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The molecular basis of lung cancer: molecular abnormalities and therapeutic implications

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Abstract

Lung cancer is the number one cause of cancer-related death in the western world. Its incidence is highly correlated with cigarette smoking, and about 10% of long-term smokers will eventually be diagnosed with lung cancer, underscoring the need for strengthened anti-tobacco policies. Among the 10% of patients who develop lung cancer without a smoking history, the environmental or inherited causes of lung cancer are usually unclear. There is no validated screening method for lung cancer even in high-risk populations and the overall five-year survival has not changed significantly in the last 20 years. However, major progress has been made in the understanding of the disease and we are beginning to see this knowledge translated into the clinic.

In this review, we will summarize the current state of knowledge regarding the cascade of events associated with lung cancer development. From subclinical DNA damage to overt invasive disease, the mechanisms leading to clinically and molecularly heterogeneous tumors are being unraveled. These lesions allow cells to escape the normal regulation of cell division, apoptosis and invasion. While all subtypes of non-small cell lung cancer have historically been treated the same, stage-for-stage, recent technological advances have allowed a better understanding of the molecular classification of the disease and provide hypotheses for molecular early detection and targeted therapeutic strategies.

Introduction

The pathogenesis of lung cancer involves the accumulation of multiple molecular abnormalities over a long period of time [1,2]. Genomic instability is universally found during accumulation of these hits [3]. The alterations can happen at the level of gene silencing through methylation, DNA sequence changes, DNA segment amplification or deletion or whole chromosome gains or losses. These changes occur early in normal-appearing tissues that do not have the characteristics of cancer cells. Microdissection of lesions of the bronchial epithelium as well as of invasive tumors has provided purified tissue for the analysis of point mutations [4], chromosomal deletions [5], microsatellite instability [6,7] and DNA methylation patterns [8].

The most common early genetic alterations in non-small cell lung cancer involve loss of genomic regions of chromosomes 3p and 9p, deletions of chromosomal arm on 5p and mutations of p53 and K-ras [9]. Loss of

chromosomal regions on chromosomes 3p and 9p have been recognized as early events [10] and identified in preinvasive lesions and in the normal appearing epithelium of smokers [11,12]. In contrast, p53 and K-ras mutations have been seen primarily in later stages of preneoplasia or frank invasive lesions [9]. Amplification of large regions on the q arm of chromosome 3 has been characterized in invasive carcinomas [13] only recently in preinvasive lesions [14].

The historical focus of much of this research has been to identify and study the role of specific genetic abnormalities in tumor cells related to chromosomal abnormalities, inactivation of specific tumor suppressor genes, the activation of specific oncogenes, the expression of hormone receptors and growth factor production associated with the development of cancer. More recently, the contribution of stromal interactions, angiogenesis, apoptosis, and epigenetic phenomena such as posttranslational modification of critical genes has been the subject of intense research. The recent completion of the first draft of the human genome sequence [15] and the availability of high throughput technologies (e.g. microarrays) have prompted investigators to propose studies to discover common genetic abnormalities in both pre- and invasive lung cancers and to test these markers for their potential use in early detection strategies. In this paper we will review the genetic basis of lung cancer progression using a stepwise approach from point mutation to invasion and address its therapeutic implications.

Early events in oncogenesis

Mutations

In the last 20 years somatic mutations have been identified and associated with the development of cancer. These mutations, involving tumor suppressor genes or oncogenes, may or may not be rate-limiting events. Epidemiological data support that groups of cells accumulate several key mutations [16]. The model of the mutator phenotype proposed by Loeb suggests that cells develop a predisposition for mutations early on [3]. This phenotype may be hereditary, yet the key genes remain to be discovered. In the lung, DNA damage can fail to be repaired, resulting in misincorporated nucleotides and therefore mutations. Spontaneous errors of replication attributed to DNA polymerase occur at a rate of 1/10,000 to 1/100,000 base pairs depending on the polymerase. These intrinsic mutations may be an important component underlying genomic instability and eventually tumor growth. We will illustrate this point by commenting on 3 classical examples: k-ras, p53 and p16.

K-ras mutations are most commonly seen in 30% of adenocarcinomas of the lung [17] but much less frequently in other subtypes. K-ras, once mutated (most frequently codon 12 G-T transversions), can transform airway epithelial cells [18,19] by activating the ERK-MAP kinase pathway. Because K-ras mutation is found early in alveolar atypical hyperplasia, a presumed precursor lesion to adenocarcinomas [20], this may be an important step in the genesis of this subtype of lung cancer. Mutant ras transgenic mice develop adenocarcinomas of the lung as well, supporting this hypothesis.

p53 is a prototype tumor suppressor gene that is the most common genetic lesion in human cancers [21] and is thus well suited for analysis of the mutational spectrum in human cancers. p53 mutations are most commonly seen in squamous carcinoma and small-cell carcinoma of the lung. Mutations predominantly represent G to T transversions consistent with causation by bulky DNA adducts such as the polycyclic hydrocarbons frequently found in the lungs of smokers [22]. The p53 tumor suppressor gene is mutated in over two thirds of lung cancers [23]. When mutated, p53 can function as an oncogene and accumulate in the cytoplasm [24]. Mutated p53 exhibits a prolonged half-life and can thus be found to be overexpressed in about 50% of lung cancers by immunohistochemistry [25]. Although not consistently associated with prognostic significance, there is little doubt that p53 mutations play a key role in tumor development by dysregulation of cell-cycle control and apoptosis.

p16, a tumor suppressor gene and critical member of the Rb pathway, is inactivated in over 40% of NSCLCs. Previous studies have demonstrated that point mutations, loss of heterozygosity on 9p21, or hypermethylation of the gene provide alternate mechanisms of inactivation in 30–50% of NSCLCs [26]. Tumors arising in smokers are found to more frequently harbor point mutations or homozygous deletions as the mechanism of loss of p16 function [27]. The relationship between tobacco and the loss of p16 points to new mechanisms involving smoking in the pathogenesis of lung cancer.

