cancer syndrome that is typified by multiple tumors at an early age of onset is characterized by inherited forms of p53 mutations. p53 genetic mutations can involve deletions, point

mutations, and overexpression. In lung cancer, the prevalent type of point mutation is a GC to TA transversion and related to adducts of benzo(a)pyrene from cigarette smoking ***(12)***. Frequency of mutations may be up to 50% in NSCLC and 80% in SCLC ***(33)***. Abnormal expression of p53 has been shown to correlate with both better and worse prognosis and further work is needed to refine these observations ***(34,35)***. In contrast to p53, the p73 gene (located on chromosome 1p36) is not dramatically altered in lung cancer, as observed by analysis of 17 lung cancer cell lines, only three of which exhibit mutations that affect the amino acid sequence ***(36)***. Finally, the p63 (located on chromosome 3p27-28) mutation was detected in the DNA-binding domain in one squamous cell carcinoma cell line of the lung but generally appears to be rare ***(37)***.

2.3.2.2. RB Gene

The RB gene, located on chromosome 13q14.11, encodes for a nuclear protein which was determined to be abnormal in patients with retinoblastoma. Knudson predicted the tumor-suppressor nature of this gene by studying inheritance patterns of familial retinoblastoma ***(38)***. The protein encoded for by RB is a 105 kDa phosphoprotein, important in regulating the cell cycle during G_0/G_1 phase. A deletion of the RB gene can be found in >90% of SCLC, and the abnormal expression of the tumor-suppressor gene RB may be an adverse prognosticator in SCLC ***(39,40)***.

In NSCLC, there is absence of normal RB mRNA in 10% of cell lines, and absence of normal RB protein in up to 30% of tumors ***(41,42)***. Most of the RB-positive lung cancer cell lines that have been tested are also negative for $p16^{INK4A}$, a kinase inhibitor of CDK4, and thus a strong inhibitor of RB phosphorylation ***(9)***. This would indicate that the pathogenesis of some lung cancers could occur by either the mutational disruption of RB protein or by the absence of the $p16^{INK4A}$ inhibitor that functions to keep RB hypophosphorylated and therefore active ***(43)***. In addition, there may be a correlation between increased abnormal RB protein expression and stage in NSCLC. For example, in one study, abnormal expression was positive at a ratio of 20% for stages I and II and 60% for stages III and IV ***(42)***.

2.3.2.3. $p16^{INK4A}$ and $p15^{INK4B}$ Genes

Certain lung cancer cells have a characteristic deletion of chromosome 9p21, thus implicating one or more tumor suppressor genes in this region as being important. From genetic analysis, $p16^{INK4A}$ (hereafter designated p16) and $p15^{INK4B}$ (designated p15) have been identified to map within 30kb of each other in this region. p16 was originally identified as a binding protein to CDK4 in a yeast two-hybrid screen, and contains four ankyrin repeats ***(44)***. p15 was

thereafter cloned from a low stringency screen using p16 probe on a human keratinocyte cDNA library, and has a very high homology with p16. In some cells stimulated by TGF-β, p15 induction is a 30-fold greater than p16 ***(45)***. Both p16 and p15 encode for proteins that inhibit CDK4-regulated cell cycle control, thereby preventing progression from G_1 to S phase. The expression of p16 has been evaluated in lung cancer, and one study reported that 18 of 27 primary NSCLC contained no detectable protein ***(46)***. The p16-negative tumors were invariably positive for RB protein expression.

3. Summary

There are multiple molecular abnormalities that can occur in lung cancer. Based on the aberrancies described previously, many investigators and drug companies are designing novel therapies. The molecular markers can also be used as prognostic variables for future clinical trials and therapeutic interventions. It will not be an easy task to make an impact on lung cancer using these methods, since multiple pathways are abnormal in its pathogenesis. It is hoped that with the advent of novel and directed therapeutics, we may soon show some impact on the survival of this devastating disease.

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