Tumor Suppressor and Immediate Early Transcription Factor Genes in Non-small Cell Lung Cancer

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Non-small lung cancer (NSCLC) is a disease that exhibits multiple genetic lesions. Lung Cancer Study Group (LCSG) 871 was designed to analyze this group of malignancies for alterations in growth factors and/or their receptors, oncogenes, tumor suppressor genes, and immediate early transcription factor genes. Immunohistochemical analysis showed that 32% of evaluable cases studied contained absent or abnormal Rb expression. Sequence analysis of the p53 gene revealed that 58% of these cancers contained structural alterations of this gene, whereas only 45% of these cases overexpressed p53 by immunohistochemical analysis. Finally, both Northern blot and immunohistochemical analysis showed that these tumors exhibited changes in the mRNA and protein expression levels respectively of the immediate early transcription factor genes c-fos, c-jun, and EGR, in that less expression of these genes was evident in the tumors compared with adjacent normal tissue. Understanding both the biologic and molecular significance of these findings may allow us to explore novel modalities for treatment of this disease.

Lung cancer will remain the leading cause of cancer death for both sexes in the United States this year. It is estimated that there will be 100,000 new cases of lung cancer in men and 72,000 new cases in women in 1994. Non-small cell lung cancer (NSCLC) comprises approximately 75% of all lung cancers, and includes adenocarcinomas, squamous cell carcinomas, bronchioalveolar carcinomas, large cell carcinomas, with the remaining consisting of combined histologic features and the uncommon pulmonary carcinoids. Several etiologic factors have been implicated in the development of lung cancer. These include smoking, which accounts for 75 to 80% of the deaths per year, exposure to hydrocarbons, radon, and asbestos. While exposure to these factors can often be controlled by changing one’s life-style, once a tumor has initiated, the prognosis is often poor because the disease usually presents at an advanced stage. The Lung Cancer Study Group (LSCG), through a cooperative effort, has been able to test different combinations of current acceptable modalities of treatment such as chemotherapy, radiation, and surgery, yet it has become increasingly clear that these treatments do not impact significantly on an increase in long-term survival of these patients. In fact, only 13% of individuals with lung cancer will be alive 5 years after diagnosis. Thus, we must begin to examine novel therapeutic approaches toward treatment of this disease. One area that has begun to be explored is the molecular biology of lung cancer and how genetic alterations impact on the pathophysiology of the disease.

How are these tumors initiated and what molecular alterations allow their progression? Although lung cancer has a pathophysiology distinct from other major cancers such as prostate, colorectal, and breast, all of these diseases appear to have common genetic alterations in both dominant oncogenes (HER-2/neu, H-ras, c-myec) and recessive or antioncogenes (p53, Rb). Thus, despite the biologic heterogeneity of these tumors, there appears to be a common denominator in terms of some of these alterations. This is encouraging because by studying one type of malignancy that contains an alteration in these genes, we may gain valuable insight into the role this mutation may play in many unrelated malignancies. Moreover, the pathway from the normal to the transformed phenotype is a multistep process, with perhaps as many as 10 or 20 of these shared genetic mutations occurring by the time lung cancer becomes clinically evident. Thus, by identifying cells that contain early lesions, it may be possible to interfere with tumor progression using very early treatment strategies. The goal of these types of studies is to understand genetic alterations that occur in tumor development, and ultimately to provide more specific and effective treatments in the form of novel therapeutics.

Alterations of Tumor Suppressor Genes in NSCLC

Oncogenes, identified some years ago as positively acting or dominant transforming genes, may cause cellular transformation through a variety of mechanisms. When overexpressed or functionally altered, they lead to uncontrolled cell proliferation and provide a growth advantage for cells. Although many dominant oncogenes have been shown to be altered in NSCLC, a growing body of evidence indicates that tumor suppressor or antioncogenes are altered as well. In contrast to dominantly transforming oncogenes, tumor suppressor genes contribute to the transformed phenotype through loss of function mutations, which result in the absence of a functional gene product. Cells that contain such mutations have a growth advantage over cells that continue to express normal gene products. Thus, it is thought that normal versions of these proteins act to confine or restrain cell growth and/or proliferation.

