

Use of Markers for the Detection and Treatment of Lung Cancer

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Abstract. The unacceptably high morbidity and mortality associated with the diagnosis of lung cancer mandates new approaches toward the early detection and treatment of this disease. Enhanced understanding of the molecular biology of the carcinogenic process is identifying many potential markers of risk of lung cancer occurrence as well as of poor prognosis. Identification of high risk populations who are at greatest risk of being diagnosed with and dying from lung cancer would allow delivery of more intensive screening and interventions to the individuals who are most likely to benefit from such strategies. This review examines the current status of markers of lung cancer risk, early detection, and prognosis, and their applicability to current standards of clinical care.

1. Introduction

Lung cancer is the leading cause of cancer deaths in the United States and one of the leading causes of cancer death in the world [1]. Barriers to reducing the mortality of this disease include the high percentage of patients presenting with advanced stage disease without effective treatment options, the lack of a proven screening method to identify patients at earlier and potentially more curable stages of disease, and the high risk of relapse and second primaries in patients who have undergone curative resection. Concerted efforts are underway in several areas to effect reductions in mortality, including attempts to identify individuals at the highest risk for the development of lung cancer or for relapse following treatment of a primary cancer in order to define the populations that are most likely to benefit from aggressive screening and treatment options. Delivery of improved screening techniques, chemopreventive interventions, and novel therapeutic approaches to the appropriate at-risk target populations is more likely to be effective than current non-targeted approaches.

Much of our knowledge of the progressive cellular changes that lead to lung cancer is derived from observational work on the bronchial epithelium of current and former smokers and non-smokers. Auerbach et al. were the first to describe preinvasive neoplastic lesions within the tracheobronchial tree of smokers and former smokers and to draw a relationship between the lesions and smoking history [2,3]. The finding of multiple and diffuse lesions throughout the tracheobronchial tree supports the concept of field cancerization, which describes the potential for multiple, carcinogen-exposed epithelial cells within an organ or body tissue to develop into invasive carcinoma as opposed to cancer developing from a single dominant clone [4]. Implicit in this concept is the understanding that a single, pre-neoplastic lesion may not itself develop into an invasive cancer but serves as a marker for the presence of other at-risk lesions, which may progress independently. Traditionally, the sequence of lung carcinogenesis has been defined strictly in terms of histological abnormalities. With improvements in molecular biological techniques, a companion genetic sequence is beginning to be identified. The recognition of the genetic abnormalities that underlie and even precede the histological changes has extended the concept of field cancerization, such that the earliest preinvasive lesions

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may be genetic and are succeeded by a combination of genetic and histological changes [5]. Thus, molecular markers offer the potential of identifying individuals at risk for lung cancer as well as serving as markers for early detection. This review will focus on recent advances in the use of markers to aid decision-making in the detection and treatment of lung cancer.

2. Markers of risk

2.1. Tobacco

Tobacco smoking, particularly cigarettes, is the leading cause of lung cancer and remains one of the strongest environmental causes of cancer [6]. Advances in our understanding of the many potent ill-health effects of tobacco smoking coupled with vigilant public health campaigns have led to overall reductions in the number of current smokers in the United States. In men, these reductions have translated into decreases in both lung cancer incidence and mortality over the last decade [7,8]. However, even following cessation, the long-term consequences of cigarette smoking still present a substantial public health burden [9].

Classically, it is estimated that approximately 10% of all smokers will develop lung cancer [10]. However, recent analysis suggests that this figure is closer to 15% in men ≥ 85 years of age with a 50 or greater year history of smoking and 8% in women ≥ 85 years of age with a comparable smoking history [11]. If the impact of competing causes of death could be minimized, the lifetime risk of lung cancer may be as high as 24% in the male smoker and 11% in the female smoker.

Lung cancer also remains a significant problem for former smokers, who account for an increasing proportion of all new diagnoses [12,13]. While prior reports emphasized a decline in lung cancer risk following smoking cessation relative to continuing smokers, several new studies have focused instead on an individual's cumulative lifetime risk of lung cancer [14]. When viewed in this way, it appears that the lifetime risk of lung cancer does not decrease following smoking cessation but rather stops rising [15]. For example, the risk of lung cancer in a 68 year old man who has smoked two packs of cigarettes per day for 50 years is 11% over the next 10 years if he quits smoking immediately, but 15% if he continues to smoke the same amount. Hence, present efforts in primary lung cancer prevention and early detection are equally focused on both current and former smokers.

2.2. Prior aerodigestive malignancy

Survivors of a prior lung cancer or head and neck cancer are at the greatest risk for a new lung cancer [16, 17]. In survivors of a prior non-small cell lung cancer, the annual risk of a new primary lung malignancy is 1–4% and the cumulative risk at 6–8 years following original diagnosis is 13–20% [16]. In survivors of a prior small cell lung cancer, the annual risk is even higher. In head and neck cancer survivors, the risk of a second primary tumor anywhere in the aerodigestive tract is 1.5–5.1% per year and at least 25% of such tumors are in the lung [17,18]. Despite the fact that many of these patients undergo regular screening and that the second primary tumors are diagnosed at earlier stages compared to first-time cancer patients, the five-year mortality remains extremely high at 80%.