Mutagens

Cigarette smoking is a major risk factor for 85% of lung cancers. Approximately one in ten life-smokers will develop lung cancer, suggesting individual differences in susceptibility [28]. The susceptibility to lung cancer is being approached by molecular epidemiology and identifying links between genes involved in DNA repair, polymorphisms in the cytochrome p450 enzymes and the metabolizing capability of glutathione s-transferase or acetylation [29,30]

The majority of lung cancers are diagnosed among ex-smokers [31]. This suggests that the accumulation of molecular damage during cigarette exposure has set a cascade of events in motion that leads to the diagnosis of

cancer often decades after smoking cessation. Risk factors for lung cancer from smoking (first publicly recognized in the 1964 Report of the U.S. Surgeon General), include total consumption, age at initiation, and years of smoking. Other risk factors include occupational and environmental exposure (asbestos, uranium, radiation), diet (vitamin A, vitamin E, cholesterol), and host (familial aggregation) and genetic factors. Some of the components of cigarette smoke implicated in lung cancer are now recognized. Cigarette smoking is a complex mixture and includes substances that are responsible for DNA adduct formation such as polycyclic aromatic hydrocarbons (PAH), aromatic amines, and tobacco-specific nitrosamines (NKK). These form DNA adducts that may escape normal adduct repair mechanisms and result in heritable alterations in DNA sequence. The resulting conversion of G-C base pairs to T-A leads to activation of the K-ras oncogene and inactivation of the p53 tumor suppressor gene [32]. The activated form of benzopyrene (BaP) is BPDE and can cause DNA adducts, and, in addition to point mutations, can also lead to single strand chromatid breaks that are more frequent in lung cancers [33]. One of the concerning facts in this process is that people who start smoking at young ages seem to be have greater amounts of permanent DNA alterations than smokers who start smoking at an older age [34].

Chromosomal changes

Cancer cells are characterized not only by mutations but also by a series of chromosomal aberrations including deletions and amplifications [35]. The chromosomal regions with frequent losses are found in regions coding for essential tumor suppressor genes and DNA repair genes that may be involved in the pathogenesis of several tumor types [36]. Large areas of deletions (e.g. chromosome 3p, 9p) or amplifications (e.g. 1q, 3q) are commonly seen across the genome of lung cancer. Higher rates of chromosomal changes as determined by loss of heterozygosity (LOH) and CGH have been found in SqCa than in adenocarcinoma of the lung [37,38].

The most common alterations involve loss of regions of chromosomes 3p21 and 9p21, deletions of chromosomal arm on 5q21 and mutations of p53 associated with LOH on 17p and K-ras point mutations [9]. Interestingly, loss of chromosomal regions on chromosomes 3p and 9p have been recognized as early events [10] and identified in preinvasive lesions and in the normal appearing epithelium of smokers [11,12]. In contrast, p53 and K-ras mutations have been seen in a high percentage of later stages progression and in early invasive lesions [9].

LOH at chromosome 3p14 was evaluated in smokers and ex-smokers and found to be more frequent in current smokers (22/25 cases) than in former smokers (5/11 cases), a high frequency that correlated with a high metaplasia index [12]. This implies that not only are these chromosomal changes frequent in normal appearing bronchial epithelia but that cells with these changes may regress after smoking cessation and be replaced by cells without this damage. The dynamics of this process is very poorly understood at this time and represents an interesting area of future research.

Lung cancer allelotypes have been investigated in detail and have recently identified new regions of allelic loss using high throughput technologies [39]. Interestingly, differences between smokers and non-smokers have shown LOH on chr. 9 and 17 targets for p16 and p53, respectively [27]. LOH and chromosomal gain is less prevalent at all sites in cancer from non-smokers [27].

Patterns of chromosomal copy number abnormalities in squamous carcinomas of the lung using CGH analysis have been published recently [40–42] and show particularly common amplified regions on chromosomal arms 1q, 3q, 5p, 8q, 11q, 12p, 17q and 20q. Among many areas of genomic abnormality, amplification of chromosomal region 3q26 was found to be the most prevalent abnormality in squamous carcinoma of the lung followed by a deletion of chromosome 3p. Limitations of chromosome-based CGH include its relatively poor genomic resolution (~10–20 MB) [43,44], lack of sensitivity for detection of aberrations involving megabase sized regions, inability to provide quantitative information about the magnitudes of genome copy number and the insensitivity of CGH to detect aberrations such as translocations that do not alter copy number. Most of these limitations can be overcome by viewing the chromosomes as the framework onto which information is mapped with high-resolution arrays of cloned probes.

Accumulation of specific chromosomal abnormalities has been correlated with clinical and pathological data in NSCLC. Chromosomal abnormalities have been recently correlated with clinical outcome for a variety of cancers [45–47], but often the genes responsible for the observed biology are unknown or only partly known. As mentioned above, 3q amplification is a common finding among many squamous carcinomas of non-lung origin. In particular, amplification of that region is seen in squamous carcinoma of the head and neck [48], esophageal cancers [49], and cervical cancers including cervical dysplasia [50]. In our recent study in NSCLC, among many amplified genes found in chromosome 3q26 (Figure 1), some are candidate oncogenes (phosphatidylinositol-3 kinase catalytic subunit, PIK3CA) or are described to be involved in tumor progression including the somatostatin gene (SST), p63 (p53 homolog gene), telomerase RNA component gene (hTER), and neutral endopeptidase (NEP) [13].