The prototypic tumor suppressor gene is the Rb or retinoblastoma gene, which was identified through its role in the development of childhood retinoblastoma. Inactivation of the Rb locus can be acquired in both a heritable and nonheritable fashion, and is recessive to the normal allele. As such, loss of function of both copies of the gene is necessary for cellular transformation and subsequent tumor development. Thus, if one inherits a single mutated Rb allele, progression may occur following the loss or inactivation of the remaining normal allele in growing retinal cells through spontaneous somatic mutations.
Although it is thought that inactivation of the Rb gene is central to the development of these ocular tumors, this gene is also altered in several other human malignancies, including osteosarcomas soft tissue sarcomas, and small cell lung cancer. As part of the LCSG 871 study, we studied the structure and expression of the Rb gene in 219 cases of primary NSCLC. This study compared sensitivities of several modes of detecting alterations in Rb at the DNA, messenger RNA (mRNA), and protein levels. It was shown that Southern (DNA) analysis revealed only 2 of 219 (0.9%) altered cases of the Rb gene. Northern analysis revealed 22 of 219 (10%), while fully 53 of 163 (32%) of evaluable cases studied by immunohistochemistry (IHC) detected absent or abnormal protein staining. These data suggest that IHC is a more sensitive technique to detect Rb alterations. However, analysis of the clinical outcome of patients whose tumors were shown to be Rb inactivated did not correlate with relapse or death.

A second tumor suppressor gene called p53 appears to be important in the pathogenesis of many unrelated tumor types, as it has been shown to be altered in almost half of all human tumors studied. It is now known that the p53 gene product, in addition to several other important functions, plays a pivotal role in negatively regulating cell growth rate. Cells normally express p53 at very low levels. However, in response to drug or x-ray-induced DNA damage, normal cells increase their p53 expression. These cells subsequently become arrested during the G1 stage of the cell cycle until the damage is repaired. Alternatively, cells will undergo apoptosis (programmed cell death through several proposed mechanisms). This function serves to prevent cells containing a growth advantage to continue to divide. In contrast to cells with normal p53 function, those that contain an altered p53 gene are not cell cycle arrested, do not undergo apoptosis, and continue to divide rapidly. In addition to regulating programmed cell death, p53 functions as a DNA binding protein. Found in the nucleus, p53 molecules form tetramers that can bind DNA at sequence specific sites and are believed to activate transcription of genes that negatively control growth and/or invasion. Thus, it is clear that the p53 gene product plays a critical role in fundamental cellular processes.

What is the mechanism through which p53 regulates the cell cycle? Recently, several independent lines of research showed that the protein encoded by p53 increases protein expression of a second gene product, a 21-kd protein. This protein, called CIP-1 (for Cdk interacting protein-1) or WAF-1 (for wild type p53-activated fragment) interacts with several members of a family of enzymes known as "cyclic-dependent kinases" or Cdk's, whose function is to regulate progression through the cell cycle. CIP-1/WAF-1 appears to inhibit a specific Cdk known as Cdk2, and through a series of biochemical events, progression through the cell cycle is blocked, thus allowing cells time to make necessary repairs in DNA damage. With this model in mind, and realizing that p53 acts to arrest DNA synthesis in damaged cells, one can imagine that p53 accumulates in response to DNA mutations to arrest replication and thus allows time for repair of damaged DNA. Tumor cells that lack intact p53 cannot carry out this G1 arrest, and thus replicate faster, adding to the instability of their genome by accumulating mutations at an increased rate. Taken together, this model helps to clarify the role of p53 as a tumor suppressor gene, because the loss of p53 function could subsequently lead to uncontrolled cell growth.

What is the molecular alteration responsible for p53 mutations? Other tumor suppressor genes such as Rb are commonly inactivated by nonsense mutations that result in an unstable or truncated protein product. However, p53 is most frequently mutated through missense mutations that result in the change of one of the amino acids. This can have several consequences, including increasing the stability of the mutant gene product, causing it to accumulate in the nucleus to the point that it can be detected by immunohistochemical staining. It has been hypothesized that as mutant p53 protein accumulates, it has a "dominant negative" effect by binding and inactivating the remaining normal p53 protein. This would result in the lack of activation by normal p53 of growth inhibitory genes, and provide a growth advantage to these cells. However, most p53 mutations in tumors involve inactivation of both alleles, casting some doubt on whether a dominant negative effect occurs in vivo.