2.3. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD), as defined by the World Health Organization, is “a disease state characterized by airflow limitation that is not fully reversible” [19]. Operationally, the diagnosis is established in an individual with a post-bronchodilator forced expiratory volume in 1 second (FEV1) $< 80\%$ of predicted and with an FEV1 to forced vital capacity (FEV1/FVC) ratio $< 70\%$. Current and former smokers with COPD have an increased risk of developing and dying from lung cancer [20]. The increased risk appears to be equally present in men and women and to correlate inversely with the FEV1 [21–23]. In a follow up analysis of participants in the first National Health and Nutrition Examination Survey, subjects at baseline enrollment with moderate or severe COPD defined as an FEV1/FVC ratio less than 0.7 and an FEV1 less than 80% of the predicted value, were found to have 2.8-fold increased risk of lung cancer after adjusting for age, gender, and smoking history when compared to subjects with normal lung function at baseline [20]. Similar findings have been reported in Northern European populations [24]. Biologically, the factors that increase lung cancer risk in patients with COPD, even when controlling for smoking history, are poorly understood, but may include common susceptibility genes, inflammation, and oxidative damage [25].

2.4. Insulin-like growth factors

The insulin-like growth factors (IGFs) are mitogenic peptide hormones that regulate cell proliferation and apoptosis [26]. In blood, IGF-I and IGF-II principally circulate bound to IGF-binding protein-3 (IGFBP-3). Plasma levels of IGF-I are associated with an increased risk for prostate, breast, and colon cancers while plasma levels of IGFBP-3 appear protective for colon cancer risk [27–29]. Case control studies testing for an association between plasma IGF and IGFBP levels and lung cancer have shown mixed results for IGF-I and IGFBP-3 and no association for IGF-II, IGF-III, IGFBP-1, and IGFBP-2 [30–33]. The Japan Collaborative Cohort Study prospectively measured serum IGF-I, IGF-II, and IGFBP-3 in over 9,500 subjects, 194 of whom developed lung cancer in over 8 years of follow up [34]. Initial analysis suggested that higher IGF-II and IGFBP-3 levels were protective against lung cancer. However, when the levels of IGF-II and IGFBP-3 were adjusted for each other, the protective effects were no longer present. Serum IGF-I levels, adjusted for IGFBP-3 levels, were predictive of lung cancer risk. The odds ratio was 1.74 (95% CI 1.08, 2.81) for subjects in the highest quartile of IGF-I levels compared to the lowest. London et al. found in a prospective study of 18,224 Chinese men in Shanghai, that serum IGF-I levels were not predictive of lung cancer risk while a protective effect for IGFBP-3 was demonstrated (OR 0.41, 95% CI 0.18, 0.92) when the analysis was restricted to ever smokers and adjusted for IGF-I levels and smoking history [35]. Therefore, while plasma or serum IGF-I levels and IGFBP-3 levels hold promise as risk markers for lung cancer, further refinements and analysis will be necessary prior to any clinical application.

2.5. DNA repair capacity

Embedded in the concept of inter-individual variation in the risk of cancer is that individuals differ in the ability of their cellular enzymatic machinery to identify and repair acquired genetic mutations [36]. The clinical laboratory correlate is the measurement of DNA repair capacity, which typically is performed by one of two means. Mutagen sensitivity is assessed by exposing *ex vivo* peripheral blood lymphocytes to a DNA mutagen such as bleomycin, arresting the cells in mitosis, and counting chromatid breaks [37]. This assay tests the enzymatic machinery involved in base excision repair. Case control studies have demonstrated that mutagen sensitivity is an independent risk factor for lung can-

cer and the magnitude of the risk correlates with the number of induced chromosomal breaks. Subjects in the highest bleomycin sensitivity quartile have a 2.81-fold increased risk of lung cancer compared to subjects in the lowest reference quartile after adjusting for age, gender, and smoking status [38]. Benzo[α]pyrene diol epoxide (BPDE) also is used as a mutagen for laboratory assays and may measure different repair mechanisms than bleomycin, as the two assays correlate only weakly [39]. Wu et al. assessed bleomycin sensitivity, BPDE sensitivity, and serum IGF-I levels in lung cancer subjects versus matched controls [31]. They found that subjects with high serum IGF-I levels and high sensitivity to each mutagen assay had the greatest risk of lung cancer (OR 17.09, 95% CI 4.16, 70.27). Hence mutagen assays may be more powerful when used in combination.

