Early Diagnosis and Screening for Lung Cancer

Humam Kadara,[1] Linh M. Tran,[2] Bin Liu,[2] Anil Vachani,[3] Shuo Li,[4] Ansam Sinjab,[1] Xianghong J. Zhou,[4] Steven M. Dubinett,[2,4,5,6,7] and Kostyantyn Krysan[2,7]

[1]Department of Translational Molecular Pathology, University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA

[2]Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California 90095, USA

[3]Pulmonary, Allergy, and Critical Care Division, Department of Medicine, University of Pennsylvania and Philadelphia VA Medical Center, Philadelphia, Pennsylvania 19104, USA

[4]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California 90095, USA

[5]Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, California 90095, USA

[6]UCLA Jonsson Comprehensive Cancer Center, Los Angeles, California 90024, USA

[7]VA Greater Los Angeles Healthcare System, Los Angeles, California 90073, USA

Correspondence: SDubinett@mednet.ucla.edu; KKrysan@mednet.ucla.edu

Cancer interception refers to actively blocking the cancer development process by preventing progression of premalignancy to invasive disease. The rate-limiting steps for effective lung cancer interception are the incomplete understanding of the earliest molecular events associated with lung carcinogenesis, the lack of preclinical models of pulmonary premalignancy, and the challenge of developing highly sensitive and specific methods for early detection. Recent advances in cancer interception are facilitated by developments in next-generation sequencing, computational methodologies, as well as the renewed emphasis in precision medicine and immuno-oncology. This review summarizes the current state of knowledge in the areas of molecular abnormalities in lung cancer continuum, preclinical human models of lung cancer pathogenesis, and the advances in early lung cancer diagnostics.

MOLECULAR ABNORMALITIES FOUND IN PRENEOPLASIA AND LUNG EPITHELIUM AT RISK

Characterizing cellular, molecular, and genetic abnormalities has been crucial to our understanding of lung tumor biology, as well as in guiding investigations of prognostic and predictive markers for the various subtypes of lung cancer. The advent of advanced molecular analysis tools, such as sequencing and proteomics, is enabling identification of potential markers for lung cancer detection at its earliest stages, including pulmonary preneoplastic lesions. This facilitates the effort to drive clinical management toward molecular-based and personalized

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targeted therapies. For example, a progressive mutational evolutionary model has been described for non-small-cell lung cancer (NSCLC) by identifying different branches of mutations using a deep sequencing approach, which may explain the temporal diversity during tumor progression (de Bruin et al. 2014; Anichini et al. 2018). Although extensive research has focused on intrinsic tumor cell abnormalities, including activated oncogenes or deactivated tumor suppressor genes, more recent attention focuses on cancer cells that are dynamically recruiting stromal, vascular, and immune cells, which in turn secrete signals and metabolites that can further promote tumor cell proliferation and immune evasion. Reciprocal interaction between developing premalignant cells and the microenvironment can modulate various steps of tumor pathogenesis, including known preneoplastic molecular events. Our overall understanding of the earliest molecular alterations in preneoplasia, in the context of the microenvironment, is vital for the eventual development of clinical interception that seeks to prevent invasive disease (Blackburn 2011; Spira et al. 2017). This section summarizes the state of knowledge of the molecular, genetic, and epigenetic changes occurring in the earliest known lesions of lung cancer. Further, exploration of the molecular aberrations of the lung "field of injury" in the cytologically normal airway, including oncogenic mutations (Kadara et al. 2019) and gene expression changes, described in further detail below, are also considered as key modulations for early pathogenesis and may be highly significant for the detection of malignancy.

KRAS Mutations

KRAS codon 12 mutations have been reported to be present in 30% to 40% of atypical adenomatous hyperplasias (AAHs) as well as in 24% to 50% of invasive lung adenocarcinomas (LUADs) (Westra et al. 1993). However, due to differences in reported base substitution patterns between AAH lesions and their respective advanced LUADs, it is plausible to surmise that AAHs and LUADs may arise independently (Westra et al. 1993; Kitamura et al. 1999). In a cohort of

AAHs and paired LUADs, *KRAS*-mutant AAHs were typical of ever-smokers and progressed to LUADs with additional driver mutations, such as *TP53*, *EGFR*, and *KRAS*, as inferred from the matched LUADs (Sivakumar et al. 2017).

BRAF Mutations

In two separate cohorts investigating AAHs and matched LUADs, the majority of AAHs displayed *BRAF* oncogene mutations (Izumchenko et al. 2015; Sivakumar et al. 2017). In one cohort, four AAHs harbored *BRAF K601E* mutation; one had *BRAF N581S* mutation, with 4/5 displaying additional driver mutations in *EGFR*, rather than *BRAF*, in their paired LUADs (Sivakumar et al. 2017). This may explain the low frequency of *BRAF* mutations in invasive LUADs (Izumchenko et al. 2015). *BRAF*-mutant AAHs were detected in both nonsmokers and ever-smokers (Sivakumar et al. 2017). Additionally, *BRAF* and *KRAS* mutations showed mutual exclusivity in AAHs and seemed to have undertaken divergent pathogenic pathways leading to LUADs (Sivakumar et al. 2017).

EGFR Mutations

AAHs could also be driven by *EGFR* mutations, namely, in-frame deletions of exon 19 and L858R and L861Q substitutions in exon 21, both of which also exist with increasing frequency throughout histological progression to more advanced adenocarcinoma in situ (AIS) lesions as well as LUADs (Yatabe 2010). Unlike their mutually exclusive counterparts (*KRAS*-mutant AAHs), *EGFR*-mutant AAHs are not associated with smoking status (Kadara et al. 2016). However, similar to *KRAS*-mutant AAHs but unlike *BRAF*-mutant AAHs, *EGFR* shares the exact mutation in both AAHs and matched LUADs (Izumchenko et al. 2015; Sivakumar et al. 2017).