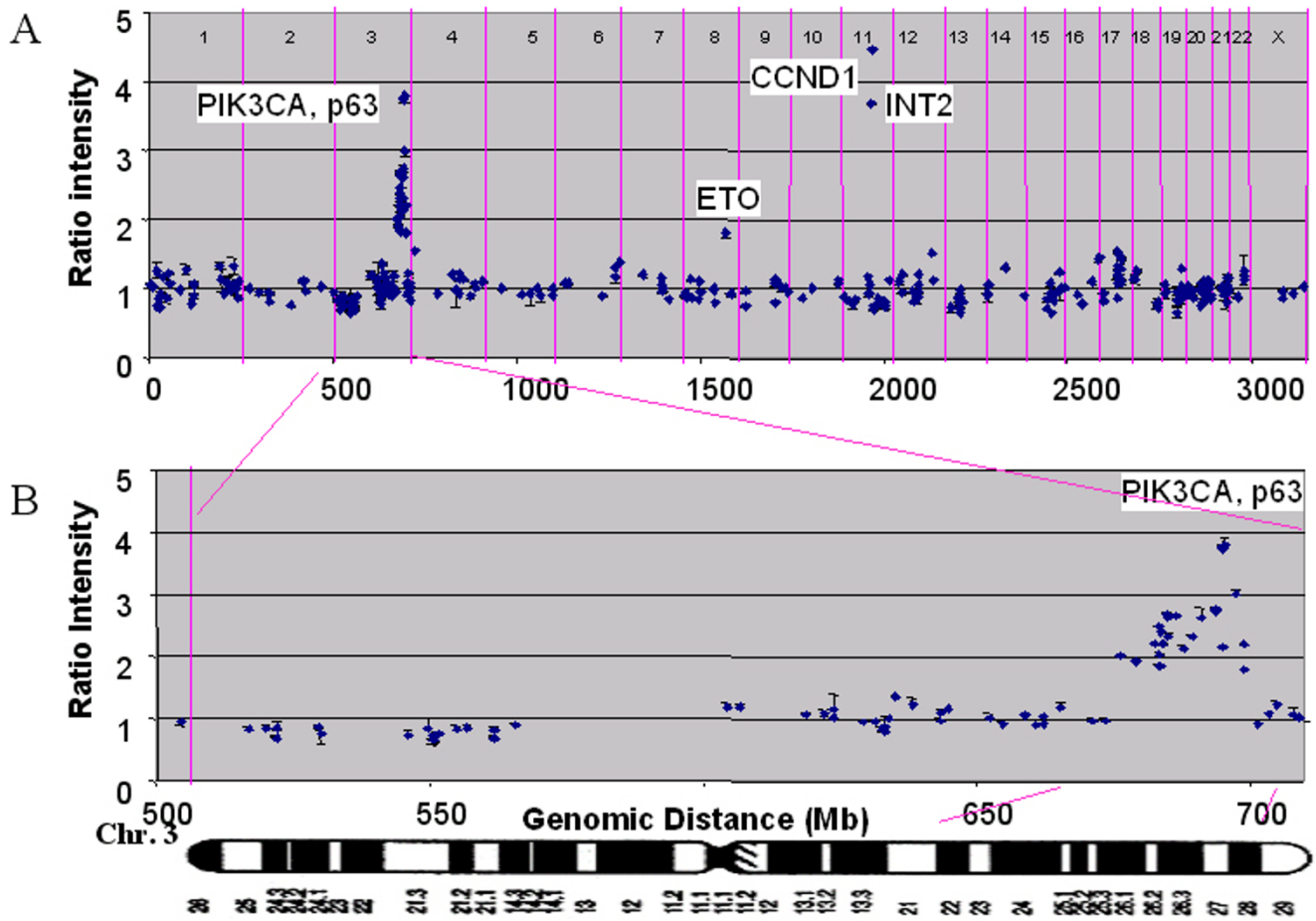

Figure 1
Array comparative genomic hybridization on a squamous carcinoma of the lung. A. array CGH profile on a squamous carcinoma of the lung labeled with Cy3 against normal DNA with Cy5. Each data point in presented mean (n = 4) ± coefficient of variance (CV=STD/Mean). B. View of Chromosome 3 array CGH profile on the same squamous carcinoma of the lung showing the size of the amplicon.

Human cytogenetic methods such as fluorescence *in situ* hybridization (FISH) are particularly useful in analyses of genomic organization, and copy number in individual cells and are applicable to tissue microarrays. Rather than look at the individual impact of isolated changes, we have begun efforts to "cluster" changes into groups of changes associated with a clinical feature. In an effort to find patterns associated with lung cancer histological subtypes based on array CGH profiling, we first identified 50 clones (most of which were on chr. 3) that best correlated with histological subtype using correlation and permutation analysis. Hierarchical clustering showed a clear pattern of gains and losses for squamous carcinoma, while the pattern for adenocarcinoma was less distinct (Figure 2). We then used an automatic classification method to assign tumor profiles to histological subtypes using a subset of 20 clones. The K-nearest-neighbor classification method correctly assigned 32/37 samples (87%) to proper histological subtype. The best multi-gene model found had a leave-one-out accuracy of 89.2%. Gene copy numbers as measured by array CGH are, collectively, an excellent indicator of histological subtype [51]. These data support the hypothesis that clusters of genes or groups of biomarkers may be more useful than single markers have been in the past as diagnostic, prognostic or predictive markers.

Specific translocation is another chromosomal abnormality, but it is much less commonly observed in lung cancer than hematologic or mesodermal tumors [52]. Chromosomal translocations modify gene function through the deregulated expression of cellular proto-oncogenes without altering the structure of the protein product or by

