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REVIEW

Molecular Genetic Abnormalities in the Pathogenesis of Human Lung Cancer

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In the past few years our knowledge of the molecular pathogenesis of lung cancer has significantly increased. There are several molecular mechanisms involved in the multistage carcinogenesis through which respiratory epithelial cells become preneoplastic and then invasive cancer. In this review we summarize some of these changes including, genomic alterations such as loss of heterozygosity and microsatellite alterations, autocrine-paracrine loops, alterations in oncogenes and tumor suppressor genes, tumor angiogenesis, aberrant promoter methylation and inherited predisposition to lung cancer. Translation of these findings to the clinic is also discussed. (Pathology Oncology Research Vol 7, No 1, 6–13, 2001)

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Introduction

Lung cancer is the major cause of cancer related mortalities in the Western world. Each year an estimated 178,100 new cases of lung cancer will be diagnosed in the United States, and 160,400 individuals will die from this deadly disease despite the best current treatment approaches. Smoking (85% of the cases are in current smokers or former smokers) plays the major role in the development of lung cancer, and the prevention of smoking initiation and efforts to aid with smoking cessation remain as the best defense against this disease.[1] Of note, 50% of all newly diagnosed lung cancers in the United States occur in people who stopped smoking 5 or more years ago. Thus damage to the respiratory epithelium appears to persist. In the past few years molecular studies have revealed that these are multiple genetic lesions in the respiratory epithelium of current and former smokers and these appear directly associated with cigarette smoking.[2,3,4]

Lung cancer is divided into two main histologic groups: small cell lung cancer (SCLC) accounting for ~20% and non-small cell lung cancer (NSCLC), which constitutes the remainder of bronchogenic carcinomas. SCLCs express properties of neuroendocrine (NE) cells; whereas most NSCLCs lack these properties and can be divided into three major subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Clinically evident lung cancers have clonal genetic changes involving mutations or expression abnormalities in multiple (~10-20) genes. If we can detect some of these genetic alterations in preneoplastic respiratory epithelial lesions before cancer develops, early intervention and chemoprevention in such high risk individuals could greatly increase survival rates.

The purpose of this review is to summarize the recently described molecular abnormalities in lung cancer pathogenesis including altered expression and function of oncogenes, loss of tumor suppressor gene (TSG) function, and epigenetic processes such as tumor acquired aberrant promoter methylation.

Genome wide scanning approaches detect multiple acquired genetic abnormalities in lung cancer

Allelotyping studies on precisely microdissected tissues including lung cancer, preneoplastic lesions, and normal respiratory epithelium, showed that loss of heterozygosity (LOH) on chromosome 3p is the earliest molecular change in the development of lung cancer, and it is observed in

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more than 90% of small cell and squamous cell lung cancers and in 50% of adenocarcinomas.[5] There are several distinct regions of frequent allele loss on 3p including 3p12, 3p14.2, 3p21.3, 3p24, and 3p25 where frequent allele loss occurs indicating the presence of multiple TSGs. By analyzing microdissected sections of normal, hyperplastic, and dysplastic epithelium, carcinoma *in situ*, and invasive cancers from the same patient, it is possible to order the progression of LOH changes from 3p→9p→8p→5q→17p.[6]

Microsatellite repeats are widely distributed throughout the genome and show a high degree of polymorphism. Microsatellite alterations are common in colon and endometrial cancers due to the replication error repair (RER+) phenotype related to mutations in the mismatch DNA repair genes. In lung cancer 35% of SCLCs and 22% of NSCLCs were found to have at least some microsatellite alterations, and correlation was made with reduced survival, younger age, and advanced tumor stage.[7-9] However the RER^+ phenotype seen in colon cancer with multiple abnormal alleles is very rare in lung cancer. So far there has been no description of frequent abnormalities in mismatch repair genes in lung cancer to explain the microsatellite alterations.