TP53 Mutations

TP53 mutations are among the well-studied lung preneoplastic molecular aberrations, which affect nearly half of all lung cancers (Rom et al. 2000). A missense mutation causes the protein

to lose its DNA binding activity due to a change in a single amino acid residue. The result is a loss of wild-type p53 activity as a regulator of genetic instability, leading to an accumulation of further genetic mutations and a cascade of oncogenic functions (Sigal and Rotter 2000). Nuclear accumulation of p53 has also been shown to be an early event in lung carcinogenesis with the potential to identify smokers who are at risk of developing lung squamous cell carcinoma (LUSC), albeit not in the estimation of survival (Piyathilake et al. 2003). While p53 aberrations have been reported as a widespread phenomenon in lung cancer, their extent varies across different lung cancer subtypes, and may change with tumor progression, as inferred from studies investigating preneoplastic lesions. For instance, while 33% of LUADs are reported to have p53 alterations (a low frequency in comparison to 70% in small-cell undifferentiated carcinoma, 47% in NSCLC, 65% in LUSC, 60% in large cell carcinoma), their precursors, AAHs, rarely display mutations of the *p53* gene.

The dynamics of the mutational events during the progression from pulmonary premalignancy to invasive disease has recently begun to be more completely understood. A recent study demonstrated an increase in the number of mutations in oncogenes and tumor suppressor genes with progression from AAH to invasive LUAD, whereas the overall mutational load did not significantly change (Krysan et al. 2019). On the contrary, another study reported a progressive increase of the total mutational burden, as well as the rate of chromosomal abnormalities, from AAH to AIS, minimally invasive adenocarcinoma (MIA), and ADC (Hu et al. 2019). Furthermore, the frequency of p53 mutations was shown to increase as AAHs progress to MIA, AIS, and invasive LUADs (Izumchenko et al. 2015; Sivakumar et al. 2017; Chen et al. 2019). Additional studies are needed to dissect the genomic evolution in the lung cancer continuum in the context of both genes and pathway deregulation.

CHROMOSOMAL ABNORMALITIES

Loss of heterozygosity (LOH) has been described as an early event in carcinogenesis across several lung cancer subtypes whereby focal chromosomal losses are commonly identified in multiple lung precursor lesions. In AAH, LOH in chromosomal regions 3p (*FHIT* gene, 18% of AAHs), 9p (*p16* gene, 13% of AAHs), 9q (tuberous sclerosis complex 1[TSC1]), 17q, and 17p (*TP53*) has been well described (Takamochi et al. 2001; Wistuba and Gazdar 2006), whereby both 3p and 9p represent loci altered in LUADs, albeit more frequently than in their respective precursors (Chmara et al. 2004). In preinvasive bronchial epithelium lesions of LUSC, multiple early allelic losses in 3p (3p21, 3p14, 3p22–24, and 3p12) are followed by LOH at 9p21 (Wistuba et al. 1999, 2000). Interestingly, LOH is detected in histologically normal bronchial mucosa of smokers, indicating that it is an early genetic aberration caused by smoking-induced damage, despite having no correlation with increased risk for development of invasive carcinoma (Wistuba et al. 1997). Allelic imbalance has been also reported in preinvasive lesions of LUSC, such as events affecting 8p21–23, 13q14 (*RB1*), and 17p13 (*TP53*) loci (Wistuba et al. 1999).

SOX2 Alterations

Amplification of distal 3q has been shown to be an early genetic event in LUSC carcinogenesis as detected in high-grade bronchial dysplastic lesions (McCaughan et al. 2010; Yuan et al. 2010). The modification translates to amplification of the *SOX2* gene, which is in fact known to be amplified in LUSC of not only the lung (~20%) but also esophagus, cervix uteri, skin, and penis (Hussenet et al. 2010). *SOX2* has been also investigated at the expression level in early LUSC where it is frequently expressed (Mukhopadhyay et al. 2014) as well as being overexpressed in 60%–90% of LUSC, further suggesting a potential role for this genetic modulation in initiation of LUSC particularly in light of its known role as a mediator in lung cancer stem cell self-renewal (Bass et al. 2009; Lu et al. 2010; Xiang et al. 2011). In LUAD pathogenesis, however, *SOX2* expression seems to be completely lost (Yuan et al. 2010).

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$P16^{INK4A}$ Aberrations

Loss of $p16^{INK4A}$ protein expression has been well documented in early stages of NSCLC whereby the *CDKN2A* (*$p16^{Ink4a}$*)/*ARF* locus at 9p21 was shown to be aberrantly methylated. This was also reported in precursor lesions of LUSC, whereby methylation of *$p16^{Ink4a}$* was demonstrated in 75% of carcinoma in situ (CIS) that is adjacent to invasive LUSC. Methylation of the *CDKN2A* promoter showed increasing frequencies during progression of LUSC from basal cell hyperplasia (17% methylation) to squamous metaplasia (24%) to CIS and invasive carcinoma (50%–75%) (Belinsky et al. 2002; Lamy et al. 2002).