A second way to assess DNA repair capacity involves the use of reporter genes [40]. A nonreplicating recombinant plasmid carrying the reporter gene of interest is exposed to a mutagen such as BPDE and then introduced into donor lymphocytes *in vitro*. A non-mutagenized plasmid is used as a control. For this assay, DNA repair capacity is reported as the ratio of activity of the reporter gene in cells transfected with the mutagenized plasmid to the activity of the reporter gene in cells transfected with the non-mutagenized, hence functional, plasmid. Cells transfected with the mutagenized plasmid should express the reporter gene only if they are capable of repairing the mutagenized plasmid. Using this technique, Spitz et al. have shown a significant inverse linear relationship between DNA repair capacity and lung cancer risk [41,42]. Subjects in the highest risk category, who have the least robust DNA repair capacity, have approximately a 2-fold increased risk of lung cancer [38]. Interestingly, lower DNA repair capacity was associated with younger age and female gender in the cases. One important limitation to this methodology is the recognition that DNA repair capacity is not static and may be influenced by exogenous factors, including psychosocial stress and tobacco smoke [43]. In the above cited study, Spitz et al. found higher DNA repair capacity in current smokers compared to former smokers and in heavy smokers compared to light smokers, suggesting high cigarette consumption may augment DNA repair capacity [38]. These observations raise serious questions about the reproducibility of such findings and underscore the need for prospective observational studies.

2.6. Lung cancer susceptibility genes

The recognition that lung cancer does not uniformly afflict all smokers has led to intense interest to identify lung cancer susceptibility genes [44]. Most directly implicated in this pursuit are the carcinogen metabolism genes. Phase I enzymes are responsible for initial catalysis and activation of carcinogens. Of these enzymes, the cytochrome P450 (CYP) gene family is the best studied. The CYP1A1 gene product is involved in the metabolism of the polyaromatic hydrocarbons from cigarette smoke. The CYP1A1 +2455A-G polymorphism results in an isoleucine to valine substitution near the heme binding site of the protein molecule, which is associated with increased activation of tobacco-smoke derived polyaromatic hydrocarbons [45]. In Japanese populations, this polymorphism is associated with a markedly increased risk for lung cancer [46]. However, comparable studies in Caucasian and African-American populations have not found such an association [47–49]. The same restriction enzyme MspI used to identify the +2455A-G polymorphism also identified a second polymorphism in intron 6, CYP1A1 +3801T-C. The functional significance of this second polymorphism is unknown. However, some studies have suggested an association with increased lung cancer risk in Caucasian populations [50]. These results require further verification.

The risk of lung cancer in carriers of the CYP1A1 MspI polymorphism is modified by the presence of a null allele for the glutathione-S-transferase M1 (GSTM1) gene [51]. GSTM1 is a phase II enzyme responsible for detoxification of polyaromatic hydrocarbons [44]. The null allele is associated with reduced gene product. Hence, the balance between carcinogen activation by a phase I enzyme such as CYP1A1 and detoxification by an enzyme such as GSTM1 may ultimately determine the amount of oxidative DNA damage. Asians bearing the CYP1A1 Msp I allele in combination with the GSTM1 null polymorphism have a 9-fold risk of lung cancer although these results require confirmation in other populations [52]. Given the complexity of carcinogen metabolism, investigations that take into account multiple enzymes and pathways are the most likely ones to be informative [53].

As previously discussed, lung cancer susceptibility may be influenced by DNA repair capacity [54]. There are four broad categories of DNA repair: base excision repair; nucleotide excision repair; mismatch repair; and double strand break repair. Mutagen assays predominantly test the integrity of nucleotide ex-

cision repair which functions to repair bulky lesions such as chemical and radiation-induced adducts. The XPD gene, named for its classification within the xeroderma pigmentosum D complementation group, produces an 80-kilodalton (kD) subunit of transcription factor IIIH (TFIIH) that is essential to nucleotide excision repair [55]. Two polymorphisms in the XPD gene have been well characterized. Homozygote carriers of the +751A-C polymorphism, which results in a glutamine substitution for lysine in the protein product, have significantly lower DNA repair capacity while heterozygotes have intermediate repair capacity. Likewise, a similar trend exists for the +312G-A polymorphism [56]. Case control studies have not shown a significant association of the +751A-C polymorphism and lung cancer risk. In contrast, Zhou et al. have demonstrated a slightly increased risk (OR 1.47, 95% CI 1.1, 2.0) of lung cancer for the Asp312Asn polymorphism [57].

The OGG1 gene product is a base excision repair enzyme that catalyzes the removal of oxidized guanine moieties [54]. The +326T-A polymorphism is associated with diminished repair capacity [58]. Le Marchand et al. demonstrated a 2-fold increase in lung cancer risk associated with this polymorphism, with the most pronounced risk being in native Hawaiians [59]. Sugimura, however, in a study of 101 Japanese case subjects and 250 control subjects, did not find an increased risk for all lung cancer histologies with the +326T-A polymorphism, although there was increased risk for squamous cell carcinoma (OR 3.0, 95% CI 1.3, 6.8) [60]. A separate study performed only in Caucasians did not show any association with lung cancer [61].