Telomeres are tandem hexanucleotide repeats (TTAGGG) at the ends of all human chromosomes. Their normal shortening occurs in all somatic cells and leads to cell senescence. By contrast, germ cells and cancer cells maintain telomere length using the enzyme telomerase, and are able to divide indefinitely. The telomere replication amplification protocol (TRAP assay), detected high telomerase activity in almost 100% of SCLCs and 80% of NSCLCs, and high telomerase activity was associated with advanced pathological stage in primary NSCLC.[10,11] Since telomerase activity is associated with malignant growth, it is a marker for lung cancer detection, and a new target for therapy. Why telomerase is re-expressed in lung cancer cells is currently unknown.

Growth factors: autocrine-paracrine loops are frequent in lung cancers

Gastrin-releasing peptide (GRP)/ bombesin (BN) autocrine loop – Many growth factors and their receptors are expressed by lung cancer cells and adjacent normal cells, thus creating the major autocrine and paracrine growth regulatory loops. The best characterized autocrine system in lung cancer is the gastrin-releasing bombesin-like peptides, (GRP/BN) and their receptors. There are three GRP/BN receptors identified GRP-, neuromedin B- (NMBR), and BN subtype-3 (BRS-3), and they all belong to the G-protein-coupled receptors subfamily. The receptors are expressed to variable degree in both SCLCs and NSCLCs.[12] Immunohistochemical studies show that 20-60% of SCLCs express GRP/BN peptide ligands for these receptors, whereas expression in NSCLCs is less frequent.[13] No mutations have been identified for GRP/BN or for their receptors, thus the mechanism of activation of this growth stimulatory autocrine loop remains unknown.[14] Neutralizing monoclonal antibodies against GRP/BN inhibits the *in vivo* and *in vitro* growth of SCLCs confirming their role in lung cancer pathogenesis. Also these antibodies are being tested in clinical trials[14,15] as new forms of therapy. Expression of gastrin releasing peptide receptor in human respiratory tissues is associated with a proliferative response of bronchial epithelial cells to GRP/BN and with prolonged cigarette smoking and may be a risk factor for development of lung cancer. There is significant evidence that women are more susceptible to tobacco smoking leading to lung cancer than men. Thus, it is of interest that the GRP receptor is located on the X chromosome but does not undergo X inactivation so that women have two but men only one gene for the GRP receptor. Women express GRP receptor more frequently than men in the absence of smoking or after shorter smoking histories.[16] These conditions may be factors in the increased susceptibility of women to tobacco induced lung cancer.[16]

The ERBB family – The protein product of the *ERBB2* proto-oncogene is a transmembrane receptor tyrosine kinase. The amplification of *ERBB2* has been implicated in a subset of breast and ovarian cancers. Abnormal expression of the ERBB2 protein was reported in ~30% of NSCLC (predominantly adenocarcinomas),[17,18] but has not been found in SCLCs. Anti-ERBB2 monoclonal antibody inhibited the *in vitro* growth of NSCLC expressing ERBB2.[19] This antibody (Herceptin™) is used in combination with conventional chemotherapy in the treatment of HER2/neu positive breast cancer , and this treatment has been recently extended to clinical trials in lung cancer.

The c-erB-1 proto-oncogene encodes ERBB1 or EGFR (epidermal growth factor receptor), which regulates cell differentiation and proliferation. The activation of ERBB1 occurs via overexpression and is more common in NSCLCs.[20,21] Monoclonal antibodies against ERBB1 can inhibit growth in overexpressing cell lines. Overexpression of EGFR is associated with adverse prognosis of NSCLC.[22] Also the new tyrosine kinase inhibitor drugs such as ZD1839 (IRESSA™ [23] inhibit EGFR activity and cause reduction of lung cancers in patients.[24] Thus, they represent a new form of targeted molecular therapy.