OTHER DEVIATIONS IN LUNG PREOPLASTIC BIOLOGY

Frequent *AKT* activation has been reported in a number of advanced cancers, including lung tumors (Tichelaar et al. 2005). *AKT* hyperactivation is mediated by the increased expression of its phosphorylated form. AKT phosphorylation and activation of downstream signaling pathways may represent an early event in lung cancer progression, based on evidence from preneoplastic lesions such as bronchial dysplasia and preneoplastic and preinvasive bronchial lesions from patients with a long history of smoking, and, in some cases, a high risk of NSCLC recurrence (Tsao et al. 2003). These studies suggest that targeting preneoplastic lesions in individuals with high risk of lung cancer and activated AKT signaling, using PI3K/AKT pathway inhibitors, may be a promising approach to chemoprevention due to the early manifestation of this activated signaling pathway (Tsao et al. 2003; Balsara et al. 2004).

Further aberrations have been described in AAHs, such as increased expression of the oncogenes coding for cyclin D1, survivin, and ERBB2 (Westra 2000; Nakanishi et al. 2003; Tominaga et al. 2003), down-regulation of the tumor suppressor gene *LKB1* also known as *STK11* (Ghaffar et al. 2003), increased expression of *NKX2-1* also known as *TTF-1* (Yatabe et al. 2002) in AAHs as well as AIS, and elevated reactive oxygen species

expression (Bonner et al. 2004). Bronchial premalignant lesions, precursors of LUSC, have been also reported to harbor elevated levels of VEGF and VEGFR, aberrant patterns of microvascularization (Keith et al. 2000; Hirsch et al. 2001; Merrick et al. 2005). There is a linear increase in proliferation and DNA repair at the earliest molecular transformation changes and up to invasive LUSC (Beane et al. 2019; Mascaux et al. 2019). There is also a transitory increase in metabolism genes (fatty acid metabolism, oxidative phosphorylation, and the citric acid cycle) (Mascaux et al. 2019).

Recent studies have explored a potential role for the immune microenvironment in neoplastic progression in the lung. One study demonstrated that an increased number of neoantigens produced by the mutated genes in AAH lesions promoted CD8^{+} T cell infiltration (Krysan et al. 2019). Furthermore, the overall neoantigen load in AAH positively correlated with CD4^{+} T-cell infiltration and PD-L1 expression. In another study, sets of normal AAH and LUAD tissues were collected and interrogated by deep sequencing (Sivakumar et al. 2017). Pathway-based enrichment analysis revealed increased activation of protumor immune pathways (Th2) as well as suppression of antitumor immune pathways (Th1) during the progression of normal lung to AAHs and further to LUADs. This was accompanied by inhibition of *IFN-γ* and *TGFB1* signaling, reduction in inflammatory responses, and increased expression of *CCL2/CCR2*, *SPP1*, and *CD27* (Sivakumar et al. 2017). Another study by Lavin and colleagues (2017) showed, by paired single-cell sequencing of early-stage LUADs and non-involved tissue, that LUADs exhibit early impairment of adaptive immunity, lending support to the concept that, early on, lung tumors develop an immunosuppressive microenvironment (Lavin et al. 2017). Indeed, the notion that immunogenomic signatures of the lung are operative in early premalignant lesions and may thus dynamically modulate classical molecular aberrations is gaining momentum (Milette et al. 2019). Mascaux et al. (2019) outlined the location and timing of the successive and coevolutionary changes occurring in preinvasive LUSC lesions (CIS) as well as their microenvironment,

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up to invasive tumors. The authors report activation of resident immune cells was detected as early as in low-grade preinvasive regions, and they highlight immune pathways modulated across early and subsequent stages, such as negative regulation of the immune system, antigen processing, and the presentation of peptide antigens (Mascaux et al. 2019). They observed negative regulation of the immune system, antigen processing, and the presentation of peptide antigen in all stages of tumor development. The earliest immune events included the down-regulation of genes that negatively regulate the immune system, including *TNFRSF14*, *CD200*, *CD59*, *TGFB3*, and *HLA-G*. In contrast, in high-grade lesions and LUSC there was an up-regulation of genes that promote immunosuppression (Mascaux et al. 2019). These findings are supported by another seminal report, which identified immune-based subtypes of bronchial premalignant lesions, highlighting a dynamic interplay between epithelial and immune pathways early on in premalignant lesions (Beane et al. 2019). Interestingly, down-regulation of genes involved in interferon signaling and T-cell-mediated immunity correlated with compromised adaptive and innate immunity and was a hallmark of progressive/persistent lesions compared to regressive lesions (Beane et al. 2019). Similarly, the early-stage lung tumors employ various ways of immune escape, including disruption of tumor antigen presentation by loss or reduction of HLA expression, mutation in antigen-presenting pathways (Chen et al. 1996), selection of mutations for low-affinity MHC binders (Wiedenfeld et al. 1994), or hypermethylation of the neoantigen promoters followed by silencing of neoantigen expression (Rosenthal et al. 2019). Taken together, these studies reveal that immune escape may occur before tumor invasion, lending support to the potential critical role of components of the premalignant immune microenvironment as determinants of lesion fate regulating progression or regression (Angelova et al. 2018). Consistent with these findings, a preexisting specific antitumor immune landscape is associated with response to immune checkpoint blockade therapy (Dhodapkar et al. 2013). Additional studies are warranted to further explore the existence of tumor microenvironment biomarkers in lung preneoplastic lesions (e.g., by characterizing immune infiltrates), as well as their potential clinical utility, such as tailoring adjuvant or neo-adjuvant immunotherapy strategies to restore specific aspects of the tumor immune microenvironment.

