

# Lung Cancer FRONTIERS

The Forum for Early Diagnosis and Treatment of Lung Cancer



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The purpose of **Lung Cancer Frontiers** is to acquire and disseminate new knowledge about lung cancer and how it can be most quickly and effectively diagnosed and treated.

## Breath Volatile Metabolic Biomarkers of Lung Cancer

By Peter Mazzone, MD, MPH, FCCP

Advances in several areas have the potential to impact the public health burden of lung cancer: avoidance of tobacco, the ability to identify those at greatest risk for lung cancer, development of effective prevention agents, identification of lung cancer in its earliest stages, improvements in our ability to characterize lung cancer, and progress in our ability to control it locally and systemically. Each of these areas is likely to benefit from the development of accurate, non-invasive biomarkers.

Biomarkers are objectively measured indicators of the state of an individual's health. They range from commonly measured vital signs to complex molecular signatures. A new biomarker can improve on current tests by being more accurate, less invasive, less expensive, and/or novel in its intent. To have clinical impact, the biomarker test result must affect a decision that will ultimately benefit the patient.

The field of lung cancer biomarkers has been dominated by studies of differences in the genome, transcriptome, and proteome of patients with lung cancer. Differences in the products of cellular metabolism are a less commonly evaluated source of biomarkers. Metabolomics usually refers to the study of non-volatile metabolic byproducts, such as carbohydrates, free fatty acids, lipids, and nucleic acids. However, abundant evidence suggests that alterations in metabolic processes exist within lung cancer tissue and/or the lung cancer host.<sup>1-3</sup> To date, small studies of lung cancer tissue, blood, and urine have revealed differences in the non-volatile metabolic signature of patients with lung cancer.<sup>4-6</sup>

In addition to non-volatile metabolic signatures, volatile organic compounds (VOCs) are produced or consumed as part of cellular metabolism and in response to cellular stress. These VOCs may travel from their site of origin and

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## Breath Volatile Metabolic Biomarkers of Lung Cancer

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be found in the bloodstream, urine, and breath. Detection and measurement of VOCs in these media can serve as a source of biomarker development. To date, evidence to support this premise has come from studies of VOC signatures of the headspace gas of cancer cell lines (ie, gas present above the cells and media in culture) and of the breath.<sup>7</sup> The VOC signatures have been detected using gas chromatography–mass spectrometry (GC–MS) and other spectrometry systems, as well as a variety of cross-responsive chemical sensors.

### Evidence of a breath VOC biomarker

A number of studies have assessed the headspace gas of lung cancer cell lines to determine if unique VOC fingerprints were present.<sup>8–10</sup> These studies have differed in the lung cancer cell lines used and thus the origin of the lung cancer, the type of control sample, and the technology used to measure the VOCs. All have shown a unique VOC signature for different cell lines, although the signatures from one study did not match those of other studies. This work has supported the premise of a volatile metabolic biomarker of lung cancer, and it has suggested that signatures of different types of lung cancer may differ from one another.

Other studies have attempted to identify breath VOC biomarkers of lung cancer through the measurement of specific VOCs with GC–MS or other spectrometry systems.<sup>11–13</sup> These studies have differed from one another in the populations of lung cancer and control groups that were included, methods of breath collection, methods of VOC analysis, strategies used to control for ambient VOCs, and the pattern-recognition statistical techniques used to analyze the findings. As an example of the studies performed using GC–MS techniques, one group of investigators collected breath samples from 65 patients with lung cancer and 31 healthy volunteers. They included in their analysis VOCs for which the concentration was at least 15% higher in the breath than in ambient air in subjects with lung cancer, but not in the control group, and they excluded VOCs felt to be environmental contaminants. They found 53 VOCs that met these criteria. A model that included four of these achieved a sensitivity of 52% for identifying lung cancer (100% specific by design). Of note, the model was not validated in

an independent cohort, the control subjects were healthy individuals, and subjects with cancer were at various stages in their treatment.<sup>11</sup>