Collectively, the studies to date that have investigated genetic susceptibility to lung cancer have been largely inconclusive. They do serve to emphasize the difficulties of conducting genetic studies in a disease with a strong environmental component. Association studies compare allelic frequencies for the gene polymorphism of interest between cases and controls. They are the most frequently performed genetic studies and often give inconsistent results. One reason for the inconsistent results is population stratification. The frequency of polymorphic alleles varies between populations. For example, the CYP1A1 MspI polymorphism is more frequent in Asians and African-Americans, where it is present in 37% and 22% of the population, respectively, than in Caucasians, where it is found in only 10% [62]. Any case control composed of heterogeneous populations may falsely demonstrate an associ-

ation of this polymorphism with the trait under study if the cases and controls are not well matched. A second problem is linkage disequilibrium. In Asians, the CYP1A1 +2455A-G polymorphism and the +3801T-C polymorphism cosegregate. Any increased risk for lung cancer attributable to the +2455A-G polymorphism may really represent an association with a related sequence which may not cosegregate with this polymorphism in non-Asian populations. Hence, verification of any association in other populations is essential.

Ideally, these problems can be mitigated by familial linkage studies. However, given the strong requirement for tobacco smoke exposure in order to produce the case phenotype (i.e., lung cancer), the relatively delayed presentation of lung cancer in cases, and the high mortality of the disease, such studies require enormous resources. The Genetic Epidemiology of Lung Cancer Consortium is a familial study of lung cancer susceptibility funded by the National Institutes of Health that hopes to overcome these difficulties through a large, multicenter, collaborative effort. This ambitious undertaking plans to screen over 70,000 incident lung cancer cases in order to identify 800 high-risk families defined by the presence of at least three affected first generation relatives. Only the most informative 100 families will be studied for linkage analysis. The investigators hope to identify a lung cancer susceptibility locus and ultimately to identify the associated genes. A separate but related approach for familial studies may be to focus on young, nonsmoking probands with lung cancer who, in theory, carry a high penetrance susceptibility gene. Yang et al. studied 257 families, encompassing more than 1,800 subjects [63]. Based on a Mendelian codominant model, they estimated that homozygous carriers of a susceptibility allele had an 85% risk of lung cancer by age 60 if male, and 74% if female. In contrast, heterozygote carriers only had an increased risk of lung cancer if they were smokers or had chronic bronchitis, suggesting that there may be an interaction between the susceptibility allele and smoking. However, the estimated allelic frequency for the general population was only 0.4%, making the attributable incidence of lung cancer in the general population negligible.

3. Early detection

3.1. Sputum analysis

Early detection methods for lung cancer need to survey two distinct anatomic compartments in the lung:

the central airways and the periphery. Both sputum cytology and airway inspection via bronchoscopy accomplish surveillance of the central airways. Saccomanno was the first to systematically study sputum cytology as a predictor of lung cancer risk. In a large cohort of uranium miners, Saccomanno documented progressive degrees of cytological atypia preceding the diagnosis of lung cancer by several years [64]. Data from the Hopkins combined sputum cytology and chest x-ray screening trial for lung cancer suggests that the risk of invasive lung cancer is approximately 10% over the next 9 years in subjects with moderate sputum atypia and > 40% in subjects with severe atypia [65]. In terms of defining subjects at higher risk for the purposes of interventional studies, sputum cytology has become an important screening mechanism [66]. The main logistical problems with sputum cytology as a screening method for lung cancer are the adequacy of sputum collection, intra- and inter-observer variability, and relatively low sensitivity of 20–30% for early lung cancer [67–69].

One means of improving the sensitivity of sputum cytology for early detection is to focus on the detection of molecular markers within expectorated epithelial cells. Belinsky et al. were able to identify promoter region hypermethylation of the tumor suppressor gene, p16INK4a, in spontaneously expectorated epithelial cells from 8 of 33 smokers with negative screening chest x-rays [70]. Three of the 8 with p16 abnormalities were found to have lung cancer. One of the other 5 subjects developed lung cancer within a year and two others had moderate epithelial atypia and marked atypia suspicious for malignancy, respectively. Overall, p16 promoter hypermethylation was associated with a 3-fold risk of an immediate diagnosis of lung cancer. In a retrospective analysis of 21 subjects with squamous cell carcinoma of the lung, the same investigators were able to identify either p16 or MGMT promoter hypermethylation in expectorated epithelial cells at the time of lung cancer diagnosis in 100% of subjects (10/10) for whom a specimen was collected at time of diagnosis [71]. Traditional sputum cytology only identified lung cancer in 40%. Furthermore, the investigators studied archival sputum specimens in 11 subjects and found that either p16 or MGMT promoter hypermethylation preceded the diagnosis of lung cancer by 5 to 36 months in 100% of subjects. In a third study, the same investigators also found p16 promoter hypermethylation of expectorated epithelial cells in 35% of cancer free smokers and former smokers referred for suspicion of lung cancer [72]. p16 promoter hyperme-

thylation was demonstrable in bronchial epithelial cell cultures from brushings in 44% of patients with lung cancer and an equal number of cancer-free control subjects, irrespective of whether they were current or former smokers. In contrast, p16 promoter hypermethylation was not present in never smokers. These results are consistent with the hypothesis that p16 promoter hypermethylation may be an early and important event in lung carcinogenesis and may predict subjects who are at greatest risk of developing lung cancer. However, further prospective study is necessary.