The *Hepatocyte growth factor (HGF)* stimulates cell growth and differentiation such as morphogenesis of the epithelial cells, and it is expressed in NSCLCs, but not in SCLCs, which suggests the existence of an autocrine loop in NSCLCs.[25,26] By contrast the receptor for HGF is c-met proto-oncogene product MET, which is expressed in both SCLCs and NSCLCs. High HGF levels were associated with poor survival in NSCLC patients.[27]

Other potential autocrine loops involved in lung cancer are IGF1 (insulin-like growth factor 1) and its receptor, IGF2 (insulin-like growth factor 2),[28] and platelet- derived growth factor (PDGF) and its receptor.[29]

Oncogenes are frequently activated in lung cancer

The dominant oncogene *RAS* plays a key role in signal transduction and cellular proliferation. In the presence of growth stimulatory signals RAS is activated, and the MAPK (mitogen activated kinase) cascade is induced. The *RAS* genes (*KRAS*, *HRAS*, *NRAS*) code for three different guanosine triphosphate (GTP) binding proteins. Hydrolysis of bound GTP to guanosine diphosphate (GDP) inactivates the RAS growth promoting signal. In the presence of oncogenic RAS mutations (nearly always point mutations in KRAS), GTP cannot be hydrolyzed to GDP, resulting in constitutively active RAS-GTP growth promoting activity. *RAS* mutations are rare or non-existant in SCLC, but are present in 15-20% of NSCLC. Up to 30% of the adenocarcinomas carry *RAS* mutations usually affecting codon 12 for *KRAS* (85% of cases), and uncommonly codon 13 of *HRAS*, and codon 61 of *NRAS*.[13] Most (70%) of these mutations are G-T transversions and are induced by bulky DNA adducts present in tobacco smoke, such as BPDE (benzopyrene diethyloxide) and nitrosamines which attach covalently to the DNA. This can explain the correlation between smoking history and *KRAS* mutations.[30] Both in early and late stage NSCLC, the presence of a *KRAS* mutation predicts a poor prognosis.[30-33]

The *MYC* proto-oncogenes (*MYC*, *MYCN*, *MYCL*) encode nuclear phosphoproteins with a helix-loop-helix domain and a leucine zipper motif at the C terminus and a trans-activating domain at the N-terminus. MYC proteins have a role in transcriptional regulation by heterodimerizing with proteins such as MAX, MAD or MX11. The MYC-MAX complex represses transcriptional activation. MAX can bind MAD and MX11, thereby MYC is released from the complex and functions as a transcriptional activator. MYC can cooperate with a mutant RAS gene to transform primary rat embryo fibroblasts to malignancy. The activation of the *MYC* genes by amplification or loss of transcriptional control resulting in protein overexpression is a major molecular mechanism in the pathogenesis of human lung cancers. *MYC* gene activation has been observed in both NSCLC and SCLC whereas *NMYC* and *LMYC* abnormalities mainly occur in SCLC. MYC amplification occurs in 15-30% of SCLCs and 5-10% of NSCLCs.[13] Lung tumor cell lines mostly derived from metastatic tumors of patients who have relapsed after chemotherapy have a high frequency of *MYC* amplification, and this probably explains the correlation of *MYC* amplification with adverse survival.[34]

The *BCL-2* proto-oncogene is a key member of the apoptotic pathway exerting an anti-apoptotic effect, and its expression is negatively regulated by the tumor-suppressor p53. BCL-2 heterodimerizes with a BCL-2 related protein BAX, an apoptosis inducing protein and downstream target of p53, thus overexpression of BCL-2 results in down-regulation of apoptosis. BCL-2 expression, detected by immunohistochemistry, is significantly higher in SCLC (75%-95%),[35] than in NSCLC, and 25% -30% of the squamous cell carcinomas and 10% of adenocarcinomas express BCL-2 protein.[36] Interestingly the response to chemotherapy which occurs by way of apoptosis is much better in SCLCs than in NSCLCs[35] despite the high BCL-2 levels in SCLCs.