PRECLINICAL HUMAN MODELS FOR THE STUDY OF LUNG CANCER PATHOGENESIS

Various preclinical murine models of lung cancer, including those using (1) chemically induced lung tumorigenesis, (2) animals with the engineered genetic backgrounds that promote the development of spontaneous lung cancer, (3) xenograft or syngeneic tumor cells (Meuwissen and Berns 2005; de Seranno and Meuwissen 2010; Kwon and Berns 2013), and (4) models for studying immuno-oncology (Olson et al. 2018), have been developed. However, deciphering the pathogenesis of lung cancer was hindered by the lack of models allowing studying the earliest stages of lung cancer initiation and the mechanisms of progression from premalignancy to metastatic disease. This is in large part due to the lack of the cell lines that could be used to mimic the early events in transformation of the pulmonary epithelium. Early studies of lung cancer pathogenesis used normal human bronchial epithelium (NHBE) cells that were explanted from surgical resection specimens (Lechner et al. 1981). NHBE cells became senescent after up to 35 doublings and required feeder cells for their growth. Despite closely resembling the normal bronchial epithelium, NHBE cells had limited usability due to the complicated growth conditions and the relatively short life span. Thus, attempts have been made to establish immortalized bronchial epithelium cells. In one of the notable experiments, NHBE cells were rendered immortal by the transfer of SV40 virus early region genes (including small and large T antigens) with or without the Rous sarcoma virus long terminal repeat (Reddel et al. 1988). This method produced a series of cell lines with various life spans with a BEAS-2B cell line that was able to proliferate continuously being the most widely used in research. This cell line expressed SV40 T antigen and was prone to spontaneous transformation (Reddel et al. 1993).

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While BEAS-2B cells were more convenient to use than NHBE cells, they had significant limitations due to the presence of viral oncoproteins and could not represent a good lung premalignancy model. The significant advances in creating such a model in the absence of viral oncoprotein expression have been made by the development of human bronchial and small airway epithelium cell lines (HBEC and HSAEC, respectively). HBEC cells were immortalized by ectopic expression of cyclin-dependent kinase 4 (CDK4) and human telomerase reverse transcriptase (hTERT) (Ramirez et al. 2004), whereas HSAEC cells were immortalized by expressing hTERT, CDK4, and a dominant-negative form of p53 (Sasai et al. 2011) without the use of viral oncoproteins. Characterization of these cells demonstrated that they were immortal, but neither grew colonies in soft agar nor formed tumors in immunocompromised mice in vivo. Genomics and transcriptomics studies confirmed that HBEC and HSAEC cells were very close to their original parental small airway epithelium cells. Thus, immortalized HBEC and HSAEC cells constitute models of premalignancy in LUAD (HSAEC) or LUSC (HBEC). Development of HBEC and HSAEC cells has opened new avenues for studying early pathogenesis of lung cancer. A seminal study demonstrated that stepwise addition of oncogenic mutations to immortalized HBEC cells (p53 knockdown [KD], introduction of oncogenic KRAS^{v12} and EGFR^{L858R} mutations) conferred the development of anchorage-independent growth (AIG) in soft agar and an invasive phenotype in a three-dimensional organotypic culture assay, but was not sufficient to transform the cells to cancer and enable tumor formation in immunocompromised mice (Sato et al. 2006). Subsequent studies have shown that manipulating the genetic background and gene expression in HBEC cells could drive epithelial-to-mesenchymal transition (EMT) and a malignant transformation. One study reported that a combination of p53 KD, KRAS^{v12}, and ectopic expression of CMYC was able to drive full malignant transformation of HBEC cells (Sato et al. 2013). In another study, ectopic expression of the transcriptional repressor Snail in p53 KD, KRAS^{v12} HBEC cells enabled tumor formation,

EMT, and metastatic growth in a murine lung cancer model (Walser et al. 2018). Furthermore, this study demonstrated that, in the context of p53 KD, KRAS^{v12}, HBEC Snail expression promoted the ALDH$^+$CD44$^+$CD24$^-$ stem cell population that in turn was required for AIG. Concordantly, ectopic expression of the transcriptional repressor Zeb1 induced malignant transformation of p53 KD, KRAS^{v12} HBEC cells (Larsen et al. 2016). These findings are consistent with previous reports indicating the multipotent capacity of HBEC cells consistent with basal cells (Delgado et al. 2011). Similarly, introduction of constitutively active PIK3CA^{H1047R}, cyclin-D1, or dominant-negative LKB1^{D194A} in combination with KRAS^{V12} induced malignant transformation and acquisition of LUAD phenotypes in HSAEC cells (Sasai et al. 2011). Moreover, exposure of HBEC cells to tobacco carcinogens induced stem-cell-like phenotypes and EMT via miR-205 and miR-200 epigenetic silencing (Tellez et al. 2011) and induced epigenetic silencing of the antiproliferative miRNA miR-196b thus increasing the proliferative capacity of the cells (Tellez et al. 2016). In a recent study, targeting of c-MYC-induced BCL6 by a small molecule drug was effective against transformed P53 KD, KRAS^{v12}, CMYC$^+$ HBEC cells, thus demonstrating the utility of HBEC cells in facilitating the identification of actionable targets for oncogene-driven lung cancer (Deb et al. 2017). Another study used an isogenic pair of LUAD and immortalized HBEC cells to assess the tumor adaptive response to various targeted and chemotherapeutic agents (Zi et al. 2020). To summarize, HBEC and HSAEC cells constitute a unique model of pulmonary premalignancy that allow evaluation of the role of different driver genes (or gene combinations) in malignant transformation both in vitro and in vivo as well as facilitating identification of actionable targets for lung cancer treatment and prevention.