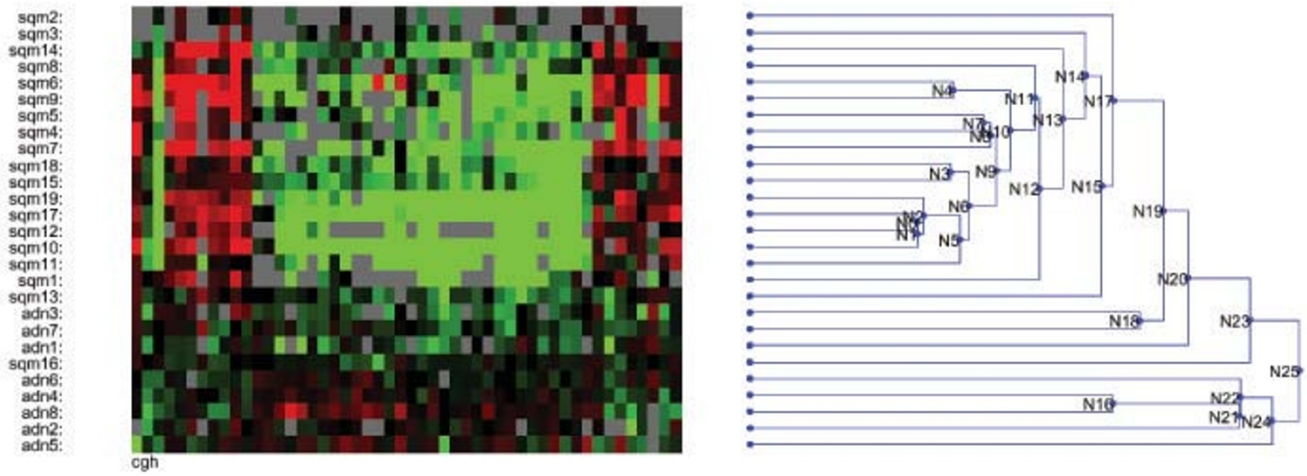

Figure 2

Hierarchical clustering analysis of NSCLCs using array comparative genomic hybridization. Cluster analysis using the 50 BAC clones closely correlated with histological subtype allowed accurate discrimination between SqCa and AdCa. K-nearest-neighbor classification was used to formally test the ability to predict subtype from array CGH profile. Cross-validation yielded 24/27 (89%) correct histological classification. Green squares: increase in copy number of a specific BAC clone, red squares: decrease in copy number.

generating and expressing a chimeric protein with growth-promoting activities. Recently, Dang et al. identified a chromosome 19–15 translocation associated with overexpression of Notch 3 [53]. The authors developed a transgenic mouse model overexpressing Notch 3 causing neonatal mortality with a phenotype suggestive of alveolar cell hyperplasia. These data suggest that Notch3 overexpression prevents epithelial differentiation and this may play a significant role in promoting oncogenesis in a subset of lung cancers [54].

Genomic instability

Genome instability is a fundamental characteristic of cancer initiation and progression. However, our understanding of the time when instability occurs during progression, the rate of instability, and the mechanisms leading to instability is far from complete. Instability can arise from different pathways. In a small fraction of lung tumors, mismatch repair deficiency leads to microsatellite instability at the nucleotide sequence level. In other tumors, abnormal chromosome number (aneuploidy) is the dominant feature [55]. The progressive accumulation of mutations, loss of apoptotic control and regulation of cell proliferation, and the appearance of aneusomy are associated with worsening dysplasia phenotypes and may reflect underlying dysregulation of mechanisms controlling genomic fidelity. Less clear than microsatellite instability is the importance of specific defects in DNA repair in lung cancer. It is known that polymorphisms in DNA repair genes XPD (codon 312 Asp/Asp vs Asp/Asn) have been found to be associated with impaired efficiency of DNA repair and apoptotic function in lung cancer [56]. New techniques, however, are allowing us to assess these changes in individual or small numbers of preneoplastic cells. Copy number changes in single cells can be assessed by FISH probes. Microdissection of dysplastic epithelium has provided purified tissue for the analysis of point mutations [4], chromosomal deletions [5], microsatellite instability [6,7] and DNA methylation patterns [8]. We thus may be able to ultimately derive a sequential pattern of development for genetic abnormalities in preneoplastic lung epithelium.

Role of viruses in lung tumorigenesis

The understanding of lung cancer molecular approaches has led to the development of transgenic models using viral antigens, including SV40 large T antigen and polyomavirus (PyV) large and middle T antigens that result in a high frequency of tumors. No common respiratory viruses have been conclusively incriminated in the development of lung cancer, but several have been implicated. Human papilloma virus (HPV), for example, has been associated with lung cancer and in particular lung cancer arising in women [57]. Simian Virus 40 has been incriminated in the development of mesothelioma [58]; Epstein Bar Virus (EBV) has been suspected to be involved in the

development of papillomas, mesotheliomas and lymphomas of the lung. Many PCR-based assays, however, have attempted to correlate bronchogenic carcinomas with respiratory viruses without success. Recent advances in proteomics may be useful in studying the role of viral infection in airway epithelial cell transformation. The proteomic analysis of tumors may allow the identification of peptide sequences specific to pathogens otherwise ignored in tumorigenesis.

Viruses have also been used (e.g., adenovirus) to facilitate gene entry into cells (adenovirus-mediated gene transfer) or in *in vivo* gene therapy of human lung cancer using wild-type p53 delivered by retrovirus [59].

Genomic instability causing lung tumorigenesis

A multistep process for clonal evolution

Genetic changes are seen in the transition from normal to intraepithelial cancer to invasive disease. The understanding of the timing of instability during progression, the rate of instability and the mechanisms leading to instability are far from complete. Chronic exposure to carcinogens initiates a process characterized by genetic abnormalities, phenotypic changes and clonal overgrowth throughout the lungs [60]. Measures of genomic instability follow rates of loss of heterozygosity [39] and accumulation of other genomic abnormalities [55]. In the airways, progressively more severe and more frequent abnormalities are seen in preinvasive lesions [61]. The progressive accumulation of genomic abnormalities associated with clonal growth among populations of tumor cells are well described and favor the clonal progression of cancer. Yet cancer remains a rare event if one considers the total number of bronchial epithelial cells and the proliferation rate of patches of clonal abnormalities [62,63].

While lung cancer originates from one or a few airway epithelial cells, it is clear that exposure of the whole airway mucosa to tobacco smoke could cause the entire bronchial tree to be at increased risk of developing lung cancer, leading to the concept of field cancerization. Field cancerization was first proposed in the fifties [64] and its molecular correlates later confirmed in the airways of human smokers [65,66]. Field cancerization is also demonstrated by the elevated Ki-67 labeling index in the airways of smokers at more than one site [67]. Although the risk of developing lung cancer increases with the presence of such preinvasive lesions, no one has identified the molecular determinants of preinvasive lesions that may predict irreversible progression to lung cancer.