There were several goals of the LCSG 871 p53 study. First, we wanted to identify the most common regions in NSCLCs in which p53 mutations occur. To address this question, we performed reverse transcriptase (RT) polymerase chain reaction (PCR) on both strands of the DNA in 154 lung tumors to sequence the p53 mRNA message encoded by exons 2 through 11 of the p53 gene. Next, we wanted to ask how this sequencing data (which we assumed to be the "gold standard") correlated with IHC staining. In analyzing these data, we assumed that most point mutations would result in stabilization of the p53 gene product, and thus be seen as positive staining by IHC analysis. However, we were unclear whether this would be the case for nonsense mutations. Indeed, we found that this was not the case for almost all nonsense mutations, including frame shift, stop codon, or splice junction mutations (all of which may result in a truncated protein product) exhibited normal or negative staining of p53. These data suggest that the p53 gene product is not stabilized by these types of mutations.

Results of this study show that 58% of the NSCLCs contained sequence mutations, whereas positive immunohistochemical staining was detected in only 45% of evaluable cases of lung tumors. Although there was some concordance between p53 missense mutations and positive immunostaining, there was poor concordance between other types of p53 mutations (including deletions, splice mutations, premature stop codons, and base additions). Of those tumors containing p53 mutations, positive staining was detected in 73% of lung tumors. These data support other studies that suggest that IHC staining is not sufficient to detect mutations in all tumors. In addition, there were 9% of lung tumors that were wild type by sequencing, yet positive for p53 by IHC, suggesting an alternative mechanism for the involvement of p53 in the pathogenesis of this disease. We are currently examining the correlation of p53

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mutation and clinical outcome of these patients in terms of prognosis. These data may give insight into the biologic relevance of these observations.

**Immediate Early Transcription Factor Genes in NSCLC**

A number of oncogene products, including those of the *myb, myc, jun, and fos* genes, are known to reside in the nucleus and function as factors that bind DNA to regulate transcription levels of responsive genes. Alterations in the expression of these genes are associated with changes in cellular growth properties. Of the members of the transcription factor family of oncogenes, *c-fos* and *c-jun* are perhaps the best studied. They are classified as immediate early transcription factors, and are strongly inducible by mitogenic stimuli such as serum, calcium ionophores, or differentiating agents without the need for de novo protein synthesis. Once activated, this class of genes rapidly increases their mRNA expression some 40- to 50-fold over baseline, and then declines.22

The proteins encoded by these genes contain regions rich in the amino acid leucine, which allow these proteins to combine into a *fos-jun* heterodimeric complex known as AP-1.23 This creates a high-affinity binding site for DNA activation (Fig 1). Other members of the immediate early transcription factor gene family include the early growth response (*EGR*) family members whose kinetic response to mitogenic stimuli resemble *fos* and *jun*. Like *fos* and *jun*, the *EGR* gene product functions as an immediate early transcription factor to bind DNA and activate transcription through a common DNA sequence.25

Finally, it appears that all three genes are important for entry of cells into the cell cycle in that growth stimuli allow these genes to increase their mRNA expression during the G0/G1 phase of the cycle.26 As this phase is critical for control of entry of cells into mitosis, these genes appear important in the regulation of cell proliferation. Thus, alterations in the normal expression patterns of these genes may be important to the development of some transforming events.