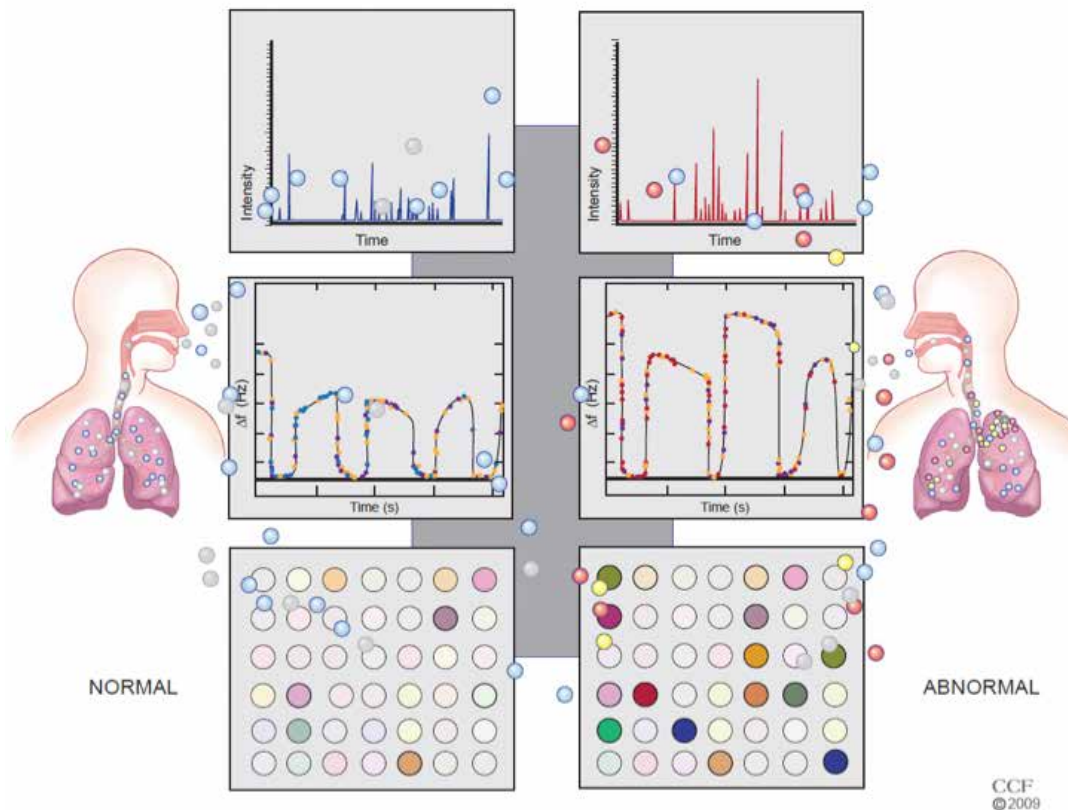
Investigators have also used cross-responsive chemical sensors to develop breath VOC fingerprints of lung cancer.<sup>14–17</sup> These sensor systems do not identify specific VOCs but instead respond to the broad mixture of VOCs present within the breath (*Figure 1*). The response varies with the type of sensor used. Examples include changes in the electrical conductance, vibration frequency, or color of the sensor. The sensor systems are often portable and reusable or disposable, making them ideal for translation into a point-of-care test. Systematic differences in the studies using sensor systems have been similar to those described in the GC–MS studies. In one study using a sensor system, breath samples from 92 subjects with lung cancer and 137 controls with smoking histories or benign lung nodules were analyzed with a colorimetric sensor array system. The output of this system was a change in the color pattern of the sensor array. The system was 75–80% accurate in distinguishing breath from subjects with non-small cell lung carcinoma from breath from controls, with accuracies increasing to 80–85% when more specific questions were evaluated (eg, adenocarcinoma vs. control, adenocarcinoma vs. squamous cell carcinoma). The results were not validated in an independent cohort.<sup>17</sup> Studies in this area highlight the promise of breath biomarker development, as well as some of the advances required for breath analysis to become a clinically useful test.

### Future directions

Breath VOC lung cancer biomarker development still has a road to travel before its promise is known. As with any biomarker, the premise and the systems must first be technically validated. For this to occur, the sensing technologies, sensors, and breath collection devices must be capable of being produced in mass quantities with quality control to ensure a uniform response whenever and wherever they are used. The technique of breath collection should be standardized. In addition, the chemical signatures must be further characterized and a better understanding of their origin is needed. Though speculation about the origin of these signatures exists, there is currently little empiric evidence to support the speculation. In 18 breath VOC

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**Figure 1.** Volatile organic compounds (VOCs) in the breath provide a window into the metabolic stresses in the body. The pattern of VOCs can be detected by gas chromatography–mass spectrometry (GC–MS) systems and a variety of cross-responsive sensors. This pattern appears to be different in lung cancer patients compared to those without lung cancer. Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 2009–2013. All Rights Reserved.

models developed with GC–MS technologies, only five VOCs have been present in at least four of the models.