Carcinogenesis in the airways has proved to be multistep and multifocal and yet clonal in nature. Multiple lines of evidence support the concept of clonal progression of tumors. First, at the chromosomal level, abnormalities found in invasive tumors and their metastases are extremely highly correlated [68,69]. Similarly, allelic losses or microsatellite abnormalities found to be in preinvasive lesions are found in similar frequencies in invasive lesions [7,63]. The issue remains complex as a small fraction of tumors appear to be truly independent synchronous primaries and different p53 mutations have been found in synchronous preinvasive lesions [70]. The prolonged, multistep nature of lung cancer development makes this disease process potentially amenable to chemopreventive interventions that should be optimally applied in the earliest preinvasive phases.

Significance of genomic instability in lung tumorigenesis

Some preinvasive lesions are committed to develop into invasive cancer [71,72]. One critical question that remains is the identification of that specific subset of the plethora of genetic changes in a given lesion that predisposes that lesion to develop into frank cancer. The literature suggests that the number of molecular abnormalities accumulated in the epithelium underlies tumor progression independent of light microscopically observable morphological abnormalities [4,73]. This observation raises the possibility that genomic instability itself may be independently predictive of tumor progression. Consistent with this hypothesis, the relationship between clonal chromosome alterations and various clinical parameters was evaluated in 70 patients with non-small cell lung cancer [47]. An increased number of marker chromosomes were observed in patients having a higher number of packs of cigarettes smoked over years.

Epigenetic alterations of gene expression in lung cancer

Gene function loss can be mediated by deletion of large chromosomal regions or by inactivation of gene function from genetic mutation, or due to epigenetic modifications of DNA such as promoter hypermethylation or histone deacetylation.

DNA adducts

One marker for significant carcinogen exposure is the level of DNA adducts in normal DNA. DNA adducts are covalent modifications of the DNA that result from exposure to specific activated carcinogens. In addition to being markers of carcinogen exposure, it is possible that these adducts may directly alter regulation of transcription of tumor suppressor or oncogenes [74]. The distribution of benzo[a]pyrene diol epoxide (BPDE) adducts along exons of the p53 gene in BPDE-treated HeLa cells and bronchial epithelial cells has been mapped at nucleotide resolution [22]. Cigarette smokers have higher adduct levels than non-smokers. Because DNA adduct levels in tumor tissue and in blood lymphocytes have been associated with lung cancer [75,76] and because these levels correlate with daily or lifetime cigarette consumption and do not reverse

after smoking cessation [77], DNA adducts have been proposed as potential biomarkers of risk for lung cancer.

In an attempt to identify risk factors associated with the level of DNA adduct accumulation, Wiencke et al. studied DNA adducts in current and former smokers and found that in current smokers the most important variable was the number of cigarettes smoked per day. In contrast, they found that in ex-smokers, the most important variable was age at initiation [34]. Mechanisms responsible for the relationship between DNA adduct levels and age of initiation are unknown, and the relative contribution of decreased adduct removal by DNA repair or cell turnover or increased adduct formation at younger ages is yet to be determined. Prospective study is needed to follow current and ex-smokers over time to determine the value of adduct levels in risk assessment.

DNA adducts have been associated with smoking status and shown to be more prevalent among women. In a matched case-control study nested within the prospective Physicians' Health Study, there was an increased level of DNA adducts in active smokers who developed lung cancer as compared to controls; a finding that was not found among former or non smokers [78]. Women smokers may be at higher risk of developing lung cancer for a given tobacco exposure and women also seem to accumulate aromatic/hydrophobic DNA adducts at a faster rate then men [79]. DNA adduct levels were higher in women even when corrected for smoking dose packs of cigarettes smoked either per day or over years.

Methylation

Among epigenetic alterations, gain of methylation in normally unmethylated CpG islands around gene transcription start sites is an increasingly recognized and important means of altered gene expression in tumors [80]. The genes affected include over half of the tumor suppressor genes that cause familial cancers when mutated in the germline, and the selective advantage for genetic and epigenetic dysfunction in these genes is very similar in sporadic cases. In contrast to genetic mutations that require two hits to inhibit both alleles, aberrant methylation is a dynamic process over multiple division cycles and may cause increasing degrees of gene function loss by increasing the density of methylation on promoter regions. "CpG islands," the targets of DNA methyltransferase, are associated with the transcription start sites in almost half of human genes [81]. Dense methylation of cytosines within CpG islands causes heritable gene silencing [82]. Aberrant methylation can begin very early in tumor progression by causing loss of cell cycle control (p16) [83], loss of mismatch repair function (MLH1) [84] and loss of cell-cell interaction (E-cadherin). The exact mechanism by which hypermethylation may cause tumor progression is still unknown. In fact, there is still debate as to whether methylation is a result rather than a cause of gene function loss [85]. Promoter region hypermethylation has been proposed as an excellent tumor marker. In lung cancer, common methylated loci were found in both tumor and sputum DNA and were detected in the sputum for up to 3 years before the diagnosis of cancer [86].

Acetylation

The dynamics of chromatin formation suggest that the association of DNA methylation and histone deacetylation may cause silencing of hypermethylated genes in tumors. During transcription, chromatin unfolds and allows ribosomal access to the DNA. Acetylation of histone tails on the nucleosome is associated with chromatin unfolding and increased regional transcriptional activity. Histone deacetylases (HDACs) modulate chromatin structure by regulating acetylation of core histone proteins. Deacetylation of histones is thus associated with compacting the DNA and transcriptional repression. In lung cancer cell lines, for example, de-acetylation of histone 3 correlated with retinoic acid refractoriness, a phenomenon related to RARbeta promoter methylation in a subset of cell lines [87]. Inhibitors of HDACs have already shown to decrease the level of a series of oncoproteins [88] suggesting a potential role as antitumor therapeutic agents.