BIOLOGICAL RATIONALE FOR EARLY DIAGNOSIS AND LUNG CANCER SCREENING

The goal of cancer screening is to increase the likelihood of cure by detecting the malignancy,

or its precursor lesion, at an early stage prior to the onset of symptoms, when treatment is most effective. Wilson and Jungner published several key principles that should be considered when evaluating the role of screening for a particular disease that provides a framework for considering the role of screening for lung cancer (Wilson and Jungner 1968). These principles include knowledge of its natural progression and the performance characteristics of available screening tests and availability of effective treatment strategies for early-stage disease.

One key principle outlined by Wilson and Junger is the need for a recognizable latent or preclinical stage of disease. At present, the most successful cancer screening approaches have been implemented in cervical and colon cancer, where studies have demonstrated a clear period of asymptomatic preclinical disease. In concert, the evidence regarding carcinogenesis in these malignancies suggests a stepwise progression to more invasive and advanced stages of cancer. In these two cancers, screening allows for the identification of precursor lesions (e.g., cervical intraepithelial neoplasia with cervical cancer screening and colonic polyps with colorectal cancer screening) allowing for early diagnosis and treatment.

Lung carcinogenesis is a complex, stepwise process that involves the acquisition of genetic mutations and epigenetic changes that alter cellular processes, such as proliferation, differentiation, invasion, immune responsiveness, and metastasis. Findings from molecular studies support a stepwise lung carcinogenesis model in which development of a field of cancerization—the accumulation of molecular abnormalities from repeated injury (e.g., cigarette smoke)—leads to genetically and epigenetically altered cells that play a central role (Gomperts et al. 2011). Early lung cancer lesions are polyclonal and driver mutations present in late-stage disease, with tumor development occurring via large-scale genomic rearrangements and copy number changes (Beane et al. 2017). Although the process is clearly complex, several important principles are apparent. In particular, there is increasing evidence that lung cancers are not uniform in their biology and that not all early lesions (such as AAH and AIS) lead to invasive cancer (Tanoue et al. 2015; Vachani et al. 2017).

CURRENT STATUS OF LUNG CANCER SCREENING INCLUDING RESULTS AND OBSTACLES

In the 1970s, the main efforts to develop screening tests for lung cancer were based on chest radiography and sputum cytology (Vachani et al. 2017). Randomized controlled trials assessing these modalities failed to show a reduction in lung cancer mortality. Because of concerns regarding limited power and contamination of the control arms, screening with chest radiography was further evaluated as a part of the Prostate, Lung, Colorectal, and Ovarian (PLCO) trial, which randomized more than 154,000 patients, including both smokers and nonsmokers, to annual radiography or usual care. Annual chest radiographic screening for up to 4 years did not have an effect on lung cancer mortality with 13 years of follow-up in the trial (Oken et al. 2011).

In the 1990s, interest in lung cancer screening turned to use of low-dose computed tomography (LDCT), which provided a more sensitive method for the detection of pulmonary nodules than chest radiography. Multiple single-arm studies went on to demonstrate two key findings: although LDCT identified a large number of noncalcified lung nodules, most of which were not malignant, it resulted in a higher proportion of early-stage lung cancers compared with historical data (Bach et al. 2003). Given these promising results, the National Cancer Institute launched the National Lung Screening Trial (NLST) in 2002, randomizing 53,454 patients at high risk (defined as age 55–74 years; tobacco pack-years 30; and, if a previous smoker, quit within 15 years) to either annual LDCT or chest radiography for 3 years (National Lung Screening Trial Research Team et al. 2011a). After 7 years of follow-up, the LDCT arm demonstrated a reduction in lung cancer–specific mortality from 1.66% to 1.33% (a 20% relative reduction) (National Lung Screening Trial Research Team et al. 2011b), as well as a reduction in all-cause mortality.

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Although the NLST represents a landmark advance in lung cancer screening, implementation of screening programs has been slow, in part due to concerns regarding the high false-positive rate and potential for harm to individuals without lung cancer. Among all LDCT scans, 24% were classified as positive (i.e., identified at least one nodule 4 mm in size), with 96% ultimately proven to be falsely positive. Although the subsequent evaluation after a positive finding was most commonly surveillance imaging, and the use of invasive procedures was relatively infrequent, 4% of LDCT participants underwent surgical lung biopsy after the baseline scan, identifying benign disease in 29% of these procedures (Tanoue et al. 2015).

In addition to NLST, a Dutch–Belgian lung cancer screening trial (Nederlands–Leuvens Longkanker Screenings Onderzoek [NELSON]) that randomized 15,822 high-risk participants to LDCT screening versus no screening, has recently been completed (de Koning et al. 2020). The NELSON trial design has several important differences when compared to the NLST, including the lack of chest X-ray in the control arm, the use of volumetric imaging for lung nodule assessment, and the inclusion of more rounds of screening. The results of this trial indicate an even more robust reduction in lung cancer–specific mortality with LDCT than what was observed in NLST (de Koning et al. 2020).

The use of current patient selection criteria for screening, based on the NLST and NELSON, results in the need to screen a large segment of the population, due to the dearth of approaches for individualized risk assessment. Use of LDCT within the vast "high-risk" population creates a higher likelihood of false-positive detection resulting in frequent diagnostic and management dilemmas for physicians, while patients face confusion, anxiety, and harm from unnecessary procedures. Recent efforts have focused on improving the ability to identify individuals who are more likely to benefit from LDCT screening. Studies have shown that the use of age and smoking history alone is less efficient than "personalized" approaches employing more complex risk models that consider other variables (e.g., COPD, family history, race), which improves the predic-

tion of future lung cancer development (Tammemägi et al. 2013, 2017; Katki et al. 2016, 2018). Although approaches to develop personalized or "precision" screening may allow for the identification of patients at higher risk of lung cancer, they may also result in screening of patients that are at risk of death from competing causes, resulting in less benefit from lung cancer screening (Rivera et al. 2018). One potential approach to improve patient identification includes the incorporation of molecular biomarkers that may identify patients at the highest risk of future disease (Seijo et al. 2019). To this end, a number of different biomarkers are in various stages of development and validation for use in risk prediction models.