Concurrently, breath-based biomarkers must be clinically validated. It is clear to the lung cancer clinician that lung cancer is not one disease, but multiple diseases. The cancer of a 50 year-old, never-smoking female is not the same as the cancer of a 75 year-old man who smoked 50 pack-years and has COPD. It is unrealistic to think that one metabolic biomarker will exist for all lung cancers. Breath biomarkers should be developed within patient and cancer phenotypes to optimize their accuracy. This will require larger studies than have been performed to date, with validation of the results in separate groups of subjects.

The validated accuracy required to be clinically useful will depend on the application of the test. For example, a test that is 80% sensitive and specific would lower from 200 to 50 the number of high-risk subjects needed to screen with

CT imaging to detect one lung cancer. This may be a useful improvement, whereas the same level of accuracy is likely not high enough to impact decisions about the management of very small lung nodules. The overall utility of breath VOC biomarker measurement in an ever-growing field of biomarkers will be based on the perception of its value. Its relative accuracy, expense, and intent, though undetermined at this time, may not ultimately differ greatly from those of other biomarkers. However, its ease of use, coupled with the potential for real-time results, will be hard for other testing methods to match in the near future.

It is an exciting time for lung cancer biomarker development, with much promise and need. Although a lot of work is required to validate breath volatile biomarkers of lung cancer—both technically and clinically—there is a substantial hope that this line of investigation will lead to a biomarker that will impact clinical care and patients' lives.

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## Lung Cancer Meetings and Symposia

### 6th International Conference on Molecular Targeted Therapies in the Treatment of Lung Cancer

April 17-20, 2013

Sorrento, Italy

Information: Fred.Hirsch@ucdenver.edu

### European Multidisciplinary Conference in Thoracic Oncology

May 9-11, 2013

Lugano, Switzerland

Information: esmo.org

### 15th World Conference on Lung Cancer

October 27-30, 2013

Sydney, Australia

Information: 2013worldlungcancer.org

## Selections from the Peer-Reviewed Literature

By Ignacio I. Wistuba, MD



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### Introduction

Lung cancer is clearly a heterogeneous disease at the clinical, pathological, molecular and genomic levels. Recent advances in understanding the complex biology of lung cancers—particularly activation of oncogenes by mutation, translocation, and amplification—have provided new treatment targets and allowed the identification of subsets of tumors with unique molecular profiles that can predict response to therapy in these diseases. The identification of specific genetic and molecular abnormalities using tumor tissue specimens, followed by administration of a specific inhibitor to the target, is the basis of personalized cancer treatment.

New technologies, such as high-throughput arrays and, more recently, next-generation sequencing (NGS), have allowed researchers to screen the whole genome, transcriptome, and proteome for new biomarkers in tumor tissue, serum, plasma, and other human body fluids and to develop genomic and proteomic profiles, or “signatures,” to better reflect the complex molecular aberrations within a single tumor.

The rapid development of technologies for large-scale sequencing or NGS of DNA and RNA has facilitated high-throughput molecular analysis that holds various advantages over traditional sequencing. These include the ability to fully sequence large numbers of genes in a single test and to simultaneously detect deletions, insertions, copy number alterations, translocations, and exome-wide base substitutions

(including known hot-spot mutations) in all known cancer-related genes.

The following three papers, published simultaneously in September 2012, highlight the rapid advances in the elucidation of the genomic abnormalities involved in the development of the most frequent types of lung cancer, namely, the two major types of non-small cell carcinoma (NSCLC): adenocarcinoma and squamous cell carcinoma. A comprehensive characterization of genomic abnormalities in the DNA and RNA of lung cancer will continue to provide novel targets for the development of additional, rational targeted therapies for all subtypes of lung cancer.