From genetic abnormalities to biomarkers for lung cancer

Lung cancer is a heterogeneous disease. The specific genetic abnormalities mentioned above have thus far proven to be of limited use individually as biomarkers for lung cancer. However, the completion of the first draft of the human genome sequence [15] and the availability of high throughput technologies (e.g. microarray) have prompted us to look in an unbiased way for complex patterns of genetic abnormalities that may be better associated with both pre- and invasive lung cancers and potential markers for use in early detection strategies.

Genomic arrays

DNA amplification and deletion in lung cancers of various histological subtypes have been analyzed by genomic approaches. We recently published the results of such analysis in a series of 37 NSCLCs [13]. With this technique, we demonstrated substantial genomic differences between squamous carcinomas and adenocarcinomas that are consistent with earlier chromosome based comparative genomic hybridization studies [40–42]. The significant difference in the total number of abnormalities between squamous carcinomas and adenocarcinomas suggests that they may differ in the level of genome instability and/or in the mechanisms by which they progress. Chromosome 3q is a common area of chromosomal gain in a variety of solid tumors. When early lesions are treated,

they are known to prevent progression to invasive cancer. As discussed above, particularly common were amplified regions on chromosomal arms 1q, 3q, 5p, 8q, 11q, 12p, 17q and 20q, but gene amplification in chromosomal region 3q26 was the most prevalent abnormality. Among many amplified genes found in this region in a variety of solid tumors, some have been called potential candidate oncogenes (phosphatidylinositol-3 kinase catalytic subunit, PIK3CA) or genes suspected to be involved in tumor progression including the somatostatin gene (SST), p63 (p53 homologue gene), telomerase RNA component gene (TERC) and neutral endopeptidase gene (NEP). These patterns may ultimately be more predictive than analysis of expression of any single genes.

Expression arrays

RNA expression patterns may be more functionally relevant than DNA copy number changes, as most of these copy number changes affect cellular behavior via altered expression of included genes. The microarray technology developed in the mid 90's offers the hope that a genetic fingerprint of these tumors can be developed associated with clinical features. Beyond the need for better classification of lung cancers, this technical revolution opens a window of understanding to the world of tumor behavior (disease progression, recurrence, response to therapy) as well as to the mechanisms of tumor development. Tumor expression profiles are also influenced by the surrounding non-malignant cells. The combination of tumor and cell line profiling allows for the study of the regulatory role of both entities [89].

Efforts in classifying lung cancers based on microarray analysis revealed subclasses of adenocarcinomas. Selected genes allow the discrimination between primary lung cancer and metastasis of extrapulmonary sites [90]. Studies of expression profiles of adenocarcinomas of the lung using different chips commercially available [90] or custom arrays [91,92] identified different classes of tumors with some overlap. Four classes of adenocarcinomas were found to have specific prognosis and molecular signature. These were characterized respectively 1) by expression of cell cycle or proliferation genes, 2) by expression of neuroendocrine markers, 3) by expression of markers of alveolar origin, and 4) by expression of ODC or glutathione S-transferase [91]. The neuroendocrine subclass was found to have outcome significantly worse than the others. The hope is that these subclass differences will point towards new molecular therapeutic opportunities for these subsets. Interestingly, when applied to neuroendocrine tumors, cDNA microarrays found poor correlations between genes expressed in carcinoid and SCLC [93], tumors that may be morphologically similar but that behave very different clinically.

Protein profiling

Recent advances in protein profiling have suggested a poor correlation between gene expression and protein expression. Perhaps more significantly, it is now well established that protein activity is often highly regulated by post-translational modifications such as proteolysis and phosphorylation. Neither protein expression levels nor post-translational modification can be assessed by genomic or cDNA microarray technologies, prompting interest in evaluation of protein expression, commonly referred to as "proteomics".

Investigators, including those at our institution, have attempted to use several proteomic methods of analysis, including 2Dgel and IHC, to identify biomarkers in tumors [94–97] in body fluids such as bronchoalveolar lavage [98] of patients with or without cancer. We recently acquired experience in this method for profiling of proteins in cancer tissue [99]. We applied MALDI-MS to 79 surgically resected lung cancers and 14 normal tissues. Software written by Dr. Jason Moore at Vanderbilt allows assignment of protein peaks in the mass spectral data across samples into unique "bins" corresponding to unique peptide species with correction for multiply charged ions. Hierarchical clustering of the resulting data has allowed the identification of patterns distinguishing between tumor and normal as well as histological subgroups. For example, to identify proteomic patterns that distinguish primary NSCLC from metastases to the lung, we compared protein expression profiles obtained from 34 primary NSCLCs with those from 7 other types of lung metastatic tumors, including 5 metastases to the lung from other sites and 2 lung metastases from previously resected NSCLC in the training cohort. We identified 24 MS signals that could discriminate all of the primary NSCLC from non-primary NSCLC in the training cohort, and were able to perfectly classify blinded samples in a test cohort [100]. Proteomic patterns from primary tumors with prognostic discriminatory power were identified as well and are potentially very useful in the clinical management of lung cancer. Although requiring prospective validation, these data bring proof of concept to an approach that may be found to be very powerful at selecting surgical candidates and other therapeutic strategies based on novel biological targets.

Identification of biomarkers

Biomarkers are needed to identify patients at high risk for lung cancer and to identify surrogate endpoints for response to chemoprevention strategies.