3.2. Fluorescence bronchoscopy

Fluorescence bronchoscopy is a relatively new technique that exploits differences in autofluorescence between dysplastic and normal tissues to allow the more sensitive detection of preinvasive neoplasia, including carcinoma-in-situ (CIS) [73,74]. Employed principally as a research tool, fluorescence bronchoscopy may allow the more rigorous examination of the large, conducting airways. Like more conventional fiberoptic bronchoscopy, the main limitation to fluorescence bronchoscopy is that most of the conducting airways, hence the at-risk epithelium, is beyond the reach of the scope. However, the ability to detect central preinvasive neoplasia may be an important means of stratifying high-risk individuals. In fact, the presence of a preinvasive lesion may be a more important risk marker for developing lung cancer at a separate, noncontiguous site than at the site of the lesion [75].

Several studies have reported the natural history of lesions found by fluorescence bronchoscopy. Bota et al. reported their experience with fluorescence bronchoscopy, over a 2 year period, following 104 high-risk subjects defined by a prior history of aerodigestive cancer, 20 pack year smoking history, or occupational asbestos exposure [76]. Low grade lesions, defined as mild or moderate dysplasia, were identified in 40% of biopsy samples while high grade lesions, defined as severe dysplasia or CIS, were identified in approximately 15% of samples. Over the follow up period, only 1 subject developed invasive cancer although three subjects developed CIS. Furthermore, none of the 59 lesions showing severe dysplasia or CIS at baseline progressed to invasive cancer, although this study is confounded by the local treatment of such lesions with ablative therapy. A separate study by Venmans et al. prospectively followed nine patients with bronchial CIS [77]. Five of the nine lesions progressed to invasive carcinoma, all within 10 months of the diagnosis

of CIS, despite aggressive local therapy. One patient had a synchronous invasive lung carcinoma at time of diagnosis of CIS and three others had moderate or severe bronchial atypia, one going on to become invasive carcinoma. Overall, six developed invasive carcinoma following the diagnosis of CIS. The prevalence of dysplasia in these studies was much higher than reported by Lam et al. in a North American population of current and former smokers with at least a 30 pack-year smoking history and atypia on sputum cytology [78]. In their study, moderate or severe epithelial dysplasia was present in 19% and CIS in 1.6%. In contrast, the study of Bota et al. was enriched by the inclusion of cancer survivors, which constituted 35% of the study population and by current cancer patients who constituted another 13% [76]. As fluorescence bronchoscopy is further employed as a tool for airway surveillance, prospective study will be needed to better define the natural history of these pre-invasive neoplastic lesions. Potentially, stratification of dysplastic tissue by associated genetic and epigenetic alterations may give further guidance in regards to which patients are at greatest risk for developing invasive lung cancer.

3.3. Helical CT

Low-dose helical computed tomography (HCT) may serve as an important adjunct to the surveillance methods of the central airways. In contrast to bronchoscopy, which relies on direct, close visualization of at-risk epithelium, HCT principally images the lung periphery. HCT is an improvement over conventional CT in that it allows the rapid and complete visualization of the lung periphery in seconds. Hence, radiation exposure and time expenditure are minimized. Henschke et al. reported their experience with HCT in an urban, North American population of current and former smokers over the age of 60 [79]. The investigators found HCT to be significantly more sensitive for the detection of non-calcified lung nodules than conventional chest x-ray. A new diagnosis of lung cancer was made in 2.7% of HCT subjects, 85% of whom had stage I disease. Mayo Clinic investigators reported a similar percentage of prevalent lung cancers detected by baseline HCT [80]. In the Mayo study, another 3 cancers were diagnosed at the one year surveillance visit and there were no lung cancer related deaths.

In view of the potential of HCT as a screening tool for the early diagnosis of lung cancer, the National Institutes of Health has initiated the National Lung Screening Trial (NLST). The NLST is a multicenter, col-

laborative effort that compares conventional screening chest radiography to HCT. Eligible subjects are men and women, aged 55–74, with at least a 30 pack year smoking history. Subjects will be randomized to either HCT or chest x-ray at baseline and then will complete annual follow up examinations for the next 2 years. All subjects will be followed for approximately four years after study termination. The primary endpoint is a reduction in lung cancer mortality. Presently, approximately 80% of incident lung cancer patients present with regionally advanced or metastatic disease [81]. However, overall five year survival is only 15% while it is > 80% for patients presenting with stage Ia disease. Therefore, it is hoped that the stage shift seen with HCT will translate into meaningful reductions in lung cancer mortality. While Henschke et al. [79] did not identify a high frequency of benign nodules in patients undergoing curative resection (< 4%), approximately 30% of subjects in the Mayo study who underwent curative resection had benign nodules [80]. Therefore, one potential problem with HCT is the high frequency of false positives although with greater experience, this number may diminish. Should the National Lung Screening Trial demonstrate a significant reduction in lung cancer mortality, further refinements will be necessary to develop the optimal frequency for screening and the best populations to target.