### **Comprehensive genomic characterization of squamous cell lung cancers**

**Cancer Genome Atlas Research Network. *Nature* 2012; 489:519-25.**

**ABSTRACT:** Lung squamous cell carcinoma is a common type of lung cancer, causing approximately 400,000 deaths per year worldwide. Genomic alterations in squamous cell lung cancers have not been comprehensively characterized, and no molecularly targeted agents have been specifically developed for its treatment. As part of The Cancer Genome Atlas, here we profile 178 lung squamous cell carcinomas to provide a comprehensive landscape of genomic and epigenomic alterations. We show that the tumour type is

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characterized by complex genomic alterations, with a mean of 360 exonic mutations, 165 genomic rearrangements, and 323 segments of copy number alteration per tumour. We find statistically recurrent mutations in 11 genes, including mutation of *TP53* in nearly all specimens. Previously unreported loss-of-function mutations are seen in the *HLA-A* class I major histocompatibility gene. Significantly altered pathways included *NFE2L2* and *KEAP1* in 34%, squamous differentiation genes in 44%, phosphatidylinositol-3-OH kinase pathway genes in 47%, and *CDKN2A* and *RBI* in 72% of tumours. We identified a potential therapeutic target in most tumours, offering new avenues of investigation for the treatment of squamous cell lung cancers.

**EDITORIAL COMMENT:** This is a landmark study of the genomic characterization of lung cancer, representing the first report on exome (DNA), epigenomic (DNA), and transcriptome (RNA) analyses of a large number of surgically resected lung squamous cell carcinoma tumor tissues. The Cancer Genome Atlas (TCGA) is a project sponsored by the US National Institutes of Health and the National Cancer Institute to perform comprehensive molecular and genomic characterization of multiple tumor types, including squamous cell carcinoma and adenocarcinoma of the lung.

There are several major discoveries reported in this paper that may impact the future of the diagnosis and treatment of squamous cell carcinoma of the lung. First, this smoking-related tumor is characterized by one of the most highly complex genomes among all tumors sequenced so far, with almost all tumors having mutations in the tumor suppressor gene *TP53*. Second, there are frequent abnormalities in several hallmark cancer pathways, such as cell cycle control, response to oxidative stress, apoptotic signaling and/or squamous cell differentiation. These are represented by alterations of groups of genes, including *CDKN2A/RBI* (75%), *NFE2L2/KEAP1/CUL3* (34%), *PI3K/AKT* (69%), and *SOX2/TP63/NOTCH1* (44%) (the percentages represent the frequency of tumors with abnormalities in any gene involved in these pathways). Third, this research confirms previously reported findings of alterations in targetable cancer pathways, including relatively high levels of gene mutations, deletions, and amplifications, as well as gene up- or down-regulation that affect, among other genes, *PI3KCA* (16%), *PTEN* (15%), *AKT3* (16%), *NF1* (11%), *EGFR* (9%), and *FGFR1* (7%) genes. Lastly, novel gene mutations were

identified in this tumor type, including a loss-of-function somatic mutation of the *HLA-A* gene, which could be involved in avoiding the immune destruction process and favor survival of malignant cells.

More studies are needed to validate the novel findings reported in this publication. Follow-up investigations are necessary to translate these discoveries into preclinical and clinical studies that explore the application of existing or newly-developed targeted drugs in squamous cell carcinomas of the lung. In addition, NGS analysis of a larger set of tumors with annotated clinical and pathological data would provide information on additional, significant genes and pathways involved in the pathogenesis of this tumor type.

In summary, this first TCGA report on lung cancer undoubtedly raises hope for subsequent reports about similar analyses in other lung cancer subtypes, including adenocarcinoma, small cell carcinoma, and other neuroendocrine tumors of the lung.

### Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing

**Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, Cho J, Suh J, Capelletti M, Sivachenko A, Sougnez C, Auclair D, Lawrence MS, Stojanov P, Cibulskis K, Choi K, de Waal L, Sharifnia T, Brooks A, Greulich H, Banerji S, Zander T, Seidel D, Leenders F, Ansén S, Ludwig C, Engel-Riedel W, Stoelben E, Wolf J, Goparju C, Thompson K, Winckler W, Kwiatkowski D, Johnson BE, Jänne PA, Miller VA, Pao W, Travis WD, Pass HI, Gabriel SB, Lander ES, Thomas RK, Garraway LA, Getz G, Meyerson M.**  
*Cell* 2012; 150:1107-20.