Despite the societal need for the early diagnosis of lung cancer, no role for biomarkers has yet been established for decision-making in intraepithelial neoplasia of the lung. Technical procedures such as tissue processing, use of

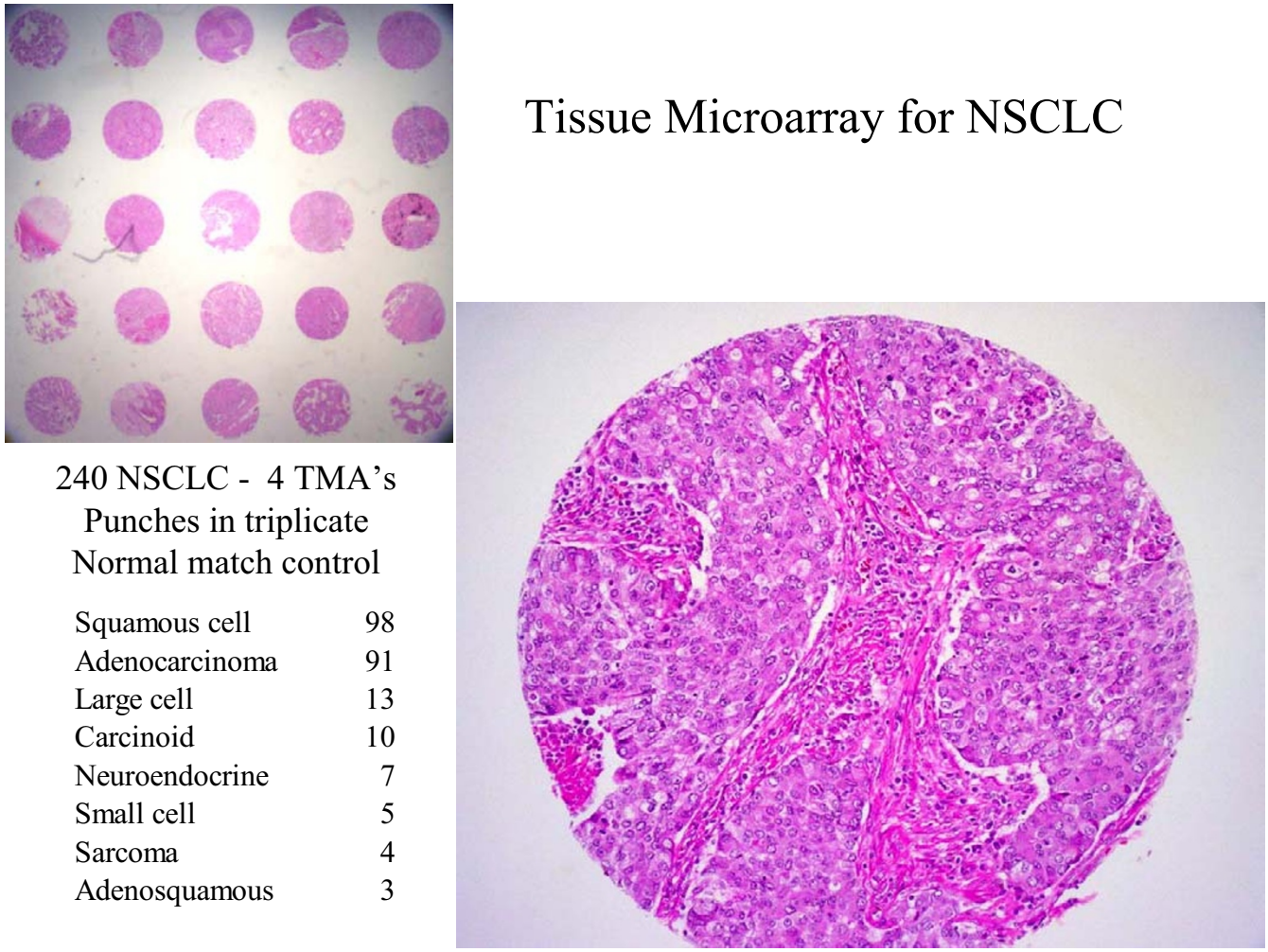

Figure 3
Tissue microarrays (TMAs) of lung cancer. TMAs are comprised of core biopsies of 0.6 mm in diameter of different tumors and of uninvolved lung from the same individuals. We retrieved 240 NSCLC tissue blocks from the pathological archives of Vanderbilt University between 1989 and 2001 and arrayed them in triplicate onto 4 separate TMAs. Tissue microarrays allow high throughput analysis of molecular markers identified in squamous lung neoplasia.

antibody reagents and data interpretation need to be developed and standardized. A comprehensive and integrated approach linking laboratory findings of IEN of the lung with clinical features holds the potential to identify clinically relevant genetic and protein markers of carcinogenesis.

The number of potential lung cancer-related genes is rapidly growing. Once identified, genes and proteins may be tested in large populations of patients by immunohistochemical or cytogenetic techniques on tissue microarrays [100]. This high throughput method allows the screening of hundreds of lung cancer samples on a single glass slide and will allow retrospective analysis of material stored with associated clinical outcome. The arrays typically comprise core biopsies 0.6 mm in diameter of different tumors and uninvolved lung from the same individuals retrieved from the pathology archives of various institutions [101] (Figure 3). A firmer understanding of the relationship of relevant protein and genetic markers to clinical and pathologic status could lead to more accurate estimates of the anatomic extent of disease, risk of recurrence, and most effective intervention.

From Genetic abnormalities to early detection and new therapies

The identification of early molecular events such as chromosomal gain or loss that predicts tumor development

suggests that early detection of lung cancer could be approached by means of molecular analysis. Sputum sample analysis for DNA methylation or chromosomal abnormalities by FISH may represent approaches suitable for early detection. The analysis of sputum, bronchial biopsies of preinvasive lesions using new detection methods such as fluorescence bronchoscopy [102], as well as exhaled breath condensate for tumor metabolites may be shown to be efficient ways of assessing high risk individuals. Early detection by low dose computed tomography scanning is being evaluated prospectively with the National Lung Cancer Screening Trial in 50,000 smokers. The addition of molecular studies may significantly increase the sensitivity and specificity of this new strategy for early detection.

Several therapeutic approaches to cancer have been developed to reduce undesirable expression of gene product or otherwise inhibit its function: (1) gene therapy (e.g. Adenovirus-p53) gene-specific ribozymes, which are able to break down specific RNA sequences, or with antisense oligonucleotides, (2) small molecule inhibition of receptor tyrosine kinases, (3) inhibition of p21(ras) farnesylation either by inhibition of farnesyl transferase or synthesis inhibition of farnesyl moieties, and (4) specific antibody approaches (e.g. anti-HER2 or anti-VEGF). We will touch on a couple of these approaches below.