4. Markers of prognosis

The poor overall survival rates for lung cancer hide the heterogeneity of outcomes in individuals with the disease, particularly if diagnosed with early stage disease. Although pathologic stage and resectability remain the most powerful predictors of clinical outcome, it is important to identify resected patients at high risk for relapse who would be most likely to benefit from further adjuvant therapy. Clinicopathologic features useful in stratifying good versus poor prognosis in patients have been reviewed previously and will not be discussed here [82,83]. Suffice it to say that although histopathologic attributes of the primary tumor, such as histologic subtype or degree of differentiation, have some impact on risk of recurrence, the strength of this association is insufficient to impact on subsequent clinical care. The focus of the following discussion therefore will be on the use of molecular markers to determine prognosis after the diagnosis of lung cancer.

4.1. Positive growth regulators

Mutations of proto-oncogenes of the ras family, particularly mutations in K-ras codons 12, 13, or 61, occur in approximately one-third of adenocarcinomas of the lung [84]. Members of this gene family encode a 21-kDa membrane-associate protein with GTP-binding activity involved in the transduction of growth signals. In a study of 69 patients with completely resected adenocarcinomas, Slebos et al. showed that patients with K-ras codon 12 mutations had a significantly worse survival than patients without mutations [85]. Sixty three percent of patients with a K-ras codon 12 mutation died within a 3 year follow up period versus 32% of patients without the mutation. However, multiple subsequent studies failed to agree on the prognostic significance of K-ras mutations, as exemplified by an ancillary study conducted by the Eastern Cooperative Oncology Group showing no prognostic significance in 197 patients with stage II or IIIA NSCLC randomized to postoperative radiotherapy plus or minus chemotherapy [86]. Huncharek et al. conducted a meta-analysis of 8 studies in the literature between 1985–1997, encompassing 881 patients with a frequency of K-ras mutations of 25%, to determine the relative risk (RR) of death at 2 years associated with the presence of K-ras mutations [84]. The RR was 2.35 (95% CI 1.61, 3.22), indicating a poor prognosis associated with K-ras mutations, but the wide heterogeneity of these studies made it impossible to determine whether this association would persist after adjusting for other well-described prognostic indicators such as stage. Although this data is suggestive, the prognostic significance of K-ras mutations awaits further prospective testing.

Similar to mutations of K-ras that activate growth stimulatory pathways, over-expression or activation of receptor tyrosine kinases has also been implicated in lung carcinogenesis. The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein whose extracellular domain binds various polypeptide growth factors, resulting in dimerization of the receptor, autophosphorylation, and transmission of the mitogenic signal [87]. It is over-expressed in 13–80% of NSCLC (24–89% of squamous cell cancers versus 23–46% of adenocarcinomas) and is the target of new therapies exemplified by drugs such as gefitinib and erlotinib; the former being approved for treatment of NSCLC after failure of both platinum-based and docetaxel chemotherapies [88]. Multiple studies have assessed the prognostic value of EGFR expression. A recent meta-analysis of 16 eligible studies de-

termined that EGFR may be a poor prognostic indicator in NSCLC, but the magnitude of the effect was small and may be subject to publication bias [87]. Of the studies examined, EGFR expression was associated with survival benefit in one trial [89], a survival disadvantage in 3 trials [90–92], and with no statistically significant effect on survival in 12 studies [93–104]. EGFR was expressed in 51.1% of the 2,810 evaluable patients with various histologic subtypes of NSCLC. EGFR was not prognostic, with the hazard ratio (HR) = 1.14, (95% CI 0.94, 1.39), when all studies were considered in the meta-analysis. When the analysis was limited only to studies employing immunohistochemistry as the primary method of detection for EGFR, the result was weakly significant (HR = 1.13, 95% CI 1.00, 1.28). However, one must keep in mind that total expression of a protein is not necessarily correlated with its functional, or active form. Kanematsu et al. found that phosphorylation, but not overexpression, of EGFR was associated with short time to progression and poor prognosis both in early and advanced NSCLC [105]. Similarly, Piyathilake et al. showed that cytoplasmic expression of EGFR, which correlated with ligand activation in *in vitro* studies, was associated with poor overall survival in 60 patients with squamous cell carcinoma, while membranous expression was not associated with survival [106]. Furthermore, co-expression of the EGFR ligand, TGF- α , with any form of EGFR (cytoplasmic or membranous) imparted a significantly worse prognosis. Studies that take into account the function of a protein may be more accurate in assessing the prognostic significance of proteins that undergo changes such as post-translational modifications before becoming fully functional.

Another tyrosine kinase receptor that has been extensively studied in lung cancer is HER-2/neu, also a member of the epidermal growth factor receptor family. HER-2/neu is a 185 kDa transmembrane receptor tyrosine kinase similar to EGFR [107]. Given the important prognostic and therapeutic implications of HER2/neu in breast cancer, multiple studies have examined the frequency of expression and biologic implications of HER2/neu in lung cancer as well. Meert et al. recently reviewed the literature and performed a meta-analysis on 30 studies of 4582 patients with lung cancer where prognosis was assessed [108]. HER-2/neu expression, as determined by a variety of different techniques including immunohistochemistry with several different antibodies, ELISA, or PCR, was detected in 31% of all patients with NSCLC, in 30% of patients with adenocarcinoma only, and in 30% of patients with small

cell lung cancer. Expression was equally frequent in locoregional disease and advanced disease (32% vs. 36%). Thirteen studies showed a significant detrimental effect of HER-2/neu on survival [94,102,104,109–118], 1 showed a positive effect [98], and 16 showed no significant correlation [83,95,97,101,119–131]. In the meta-analysis (20 published studies with sufficient information available), HER-2/neu was found to be significantly associated with poorer survival (HR = 1.55, 95% CI 1.29, 1.86). However, the technical limitations due to heterogeneity of study designs and publication bias toward positive studies point to a need for a prospective study to adequately assess the impact of HER2/neu on lung cancer prognosis.