**ABSTRACT:** Lung adenocarcinoma, the most common subtype of non-small cell lung cancer, is responsible for more than 500,000 deaths per year worldwide. Here, we report exome and genome sequences of 183 lung adenocarcinoma tumor/normal DNA pairs. These analyses revealed a mean exonic somatic mutation rate of 12.0 events/megabase and identified the majority of genes previously reported as significantly mutated in lung adenocarcinoma. In addition, we identified statistically recurrent somatic mutations in the splicing factor gene *U2AF1* and truncating mutations affecting *RBM10* and *ARID1A*. Analysis of nucleotide

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context-specific mutation signatures grouped the sample set into distinct clusters that correlated with smoking history and alterations of reported lung adenocarcinoma genes. Whole-genome sequence analysis revealed frequent structural rearrangements, including in-frame exonic alterations within *EGFR* and *SIK2* kinases. The candidate genes identified in this study are attractive targets for biological characterization and therapeutic targeting of lung adenocarcinoma.

**EDITORIAL COMMENT:** Diagnostic genotyping of *EGFR* mutations and *ALK* translocations in lung adenocarcinomas is now routinely used to guide treatment of this disease. In addition, previous reports on genomic characterization of lung adenocarcinoma have shown that this lung cancer subtype harbors frequent activating mutations of *KRAS*, *BRAF*, *HER2*, *PI3KCA*, and translocations in *ROS1* and *RET*, most of which are being actively pursued as targets in ongoing clinical trials. This study, utilizing NGS methodologies to sequence the exomes and/or whole genomes of DNA of a large number of surgically resected adenocarcinoma tumors, verified the genes with frequent somatic mutations previously reported in this tumor type. Importantly, this study also identified novel mutated genes likely involved in the pathogenesis of lung adenocarcinoma, and its findings represent a significant advance towards a comprehensive characterization of potentially actionable somatic alteration in this tumor type.

Among other important findings, this study revealed that 25 oncogenes and tumor suppressor genes were significantly mutated in adenocarcinoma tumors, including a new set of genes (*SMARCA4*, *U2AF1*, *ARID1A*, *RBM10*, *SETD2*, and *BRD3*) involved in epigenetic and splicing (RNA) regulation, which may represent a novel hallmark of cancer in up to 10% of lung adenocarcinomas. Notably, this study did not identify mutation of oncogenes in every tumor specimen and failed to statistically nominate several important, but rarely mutated, genes in lung adenocarcinoma, including in-frame translocation involving kinase fusion genes such as *ALK*, *RET1*, and *ROS1*. These shortcomings suggest that additional NGS studies combining DNA and RNA sequencing of a larger number of tumors with well-annotated clinical and pathological information are warranted to better understand the genomic pathogenesis and progression of this tumor type.

### Genomic landscape of non-small cell lung cancer in smokers and never-smokers

**Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, Maher CA, Fulton R, Fulton L, Wallis J, Chen K, Walker J, McDonald S, Bose R, Ornitz D, Xiong D, You M, Dooling DJ, Watson M, Mardis ER, Wilson RK. *Cell* 2012; 150:1121-34.**

**ABSTRACT:** We report the results of whole-genome and transcriptome sequencing of tumor and adjacent normal tissue samples from 17 patients with non-small cell lung carcinoma (NSCLC). We identified 3,726 point mutations and more than 90 indels in the coding sequence, with an average mutation frequency more than 10-fold higher in smokers than in never-smokers. Novel alterations in genes involved in chromatin modification and DNA repair pathways were identified, along with *DACH1*, *CFTR*, *RELN*, *ABCB5*, and *HGF*. Deep digital sequencing revealed diverse clonality patterns in both never-smokers and smokers. All validated *EFGR* and *KRAS* mutations were present in the founder clones, suggesting possible roles in cancer initiation. Analysis revealed 14 fusions, including *ROS1* and *ALK*, as well as novel metabolic enzymes. Cell-cycle and JAK-STAT pathways are significantly altered in lung cancer, along with perturbations in 54 genes that are potentially targetable with currently available drugs.