Screening with LDCT with currently accepted patient selection criteria has several other important shortcomings. The significant molecular heterogeneity among lung cancers results in a failure to detect clinically relevant cancer early enough to intervene. Conversely, screening also results in the identification of some proportion of clinically insignificant cancers; these indolent lesions, frequently manifesting as smaller ground glass pulmonary nodules that represent AAH or AIS lesions, may never progress to invasive cancers but can still result in the need for additional imaging and invasive procedures. Finally, limiting screening to individuals meeting selection criteria employed in the NLST and NELSON results in the inability to identify cancers early in those who fall outside of these criteria. Evidence from the United States suggests that at least half of all new lung cancers occur in patients not currently meeting these criteria, which serve as the basis for patient selection in various current guidelines (Wang et al. 2015). The development of molecular biomarkers that identify higher risk individuals may facilitate approaches that allow for LDCT screening of a broader population resulting in a greater reduction in lung cancer mortality at the population level.

In spite of these limitations, LDCT screening represents a proven path for reduction in lung cancer mortality in the high-risk smoking subset of patients. Even though only a subset of lung cancers arise in the screen-eligible population, they are clearly at higher risk and only a

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small subset of these patients eligible to be screened currently do so. Additional efforts on education are needed (Pham et al. 2018).

SPUTUM AND BLOOD MARKERS FOR LUNG CANCER RISK ASSESSMENT (DNA METHYLATION, PROTEOMICS, CELL-FREE DNA)

Risk assessment is fundamental to increase opportunities for curative treatments in lung cancer patients. Recently, there has been much effort expended to develop sputum and blood markers for lung cancer assessment. Aberration of DNA methylation is an important characteristic of tumor tissue. Its prevalence and early occurrence in tumor have stimulated the development of lung cancer screening approaches using methylation of cell-free DNA (cfDNA). Leng et al. (2017) employed an eight-gene methylation panel to build a classifier in sputum for lung cancer risk among CT-screen eligible smokers (Leng et al. 2017). The cfDNA methylation panel achieved an accuracy of 82%–86% for lung cancer prediction. Combined with clinical variables, the accuracy has been further improved to 87%–90%. Comparing the prediction based only on clinical variables to its inclusion with methylation, the specificity increased from 25% to 54% at a sensitivity of 95%. Hulbert et al. (2017) described a three-gene model discriminating individuals with suspicious nodules on CT imaging with 98% sensitivity and 71% specificity using sputum samples ($N = 210$) from stage I or II NSCLC patients and controls (Hulbert et al. 2017). Blood cfDNA methylation is also widely used for lung cancer risk assessment. In addition to sputum samples, Hulbert et al. (2017) reached a 93% sensitivity and 62% specificity using plasma samples. Genome-wide methylation profiling is perhaps the most powerful tool to explore methylome and has been used in early detection of lung cancer. Kang et al. (2017) has proposed a model that simultaneously infers the proportion and tissue of origin based on cfDNA methylation (Kang et al. 2017). Their methods showed promising results in classification of plasma samples from liver and lung cancer patients. Another method based on

haplotype blocks of cfDNA methylation was developed by Guo et al. (2017) for the detection of various kinds of cancer, including lung cancer (Guo et al. 2017). Shen et al. (2018) developed an immunoprecipitation-based protocol to profile cfDNA methylome, and showed a high AUC of 0.975 on 32 stage I-II lung cancer patients.

Apart from cfDNA methylation, proteins are promising markers for lung cancer diagnosis. Integrative Analysis of Lung Cancer Etiology and Risk (INTEGRAL) Consortium for Early Detection of Lung Cancer developed a biomarker risk score based on four circulating proteins (CA125, CEA, CYFRA 21-1, and Pro-SFTPB). An integrated risk prediction model that combined smoking exposure with the biomarker risk score yielded an AUC of 0.83 in the validation study of 63 ever-smoking patients with lung cancer and 90 matched controls (INTEGRAL Consortium for Early Detection of Lung Cancer et al. 2018). The CancerSEEK test combined both somatic mutations and a panel of protein markers to detect and locate cancer. It showed a sensitivity of ~60% on 104 stage I-III lung cancer patients (Cohen et al. 2018). Several studies explored the presence and expression of tumor-related proteins in sputum. Sun et al. (2009) found a significant elevation in expression of a proliferation-inducing ligand in sputum of lung cancer patients compared with controls (82% vs. 3%) (Sun et al. 2009). Pio et al. (2010) showed increased levels of complement factor H in sputum of lung cancer patients (Pio et al. 2010).

EARLY DETECTION OF RELAPSE/ RECURRENCE

Circulating Tumor Cells

Liquid biopsy analyzing circulating tumor cells (CTCs) has been explored as an approach for early detection of relapse and recurrence in lung cancer (Gallo et al. 2017; Blackhall et al. 2018; Kapeleris et al. 2018). CTCs represent disseminated tumor cells from the primary site into the circulation with the potential to metastasize at distant organs. CTCs can be obtained noninvasively at multiple time points, which potentially could provide a real-time assessment of

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patients' disease progression and clinical response to therapy. The detection of CTCs could precede the onset of clinical or radiological signs of metastasis or local recurrence, thus providing an opportunity to stratify patients for the most effective treatment option at a much earlier course in the disease. Regional tumor biopsy may not reflect intratumoral heterogeneity of lung cancer as well as heterogeneity at the sites of micrometastases. Therefore, it has been hypothesized that assessing CTCs could overcome this problem by revealing clonal diversity as tumor cells in the blood are originating from primary as well as metastatic tumor sites.