4.2. Cell cycle regulators

Multiple studies have examined the correlation between prognosis and proliferative indices or cell cycle regulatory proteins that are involved in controlling cell replication. Higher expression of proteins expressed primarily during cell division, such as Ki-67, proliferating cell nuclear antigen (PCNA), and minichromosome maintenance protein 2 (MCM2), has been associated with poorer long term survival in many, but not all, studies [112,132,133]. When both Ki-67 and MCM2 were evaluated by immunohistochemistry in a study of 221 NSCLC cancer, lower MCM2 was associated with longer survival (46 versus 31 months, $p = 0.039$) and a lower relative risk of death (0.55, 95% CI 0.34, 0.88), while Ki-67 was not prognostic [134].

In an effort to further refine the studies on cell proliferation, recent studies have begun to examine various proteins that are positive and negative regulators of critical junctions during cell cycle progression. Cell cycle progression is governed by cyclin dependent kinases (cdks) whose activity is regulated by binding with positive effectors (cyclins) and negative effectors (cdk inhibitors). The D-type cyclins and cyclin E regulate the transition from G1 to S. Dosaka-Akita found that high-level cyclin E expression was found in 53% of 217 NSCLCs and was associated with higher Ki-67 labeling as well as significantly lower 5-year survival (81% vs. 57%, $p = 0.007$) [135]. Cyclin D1, on the other hand, was not associated with Ki-67 or survival. Cyclin A, which is expressed in S phase, has also been found to be associated with prognosis in studies where expression was measured by immunohistochemistry but not when expression was measured by RT-PCR [136–138]. Cyclin B2, which is a key modulator of the G2-M transition, was found to have high level expression in 22%

of 77 NSCLC and was associated with poor survival in squamous cell carcinomas only [139].

Negative regulators of the cell cycle include p21/Waf1 and p27, which inhibit cyclin-cdk complexes and prevent cell cycle transit. Shoji et al. found that p21 was expressed in 51.5% of 120 tumors from patients with NSCLC, and p21 loss was associated with significantly worse 5-year survival (73.8% vs. 60.7%, $p = 0.006$) [140]. Similarly, a study of 98 patients with NSCLC demonstrated that p27 was present in 42% of cases and loss was associated with poor survival [141]. Furthermore, the combination of high cyclin E and low p27 expression identified a particularly poor prognosis group.

4.3. Negative growth regulators

In addition to dysregulation of proliferative signals, carcinogenesis is also characterized by loss of negative regulators of cell proliferation. The tumor suppressor gene p53 is a major determinant of cell survival and maintains the integrity of the human genome [142]. It is a nuclear phosphoprotein with transcriptional activation capabilities that is inactivated in over half of all human cancers, including lung cancer. Inactivation of p53 by missense mutation usually leads to a prolonged protein half-life with nuclear accumulation that can be detected by immunohistochemistry, although immunohistochemical detection of p53 is not always correlated with genetic alterations.

There is no consensus regarding the association between p53 mutation or immunohistochemical expression and prognosis. Ahrendt et al. conducted a prospective study of 188 patients with operable NSCLC, stages I-IIIa, using direct dideoxynucleotide sequencing and p53 GeneChip analysis to look for p53 mutations [143]. p53 mutations were found in 55% of tumors, with a significant negative prognostic effect in stage I tumors (HR = 2.8, 95% CI 1.4, 5.6) but not in higher stage cancers. Schiller et al. also found no prognostic significance to p53 mutation as determined by single-stranded conformational polymorphism or overexpression as determined by immunohistochemistry in a prospective ancillary study of 197 patients with stage II or IIIA NSCLC randomized to postoperative radiotherapy plus or minus chemotherapy [86]. Mitsudomi et al. reviewed the extensive p53 literature and conducted a meta-analysis to determine the prognostic significance of p53 mutation or protein over-expression [144]. The incidence of p53 alteration in DNA studies was lower than in protein studies (37%

vs. 48%, $p < 0.0001$), and the incidence of p53 over-expression or mutation was lower in adenocarcinomas than squamous cell carcinomas (36% and 34% vs. 54% and 52% respectively, $p < 0.0001$). p53 imparted a significantly worse prognosis in patients with adenocarcinoma but not squamous cell carcinoma, with p53 gene mutations imparting a worse prognosis than immunohistochemical over-expression. Specifically, for adenocarcinoma, the combined survival differences at 5 years, calculated as the difference in survival between patients with alterations in p53 genetics or protein expression and patients without any such alterations, was -21.8% by protein studies ($p = 0.000004$) and -48.0% by DNA studies ($p = 0.00003$). For patients with squamous cell carcinoma, the combined survival difference was -15.6% for protein studies ($p = 0.42$) and -2.0% for DNA studies ($p = 0.89$). Taken together, these data suggest that p53 status may yield important prognostic information in certain subgroups such as patients with early stage cancers or adenocarcinomas, but whether this information should be integrated into clinical practice remains to be determined.