Tumor-suppressor genes are frequently inactivated in lung cancer

Candidate tumor suppressor genes at chromosome 3p – As discussed above, allelic loss at chromosome 3p is a frequent event in both SCLCs (>90%) and NSCLCs (>80%).[5,37-39,40] This and the existence of homozygous deletions in multiple lung cancer cell lines and tumors are strong indications that there is one or more TSGs on chromosome 3p.[37,41,42] There are several 3p homozygously deleted regions: 3p12, 3p14.2 (*FHIT* region), and at least three distict regions at 3p21.3.[43,44] More than 25 genes were identified in the sequenced 3p21.3 region[45] homozygous deletion,and extensive functional studies are in progress to identify the lung tumor-suppressor gene(s) which reside at this locus. Recent studies indicate that several of these genes inhibit anchorage-independent growth and tumor formation in nude mice. Among these are the calcium channel auxiliary subunit, alpha (2) delta-2 (CACNA2D2),[46] the SEMA3B protein,[47] which triggers apoptosis in neuronal cells,[48] and RASSF1A, a protein with a RAS association and a DAG binding domain.[49,50]

Other candidate TSGs at 3p21 are *BAP1* (BRCA1 binding protein), and the *hMLH1* mismatch DNA repair gene. A few lung cancer cell lines were found to have deletions and mutations for BAP1.[51] Mutations of the hMLH1 gene have been reported in colorectal cancer, but there are no reports in lung cancers.[52] The *DUTT1* (Deleted in U2020) gene belongs to the neuronal cell adhesion molecules. It is localized at 3p12, and is homozygously deleted in two lung cancer and one breast cancer cell lines,[53] but no mutations were found when the ORF was screened on a panel of SCLCs and NSCLCs (unpublished data). The *FHIT* gene encodes a dinucleoside 5', 5'''-P^1-P^3-triphosphate hydrolase, maps to the 3p14.2 region, and the loss of the gene could result in the accumulation of diadenosine tetraphosphate thus stimulating DNA synthesis and proliferation. Immunohistochemistry detected the absence of

the FHIT protein in ~50% of primary lung tumors.[44,54,55] Reintroduction of wild type FHIT suppressed *in vitro* growth in a lung cancer cell line and *in vivo* tumorigenecity of lung cancer cells in nude mice.[56,57] The *VHL* (Von Hippel-Lindau) gene at 3p25 is frequently mutated in renal cell carcinoma, but mutations in lung cancer are rare.[58] The hOGG1 gene encodes a DNA glycosylase which repairs oxidatively damaged DNA. Two missense mutations were identified in a mutational screening of 25 SCLC tumors.[59] The RARβ (retinoic acid receptor beta) gene, located at 3p24 is a strong TSG candidate. Low or absent RARβ mRNA expression was detected with high frequency in lung cancer cell lines and primary lung tumors. It appears to result from aberrant promoter methylation of the RARβ and was observed in ~40% of primary SCLCs.[60,61]

P16-cyclin D1-CDK4-RB pathway – The *RB* gene is located on chromosome 13q14, and its protein product is a nuclear phosphoprotein initially identified as a TSG in childhood retinoblastomas.[62] The phosphorylation status of the RB protein and its interaction with transcription factor E2F is one of the most important determinants in the regulation of G0/G1 transition.[63]

When RB is dephosphorylated it suppresses the G1 to S phase transition. During G1 cyclin D1 is associated with cyclin-dependent–kinases (CDK2 and CDK4); this results in the phosphorylation, and activation of RB. Hypophosphorylated RB binds the E2F transcription factor, thus blocking the transcription of genes regulating the cell cycle. When RB is phosphorylated E2F dissociates and activates the transcription, thus facilitating S phase entry. Abnormalities of the RB gene in lung cancer include deletions, nonsense mutations and pathogenic splicing variations. More than 90% of the SCLCs, and 15-30% of the NSCLCs have abnormal or no RB expression.[64-66] The absence of RB expression was associated with poor prognosis in stage I and II NSCLCs,[67] but other studies did not support this finding.[68,69] In addition germline carriers of an *RB* mutation are 15 times more likely to die from lung cancer than unaffected individuals.[70]