Specific molecular targets

p53

Recently several phase I studies have evaluated the safety, biological effect and different routes of administration of adenoviral-mediated p53 gene therapy in various tumor types. These studies indicate that adenovirus-mediated p53 gene therapy and introduction of wild-type p53 into tumor cells represents a potentially valuable tool for the therapy of many types of human cancers [103] mainly by causing cell-cycle arrest or apoptosis [104,105]. When injected intra-tumorally, wt-p53 has shown to be expressed in patients with p53 mutations and 3/7 patients showed regression of tumor size [106]. Using the wild-type p53 recombinant adenovirus, the same group of investigators showed in phase I trial that 16/25 had stabilization of disease and 2 had partial remissions [107]. One of the major limitations of the intra-tumoral approach is the inefficient delivery of genes of interest within the tumor mass. We have shown that intra-alveolar delivery of the gene in patients with bronchioloalveolar carcinoma led to objective responses.

GFR antagonists

Several epithelial tumors express EGFR with and without EGFR amplification [108]. This EGFR overexpression is associated with increased ligand production and hyperactive receptor function. About a third or more of NSCLC showed overexpressed EFGR [109]. Overexpression of EFGR was also associated with poor prognosis of patients with NSCLC [110]. Low-grade bronchial preinvasive lesions have also been shown to overexpress EGFR [111]. EGFR expression has been found to be elevated in metaplastic biopsies when compared to normal biopsies in active smokers [112] and that when co-expressed with p53 may predict squamous cell carcinoma development. Interruption of this autocrine pathway with receptor antibodies (extracellular domain of the protein) or tyrosine kinase inhibitors (competition with the kinase ATP binding site) can cause tumor regression [113,114]. ZD1839, IRESSA and OSI-774 (Tarceva) are potent and specific inhibitors of the tyrosine kinase moiety of EGFR. Response rates in heavily pretreated patients with NSCLC vary between 10–18% in the IDEAL trials [115,116], which may seem low but is actually far higher than any standard chemotherapy and represents a major benefit for these low-toxicity oral agents. Studies are proposed to investigate the value of EGFR inhibition in combination therapy, in earlier stage NSCLC and in lung cancer chemoprevention (STOP trials, SPORE Trial of Lung Cancer Prevention). Such chemoprevention trials with molecular and morphologic (preinvasive lesions) surrogate endpoints may suggest reversibility of lesions. However, the rate of spontaneous regression of these preinvasive lesions is, as yet, poorly characterized.

Kras: Farnesyl transferase inhibitor Zarnestra

K-ras was one of the first oncogenes implicated in human cancer. Development of retroviral vectors containing anitsense K-ras constructs or inhibitors of ras function may reduce proliferation or tumorigenicity. Farnesyltransferase enzyme activity is required to transfer farnesyl isoprenoid to the Ras c-terminus to anchor it to the cell membrane. This step is critical for Ras activation as an oncogene. The ras protein is known to undergo a series of post-translational modifications at the c-terminal CAAX motif, which forms a thioether bond of p21 ras with farnesyl and ties it to the plasma membrane [117]. At the cell surface, ras relays growth regulatory signals from receptor tyrosine kinases to various pathways of cell signal transduction. Unfortunately the currently available inhibitors work best with activated H-ras, a rare finding in lung cancer rather than the more common K-ras activation. Also not well explained is the observation that antitumor activity is very poorly correlated with measurable activation of any of the ras genes. However, several farnesyltransferase inhibitors are currently being tested in the clinic. R115777-Zarnestra is also being proposed in the clinic in a secondary chemoprevention trial. This trial is essentially based on the efficacy of FTI-276 on established lung adenomas (considered to be premalignant lesions of the lung) from A/J mice exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-related carcinogen

[118]. Analysis of the tumors showed a 60% reduction in tumor multiplicity and a 42% reduction in tumor incidence as well as a significant reduction in tumor volume (approximately 58%).

COX-2 inhibition

Cyclooxygenase-2 (COX-2) is an inducible enzyme that catalyzes the production of prostanoids. COX-2 can activate carcinogens in tobacco smoke [119], and COX-2 expression may play a role in angiogenesis by correlating with VEGF levels [120]. In addition, COX-2 activity may have a role in inhibiting apoptosis and modulating immune responses [121]. While nonsteroidal anti-inflammatory drugs have shown to reduce the risk of colorectal cancer, no such evidence yet exists in lung cancer. COX-2 inhibition has proven to reduce lung cancer cell growth *in vitro* [122]. *In vivo*, COX-2 has shown to cause persistent remission in patients otherwise refractory to lung cancer. COX-2 overexpression is a marker of poor prognosis in early stage NSCLC [123,124]. COX-2 inhibitors are being evaluated in combination therapy for chemoprevention and therapy for lung cancer.

Other targeted strategies

Other targets include antibodies against VEGF ligand, EGFr or HER2 and inhibition of proteosome activity to counteract NFκB activation. All of these are currently in large scale clinical trials. Markers identified as being overexpressed in lung cancers represent potential immunotherapy targets even if no significant function can be found for the marker protein. An example is the recent identification of frequent overexpression of the cancer testis antigens from the microarray studies [125]. These genes are already being tested as vaccine targets in melanoma, and are only recently recognized as being overexpressed in the majority of non-small cell lung cancers.

Conclusions

A large number of genetic pathways associated with cancer development are being discovered at a rapid pace. The clinical impact of this recent knowledge on disease management is still relatively small, but real and growing. Little progress has been made in lung cancer chemoprevention, yet preventing, inhibiting and reversing the preneoplastic changes leading to cancer may ultimately prove a much more tractable goal than treating advanced disease. The slow process of carcinogenesis makes this period an open window for chemoprevention so that the intervention occurs when genetic instability is still controllable.

Abbreviations

CGH: comparative genomic hybridization, LOH: loss of heterozygosity, FISH: fluorescence in situ hybridization, TMA: tissue microarray, NSCLC: non small cell lung cancer

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