Isolation of CTCs

CTCs can be enriched and isolated from whole blood by cell surface marker-dependent or -independent approaches (Pantel and Alix-Panabieres 2019). The marker-dependent approach exploits the differential expression of surface proteins between tumor cells and blood cells, either by positive or negative selection. For example, the epithelial cell adhesion molecule (EpCAM) is the most frequently used marker for positive selection, which is the basis for the FDA-approved CellSearch system (Riethdorf et al. 2007). Of note, CTCs are phenotypically heterogeneous and may not express a chosen marker. In addition, CTCs from NSCLC often exhibit EMT characteristics and thus are EpCAM-negative. Using multiple surface markers to capture CTCs could alleviate these problems. Winer-Jones et al. (2014) used a mixture of antibodies to EpCAM, Her2, MelCAM, Muc1, and Trop2 to efficiently recover tumor cells from blood. Furthermore, the use of negative selection whereby nonmalignant cells are depleted by staining with lineage-specific markers, such as CD45 for leukocytes, CD146, or CD31 for endothelial cells, and CD34 for hematopoietic stem cells, may be used to capture the subsets of CTCs that do not express the established CTC surface markers (He et al. 2008). Alternatively, CTCs can be isolated in a marker-independent fashion based on their physical properties. This can include size (the isolation by size of epithelial tumor cells [ISET]) (Vona et al. 2000), density (Weitz et al. 1998), electric

charge (Abonnenc et al. 2013; Peeters et al. 2013), and deformability (Aghaamoo et al. 2015). A study that compared ISET and CellSearch approaches demonstrated that ISET had higher sensitivity in detecting CTCs, consistent with the concept that CTCs in NSCLC may undergo EMT and lose the EpCAM expression (Krebs et al. 2012).

Characterization of CTCs

Purified CTCs can be characterized at DNA, RNA, and protein levels by various approaches (Pantel and Alix-Panabieres 2019; Kolinsky et al. 2020). Chromosomal aberrations, copy number variation, and mutations in CTCs can be identified by fluorescence in situ hybridization (FISH) and targeted by whole exome sequencing (WES); sequencing usually requires prior whole-genome amplification (Lin et al. 2017; Pailler et al. 2017; Faugeroux et al. 2020). Expression of individual genes and the transcriptome of CTCs can be assessed by quantitative RT-PCR (Markou et al. 2018) or RNA sequencing at bulk or single-cell levels. Multiplex immunophenotyping and proteomics can provide insights at the protein level (Matthew et al. 2016; Rugo et al. 2018). In addition, functional assays such as Epithelial ImmunoSPOT (EPISPOT) and EPISPOT in a drop (EPIDROP) are being developed to detect specific proteins secreted or released by CTCs as a readout for viable CTCs in short-term culture (Kuske et al. 2016; Eyer et al. 2017). Last, recent advances in the culture of CTCs and in vivo xenotransplantation of CTCs have made it feasible to perform functional and mechanistic studies of CTCs in mediating metastasis and response or resistance to treatments (Hodgkinson et al. 2014; Zhang et al. 2014b; Hamilton et al. 2015; Drapkin et al. 2018).

CTCs in Early-Stage NSCLC

As a result of improvements in the sensitivity of detecting CTCs, it has been revealed that CTCs can be used as prognostic and predictive markers in NSCLC (Gallo et al. 2017). Studies have addressed the prognostic value of CTCs in early-stage NSCLC. Different mRNA markers, in-

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cluding carcinoembryonic antigen (CEA), survivin, thyroid transcription factor-1 (TTF-1), and lung-specific X protein (LUNX), have been assessed in CTCs in pre- and postoperative peripheral blood samples from stage I-III NSCLC patients. CTCs expressing these mRNAs were identified as independent prognostic markers associated with reduced overall survival (OS) and shorter disease-free survival (DFS) (Yamashita et al. 2002; Yie et al. 2009; Yoon et al. 2011; Li et al. 2014). Using ISET technology, CTCs were found in presurgery peripheral blood samples in 49% of NSCLC patients undergoing curative surgery, and a CTC count of ≥50 was significantly associated with shorter OS and DFS (Hofman et al. 2011). In addition, CTCs were identified by immunocytochemical methods in tumor-draining pulmonary vein or peripheral blood samples of NSCLC patients postsurgery, which was associated with a shorter DFS (Sienel et al. 2003). A recent clinical trial monitoring CTCs in stage I NSCLC treated with stereotactic body radiation therapy (SBRT) revealed that positive CTC counts following therapy and predating clinical progression are associated with disease recurrence, while negative CTCs correlated with disease control (Frick et al. 2018). A meta-analysis of five studies in which early-stage NSCLC patients underwent surgical resection, demonstrated that positive CTCs postsurgery indicate a poor prognosis for DFS (Liang et al. 2018). A recent study using single-cell transcriptome of CTCs revealed a refined signature of gene expression as a predictive biomarker of metastatic risk in early-stage NSCLC with high risk of recurrence (Lim et al. 2019).