**EDITORIAL COMMENT:** It has been established that two major, distinct types pathways are involved in the development of adenocarcinoma of the lung: smoking-related and non-smoking related. This study, although it examined a limited number of cases (16 adenocarcinomas and 1 large cell carcinoma), applied for the first time comprehensive DNA whole genome (NGS) analysis on surgically resected NSCLC tumors obtained from patients with never- (5 cases) and ever-smoking (1 former light smoker and 11 smokers) histories.

This study confirmed the markedly distinct genomic landscape of lung NSCLC, mostly adenocarcinomas, arising in never-smokers compared with smokers. Among other findings, this paper reported a significantly higher level of somatic mutations in tumors from smokers. The authors detected a different spectrum of DNA mutations in patients with a smoking history, with C:G to A:T transversions and C:G to T:A transitions found predominantly in never-smokers and ever-smokers, respectively. In addition, they confirmed

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and identified distinctive sets of known and relatively novel mutations in tumors from never-smokers (*EGFR* mutations, *ALK* and *ROS1* translocations) and ever-smokers (*KRAS*, *TP53*, *BRAF*, *JAK2*, *JAK3*, and mismatch repair genes mutations). Lastly, a number of translocations apart from kinases (*ALK*, *ROS*, *RET*) were identified, involving the genes *RASSF1A-TTYH2* and *FZRI-NFIC*, which are involved in several important cancer metabolic pathways.

The aberrations in DNA repair pathways, chromatin modification genes, and novel translocations identified in this study represent new therapeutic opportunities for lung adenocarcinoma. However, as with previous studies reviewed in this section, the initial findings must be validated in a larger set of tumor specimens from never- and ever- smokers, including tumors with extensive clinical and pathological annotations, and hopefully obtained from patients with diverse ethnic backgrounds, including an East Asian population with a higher incidence of lung cancer in never-smokers.

### Final comment

In lung cancer, most biomarkers discovered and used in clinical applications to date consist of a single genetic mutation, gene amplification, or translocation. However, in many patients or cancer types, these single biomarkers are not sufficient to select patients for targeted therapies. One reason might be that, in many cases, multiple changes in tumor cells, rather than a single modification, lead to activation of selective and often interactive molecular pathways promoting tumor growth and survival. In addition, various targeted treatment regimens have been shown to result in the activation of alternative, compensatory molecular pathways that continue to promote cancer cell survival. Therefore, the continuing elucidation of the complex molecular and genomic abnormalities involved in the pathogenesis of lung cancer subtypes (eg, squamous cell carcinoma, adenocarcinoma, etc.) is essential to advance the field of biomarker development, not only to determine prognosis and predict treatment response, but also to develop markers for early detection, accurate diagnosis, and targeted chemoprevention.

The three papers commented upon above examined DNA and/or RNA extracted from lung cancer tumor specimens obtained from surgical resections, mostly TNM stages I to III. Recently developed NGS methodologies initially required a relatively large amount of nucleic acids for analysis, usually obtainable from surgically resected fresh tumor tissues. The continuing improvement of NGS methodologies will soon allow application of comprehensive exome and whole genome (DNA) and transcriptome (RNA) analyses to small diagnostic tissue specimens (eg, core needle biopsies, bronchoscopic and transbronchial biopsy samples, etc.), which will permit the examination of lung tumors from patients with advanced metastatic and refractory lung tumors. This will afford a better understanding of the genomic alterations involved in lung tumor progression and mechanisms involved in resistance to therapy.

The amount of starting material (DNA or RNA) needed for the newest NGS applications is getting smaller. Currently, the analysis of a limited panel (~400) of gene mutations, amplifications, and translocations can be performed even in DNA extracted from formalin-fixed and paraffin-embedded tumor tissue specimens. However, one of the potential barriers in this process is the large computing capacity needed to manage the billions of small sequence readouts generated, and to assemble those with large databases to interpret the raw data. Another challenge for NGS is the identification of meaningful driver mutations and the separation of “true” mutations from a background of intrinsic sequence variations. In addition, verifying and validating the driver-discovered somatic mutations in lung cancer will require experimental and detailed classical molecular pathology studies to bring NGS into a clinical context.

### Disclosures

Dr. Wistuba submitted an ICMJE Disclosure Form to *Lung Cancer Frontiers*. He discloses no significant conflicts of interest exist with any companies whose products or services are discussed in this article.



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