$p16^{INK4}$ is a kinase inhibitor of CDK4 and thus is an inhibitor of RB phosphorylation making it also a TSG.[71] The $p16^{INK4}$ gene is most commonly altered in NSCLCs by aberrant promoter rmethylation (25%)[60,72] and homozygous deletions or point mutations (10%-40%).[73-76] It is not clear whether loss of $p16^{INK4}$ results in poor prognosis in NSCLCs.[77,78]

$p19^{ARF}$ is a p16 splice variant with a common nucleotide, but different amino acid sequence, and therefore an altered reading frame from $p16^{INK4}$. $p19^{ARF}$ was shown to play an important role in tumor-suppression with binding to the MDM2-p53 complex and thus preventing p53 degradation. $p19^{ARF}$ was found more frequently lost in lung tumors with neuroendocrine features[79] Thus the $p16^{INK4}$ $p19^{ARF}$ locus products interact with both the Rb and p53 pathways.

The p53 pathway – The *p53* gene is located at 17p13.1, and encodes a 53 kd nuclear protein that acts as a transcription factor, blocks the cell cycle at late G1, and also can trigger apoptosis. Thus p53 has a role in maintaining the stability of the genome during cellular stress from DNA damage, hypoxia, and activated oncogenes.[80] p53 activates the transcription of the downstream target genes *p21, MDM2, GADD45* and *BAX*.[7] Loss of heterozygosity at 17p13 is very frequent in lung cancer, and the other p53 allele is inactivated by mutations in different types of cancers including lung cancer. Mutational inactivation of p53 occurs in >75% of SCLCs, and 50% of NSCLCs.[81-83] The majority of the mutations are G to T transversions, and mutational hot spots can be correlated with BPDE adducts, a carcinogen present in tobacco smoke.[4] p53 missense mutations usually lead to increased protein half–life, thus overexpression of the p53 protein can be easily detected by immunohistochemistry.[84] Abnormal p53 expression has been correlated with a better, but in some cases poor, prognosis.[85] Wild type p53 is delivered by adenoviral or retroviral gene therapy into tumors by local injection and induce tumor regression in ongoing clinical trials.[86,87]

PTEN (Phosphatase Tensin Homolog Deleted on Chromosome Ten) gene is located at chromosome 10q23 and its protein product is a lipid phosphatase, which dephosphorylates PIP3 and has been shown to have tumor suppressor activity *in vitro* and *in vivo*. A few lung cancer cell lines and primary tumors have mutations or deletions of the *PTEN* gene.[88] Another candidate TSG on chromosome 10q25-26 is *DMBT1*. It is frequently downregulated and occasionally homozygously deleted in lung cancer.[89]

Aberrant promoter methylation frequently occurs and extinguishes gene expression in lung cancer

Gene expression can be turned off by aberrant promoter methylation of the promoter region acquired in tumors. In human neoplasias tumor acquired promoter methylation has been found to be a major alternative mechanism to mutations in inactivating TSGs and silencing the expression of other cancer related genes. Abnormalities in 5' CpG island methylation have been described previously for several genes in lung cancer.[90] In a recent study, 107 resected primary NSCLCs and 104 corresponding nonmalignant lung tissues were analyzed by MSP (methylation specific PCR) for aberrant promoter methylation in eight different genes. Aberrant methylation in the tumor samples was detected in 40% for RARβ, 26% for metalloproteinase-3 inhibitor (TIMP-3), 25% for $p16^{INK4a}$, 21% for O^6-methylguanine-DNA-methyltransferase (MGMT),

19% for death-associated protein kinase (DAPK), 18% for E-cadherin (ECAD), 8% for $p14^{ARF}$, and 7% for glutathione-S-transferase P1. Promoter hypermethylation of these genes was not significantly correlated with patient survival.[60]