CTCs in Advanced NSCLC

The role of CTC as a prognostic biomarker in advanced NSCLC patients has been demonstrated by multiple studies (Gallo et al. 2017). Using the CellSearch system in chemo-naive advanced disease or metastatic NSCLC patients who received first-line chemotherapy, it was shown that a baseline count of CTC ≥5 was significantly associated with a shorter progression-free survival (PFS) and OS (Krebs et al. 2011; Muinelo-

Romay et al. 2014). A reduction in CTC postchemotherapy was predictive of longer PFS and OS. Additional studies using alternative approaches for CTC isolation, such as gradient centrifugation or using survivin mRNA as a surrogate marker of CTCs, revealed the correlation between low-baseline CTCs and improved PFS and OS (Du et al. 2014; Zhang et al. 2016). Consistently, detection of CTCs by multiple markers increased the sensitivity and confirmed that high CTC load correlated with a shortened OS in advanced NSCLC patients receiving chemotherapy (Sher et al. 2005; Liu et al. 2008). Similarly, a recent study using an adenoviral probe that detects elevated telomerase activity to track CTCs in patients with locally advanced NSCLC receiving chemoradiation therapy revealed that increased CTCs post-therapy correlated with disease recurrence, which preceded radiographic evidence of recurrence (Chinniah et al. 2019). CTC count has been evaluated in targeted therapy of EGFR tyrosine kinase inhibitors (TKIs) in NSCLC. A high baseline CTC count by a CellSearch test correlated with response to EGFR TKI treatment, whereas a decrease in CTC following treatment predicted a longer PFS (Punnoose et al. 2012). In a separate study using flow cytometry to detect CTC in EGFR-mutant advanced NSCLC, patients with low CTCs had a superior EGFR TKI response rate, PFS, and OS (He et al. 2016). PD-L1 expression on CTCs has been recently evaluated as a potential biomarker to predict response and relapse for NSCLC patients undergoing immune checkpoint inhibitor (ICI) immunotherapy (Hofman et al. 2019). Studies suggested that PD-L1 expression on CTCs could serve as a surrogate for tracking changes following ICI treatment (Adams et al. 2017; Ilié et al. 2018). Pretreatment PD-L1[+] CTCs were found to be associated with poor prognosis in patients treated with PD-1 inhibitors (Guibert et al. 2018; Kallergi et al. 2018), and persistence of PD-L1[+] CTCs post-ICI treatment might mirror resistance to therapy (Nicolazzo et al. 2016).

CTCs in SCLC

The prognostic value of CTCs has also been assessed in small-cell lung cancer (SCLC). SCLC is

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a highly aggressive neuroendocrine cancer with early metastatic potential and poor outcomes (Gazdar et al. 2017). CTC counts are considerably higher in patients with SCLC than those with NSCLC, indicating a pronounced disseminative activity of SCLC (Blackhall et al. 2018). CTC analyses in SCLC patients revealed that CTC numbers fell rapidly after one cycle of chemotherapy, and that CTC counts ≥50 before or persisting post-chemotherapy is associated with reduced OS (Hou et al. 2009, 2012). Several other studies have independently confirmed that baseline CTC counts correlate with OS, although no consensus has been reached for an optimal cutoff threshold (Hiltermann et al. 2012; Naito et al. 2012; Zhang et al. 2014a; Cheng et al. 2016). Changes in CTC counts during treatment provide further prognostic information (Normanno et al. 2014).

Cell-Free DNA

Following surgery, many lung cancer patients may have minimal residual disease (MRD), which can result in recurrence. The early detection of recurrence could offer new therapeutic approaches for improving outcomes following surgery (Luskin et al. 2018). In a landmark study, next-generation sequencing (NGS)-based and patient-specific mutational panel assays comprising 12–30 single-nucleotide variants (SNVs), detected from phylogenetic tumor cfDNA profiling, were developed and used in 25 patients enrolled in the TRACERx lung cancer study (Abbosh et al. 2017). The detection of SNVs in tumor cfDNA showed correlation with clinical evidence of NSCLC recurrence. The threshold of tumor cfDNA detection was <0.1%, and the median lead time to clinical and radiological detection of relapse was 70 days (10–346 days) (Abbosh et al. 2018). Another study showed that a panel of 128 genes targeted by CAPP-Seq can detect tumor cfDNA in the first posttreatment blood sample, in which tumor cfDNA was detected before radiological recurrence with an average lead time of 5.2 months in 72% of patients (Chaudhuri et al. 2017). This study concluded that tumor cfDNA analysis can predict relapse prior to clinically evident recurrence.

In addition to detecting tumor-specific DNA mutations, gene methylation in liquid biopsies has been used to evaluate risk of recurrence in lung cancer. Belinsky et al. (2017) showed the potential use of an eight-gene methylation panel in plasma and sputum to predict lung cancer recurrence following resection for stage I NSCLC. These results demonstrated that patients with one or more methylated genes in the plasma had an increased risk of recurrence with a significant hazard ratio of 1.5.

Longitudinal cfDNA analyses can provide not only opportunity for posttreatment monitoring but also insights into tumor evolution (Abbosh et al. 2017). Subclonal structures, which seed relapse, can be identified by mapping SNVs detected in posttreatment plasma back to pretreatment data. This may facilitate treatments that target the evolving tumor subclones.

CONCLUSIONS

Understanding of the molecular basis for lung cancer pathogenesis and technologies for molecular early detection are improving rapidly, as are technologies for monitoring of disease burden and noninvasive assessment of prognosis and prediction of treatment response. Clinical application of LDCT screening is coming into clinical practice, but an improved understanding of molecular risk factors and noninvasive diagnostic assays to assist in management of indeterminate pulmonary nodules is needed.

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