Since these genes are involved in early regulation of cellular growth properties and at least two (*c-jun* and *c-fos*) can act as oncogenes, we wished to determine whether their expression levels were altered in human lung cancers compared with normal lung tissue. To address this, Northern blot analyses were performed using *c-fos*, *c-jun*, and *EGR* probes on RNA extracted from a cohort of NSCLCs and their adjacent normal matched tissue. Analysis of this cohort revealed that 73% of the tumors demonstrate markedly lower expression of these transcription factors compared with adjacent normal tissue. Moreover, this expression pattern appeared to be coordinate for all three genes in most cases. This differential expression pattern of the mRNA transcripts was confirmed at the protein level using an immunohistochemical approach with antibodies directed against the *c-jun*, *c-fos*, and *EGR* gene products. Southern blot analyses did not reveal any gross alterations of these genes at the DNA level, indicating that the observed differential expression pattern was not due to gross structural changes in the genes. These data suggest that downregulation of these genes may be involved in the pathogenesis of lung cancer, although the mechanism through which this occurs remains unclear.

**Prospects for the Future**

Several molecular alterations in NSCLC have been identified, and it is clear that many of the genes affected are important in the regulation of cell proliferation. We have gained much knowledge about how these genes are regulated throughout the cell cycle, and are exploring ways in which to control the deregulation of this fundamental process when these genes become altered (Fig 2). Many of these alterations, however, may not contribute directly to the pathogenesis of this disease. Thus, it is clear we need to understand the biologic relevance of these observations before we begin to think about restoring their actions. Looking at what regulates these altered genes rather than the genes themselves may give us critical information as to how an alteration results in malignancy. For example, while loss of the Rb protein does not correlate with poor prognosis, it may be that we need to look further along the signal transduction pathway of this gene product to pinpoint a more critical target candidate. Downregulation of the immediate early transcription factor genes *fos, jun,*

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**Figure 1.** When stimulated, the immediate early transcription factors *fos* and *jun* combine to form a heterodimer called AP-1, which binds specific DNA sequences to activate responsive genes.

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Figure 2. Cell cycle regulation of genes implicated in NSCLC. Immediate early genes are upregulated during the G0/G1 stage of the cycle to regulate admission and proliferation. p53 and CIP-1/WAF-1 increase their expression during the G1/S transition to regulate DNA synthesis. The Rb protein is phosphorylated during the cell cycle. In its unphosphorylated form, it acts to block passage from G1 to S by complexing with the transcription factor E2F.

and EGR appears to be almost universal in NSCLC; thus, it is difficult to understand the biologic significance of this observation. It may be that genes that are subsequently regulated by these transcription factors are themselves critical and thus specifically responsible for the pathogenesis of this disease. Finally, a wealth of data suggests that the p53 gene is biologically important in that it plays a pivotal role in the regulation of cell growth and proliferation. Taken together, it is clear that defining the molecular alterations of a malignancy is the first step. We must now, as biologists, evaluate whether these alterations are responsible in some way for the pathogenesis of this disease. It is then that we can begin to design rational molecular therapeutics to correct these genetic alterations.

The great excitement of applied research is the idea of taking basic scientific observations made in the laboratory to the bedside in the form of novel therapeutics. This fundamental approach proceeds from an understanding of the most basic fact about cancer: that it is simply uncontrolled growth. An understanding of how this occurs on a molecular level may ultimately lead to clues as to ways in which to halt its progression. For example, there are several NSCLC cell lines that express very low levels of immediate early transcription factor genes and grow tumors in animal models. It would be of biologic relevance to ask if tumor growth can be retarded in these animals when expression of these gene products is restored to normal (or higher) levels in the cell lines studied. As a tumor suppressor gene, mutant p53 contributes either directly or indirectly to the transformed phenotype through its interactions with associated proteins. Thus, replacing an altered p53 gene or trying to correct a point mutation through genetic engineering may allow for restoration of its role in suppressing growth. Other ideas involve proteins that are able to bind mutant p53 and change the conformation of the mutant protein back to the shape of the normal molecule, thus restoring function.

Already we have many diagnostic tools that enable the clinician to diagnose certain cancers at an earlier stage than was ever possible before. We even have several molecular markers for specific malignancies that allow physicians to identify a more clinically aggressive tumor, allowing them to treat this disease in a more effective manner.27 We are gaining the capacity to explore the biologic relevance of these alterations to determine their contributions to the pathophysiology of these diseases. The possibilities for correcting critical genetic alterations are limited only by a rapidly expanding technology and the creativity of the scientific mind.

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