Tumor angiogenesis effects the prognosis of lung cancer patients

Tumor growth requires angiogenesis, the development of new blood vessels. There are multiple angiogenic factors, inducers and inhibitors regulating endothelial cell proliferation and migration. Various growth factors have been shown to stimulate angiogenesis including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived endothelial cell growth factor (PD-ECGF). Expression of angiogenesis and these factors influence the clinical behavior of lung cancer. VEGF expression was associated with new blood vessel formation, decreased overall and disease free survival, and was an independent prognostic factor in NSCLC patients.[91] Expression of the basic fibroblast growth factor (bFGF) was found to be a prognostic indicator in pulmonary adenocarcinoma, since the 5 year survival rate was significantly lower for bFGF positive patients.[92] Upregulation of the platelet-derived endothelial growth factor (PDGF) was associated with a more aggressive tumor phenotype in patients with node negative disease.[93] There are new lung cancer treatment strategies against angiogenesis, particularly targeting VEGF and its receptors. The VEGF receptors KDR and FLT have tyrosine kinase activity and occur frequently in smoking damaged lung. Clinical trials in lung cancer are in process with a "humanized" monoclonal antibody against VEGF which blocks its binding to its receptor and with tyrosine kinase inhibitor drugs.

Preneoplastic lesions occur frequently in the smoking damaged lung

Detection of genetic abnormalities in the smoking damaged lung epithelium may be useful in the identification of patients at high risk for developing lung cancer. Preinvasive preneoplastic lesions such as hyperplasia, dysplasia, and carcinoma *in situ*, contain genetic changes such as allele loss at 3p, 9p, 8p, 17p, MYC, RAS and p53 mutations.[7] As described previously there is a sequential order of how these alterations occur. Recent studies suggest that the above changes can be observed even in normal appearing bronchial epithelium of former and current smokers with 3p allele loss being the earliest event.[2] These abnormalities could be candidate biomarkers for the detection of early lung cancer and the identification of individuals at high risk of developing lung cancer.

Inherited lung cancer susceptibility

While the majority of the lung cancer cases are due to cigarette smoking, epidemiologic studies suggest that lung cancer aggregates in families. There is an increased risk for developing lung cancer for first degree relatives of younger age, non-smoking lung cancer cases. There are a number of inherited susceptibility polymorphisms that are thought to be important in the risk of developing lung cancer. These include polymorphisms in the cytochrome P450 enzymes (phase I and phase II enzymes), and phase III, glutathione-S-transferases, which play a role in eliminating certain carcinogenes including polycyclic aromatic hydrocarbons.[94] Individuals also vary in their susceptibility to carcinogen and mutagen induced chromosomal breaks.[95] Increased number in peripherial blood lymphocytes after *in vitro* carcinogen treatment is associated with increased lung cancer risk. This is particularly true of BPDE induced breaks in the 3p21.3 region.[96]

Conclusion

In the past few years, our understanding of the molecular pathology of lung cancer has advanced rapidly. Several genes and their corresponding protein products have been identified, including oncogenes, tumor suppressor genes, growth factors and their receptors, and genes involved in DNA repair.

Genetic changes can occur at a very early „preneoplastic/preinvasive" stage, therefore detection of genetic lesions in preneoplastic tissues combined with new radiographic screening methods, such as spiral CT scans, will open new doors for early diagnosis, identifying individuals at highest risk of developing lung cancer, and directing intervention with chemoprevention. These new findings of molecular pathology will lead to new treatment strategies, including drugs designed to inhibit enzyme activity, the use of monoclonal antibodies against growth factors and their receptors, immunization against tumor specific mutant peptides, blocking the expression of activated oncogenes with antisense agents, the replacement of defective tumor-suppressor genes (gene therapy), and application of apoptosis modulators. These new tools will usher in a new era in the diagnosis, prevention and treatment of lung cancer.

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