The prognostic significance of a number of other negative growth regulators has also been studied in NSCLC. The p16-cyclin D1-CDK4-RB pathway is critical to controlling the G1 to S transition of the cell cycle. p16 binds to and inhibits cyclin D-CDK4 complexes and thus prevents phosphorylation of the retinoblastoma protein, thereby leading to growth arrest. p16 mutation or loss of expression through promoter hypermethylation occurs in 30-70% of NSCLC [145]. In a study of 98 patients with NSCLC stages I-IV, the 3-year survival probability was 46% in patients without p16 expression compared with 88.2% in patients whose tumors expressed p16 [146]. Loss of expression of other growth-suppressing genes that are inactivated by aberrant promoter hypermethylation, including RASSF1A, DAP kinase, and APC, is also associated with poor survival [147-149]. In contrast, loss of expression of the retinoic acid receptor- β , a member of the steroid receptor superfamily whose expression is frequently down-regulated during aerodigestive carcinogenesis, correlated with better outcome in stage I NSCLC [150].

4.4. Cell death and replicative potential

Other characteristics of cancer cells are evasion of programmed cell death (apoptosis) and limitless replicative potential. The bcl-2 gene is an inhibitor of apoptosis due to a wide range of insults, includ-

ing growth factor depletion, radiation, and chemotherapeutic agents. Martin et al. performed a meta-analysis of 25 trials, comprised of 3370 patients, that addressed the prognostic significance of Bcl-2 expression in NSCLC [151]. Bcl-2 was expressed in 39% of lung tumors, with expression most frequent in SCLC (71%) and less frequent in NSCLC (35%). 32% of squamous cell carcinomas and 61% of adenocarcinomas expressed Bcl-2. Positive Bcl-2 expression was associated with improved survival, HR = 0.70 (95% CI 0.57, 0.86) in stage I-II NSCLC. The biology responsible for this improvement in survival is not clear. In contrast, expression of telomerase, which is a ribonucleoprotein that maintains the integrity of chromosomes by lengthening the ends that have become shortened during successive cell division cycles, is associated with shorter overall survival and disease-free survival in stage I NSCLC [152]. The catalytic protein subunit of telomerase, hTERT, was expressed in 33% of patients, and the 5-year survival for patients with hTERT positive tumors was 42.7%, compared with 62.9% (95% CI 54.1, 73.1%) for patients with hTERT negative tumors.

4.5. Genomic and proteomic markers

As is evident from the above discussion, many individual markers have been shown to have prognostic significance in lung cancer, but with limited impact on clinical decision-making. Approaches that integrate information about multiple different markers could potentially be more informative. Application of genomic and proteomic strategies to prognostication is only now beginning to emerge. Beer et al. recently showed that gene-expression profiles, using a risk index based on 50 genes, could identify low-risk and high-risk stage I lung adenocarcinomas that differ significantly with respect to survival [153]. Similarly, Yanagisawa et al. used proteomic patterns obtained from tissue samples to classify lung cancer histologies, distinguish primary tumors from metastases, and classify nodal involvement [154]. A proteomic pattern comprised of 15 distinct mass spectrometry peaks allowed the differentiation of patients with resected NSCLC with poor prognosis (median survival 6 months) from patients with good prognosis (median survival of 33 months, $p < 0.0001$). Although the number of patients studied was relatively small in both of these studies, these techniques have tremendous potential to improve our current approaches to prognostication.

5. Implications for clinical care

Advances in technology and in our understanding of the molecular biology of lung cancer are revealing new targets for risk assessment, early diagnosis, prevention, and treatment, with the potential to significantly alter our approach to the patient at risk for or with newly diagnosed lung cancer. Decision making based on risk is already available for application to the patient at high risk for breast cancer (using the Gail model to assess risk) or for determining the need for adjuvant chemotherapy after resectable breast cancer (based on stage of disease, histology, and attributes of the primary tumor). Despite the overwhelming contribution of tobacco exposure to the genesis of lung cancer, our ability to identify the person at highest risk for lung cancer who would be the ideal candidate for intensive screening and preventive measures remains limited. However, it is imperative that adequate models to identify the appropriate candidates for intervention at various stages of lung carcinogenesis be developed, since all interventions are associated with some degree of actual or potential harm. Interventions, including screening, must be tailored to the risk of the candidate populations so that the benefits outweigh the negative consequences of the intervention. Molecular markers that provide additional insight into an individual's risk of having or dying from cancer offer the hope of individualizing interventions such that lung cancer will no longer be the major cause of deaths in the United States.

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