Molecular Diagnostic Testing in Non-Small Cell Lung Cancer

Jarushka Naidoo, MB, BCh, BAO, MRCPI, and Alexander Drilon, MD

Abstract

The discovery of targetable genomic alterations has revolutionized the field of personalized medicine in non-small cell lung cancer (NSCLC). As the number of clinically actionable drivers continues to expand, a thorough understanding of the molecular diagnostic platforms that are available for the detection of these changes is required to select the most appropriate test or group of tests in the clinic. This review summarizes the common oncogenic aberrations that occur in NSCLC and the diagnostic assays that are poised to detect them.

Molecular diagnostic algorithms have undergone a significant evolution over time, moving from a "one-gene, one-test" paradigm to the inclusion of multiplex assays for common hotspot point mutations, and insertions and deletions. While current testing in most centers is characterized by a combination of several different single-gene or multiplex diagnostic assays, the advent of next-generation sequencing has provided a means of interrogating mutations, rearrangements, and copy number changes across a variety of therapeutically relevant oncogenes and tumor suppressor genes in a single test. As the cost of next-generation sequencing continues to decrease, this platform is likely to become the diagnostic test of choice for clinicians treating patients with advanced NSCLC.

Introduction

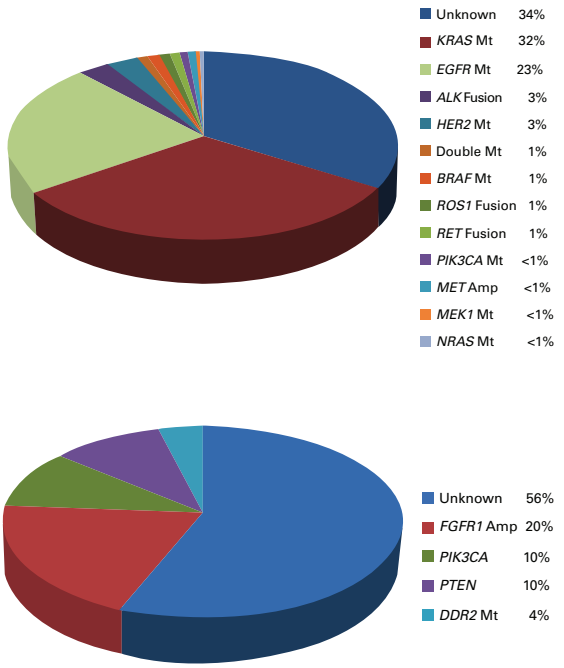
Over the past decade, the oncology community has witnessed a revolution in our understanding of the biology of lung cancer with the identification of a significant proportion of patients whose tumors harbor targetable molecular changes. These recurrent genomic alterations include mutations, gene rearrangements, and copy number changes in relevant lung cancer genes (**Figure 1**). A variety of diagnostic assays can be used to identify these abnormalities, which act as predictive biomarkers of benefit from a corresponding targeted therapy. This review summarizes the current state of knowledge regarding molecular drivers in lung cancer, available platforms to diagnose these genomic alterations, and factors affecting the choice and interpretation of diagnostic assays.

Genomic Diversity in NSCLC

Recurrent mutations in proto-oncogenes comprise a large proportion of therapeutically targetable alterations in lung cancer. *EGFR* mutations in non-small cell lung cancer (NSCLC) were first discovered in 2004 and are present in 10% to 15% of Caucasian patients with advanced disease. *EGFR* exon 19 deletions and the exon 21 *L858R* point mutation make up the majority of sensitizing mutations that confer increased responsiveness to tyrosine kinase inhibitors (TKIs).[1,2] Multiple randomized studies have compared first-line EGFR TKIs with standard chemotherapy in populations clinically or molecularly enriched for patients with *EGFR*-mutant lung cancers.[3-5] These studies have consistently demonstrated the superiority of EGFR TKIs over chemotherapy in terms of response, progression-free survival (PFS), tolerability, and quality of life, resulting in the approval of these agents for the treatment of *EGFR*-mutant lung cancers.[6,7]

Since the discovery of *EGFR*-mutant lung cancers, a number of other driver mutations have been identified in lung adenocarcinomas, including mutations in *BRAF*,[8,9] *KRAS*,[10,11] *HER2*,[12-14] *PTEN*, *AKT*, and *PIK3CA*.[15] In squamous cell lung cancers, a variety of actionable alterations also have been discovered,[4] including *DDR2*, *PIK3CA*, *PTEN*, *AKT*, *KEAP1*, and

FIGURE 1. Molecular Alterations in NSCLC



Representative pie charts from molecular diagnostic testing of NSCLC using a combination of assays at Memorial Sloan Kettering Cancer Center (MSKCC). Sanger sequencing, IHC, FISH, multiplex hotspot mutational testing, and multiplex sizing assays were used as part of a diagnostic algorithm for lung adenocarcinomas (top) and targeted next-generation sequencing used for squamous cell carcinomas (bottom). The percentage of each actionable alteration is shown.

Practical Application

- An overview of the molecular drivers of NSCLC
- A summary of the molecular assays used to identify drivers in tumor samples
- How to interpret the commonly used molecular tests in NSCLC
- Practical considerations when ordering molecular tests in the clinic
- Future directions in molecular diagnostic testing in NSCLC

NFE2L2 mutations.[16] Many of these mutations cluster around the catalytic tyrosine kinase domain of the gene of interest and result in constitutive activation of the mutant protein and downstream pathways.

Recurrent gene rearrangements involving *ALK, ROS1, RET,* and *NTRK* have emerged as important drivers of tumor growth in lung cancer.[17-19] *ALK* rearrangements occur in approximately 3% to 5% of lung adenocarcinomas and are associated with response rates of 60% to 80% with the ALK inhibitor crizotinib.[17,20] Similar to *EGFR*-mutant lung cancers, for patients with *ALK* fusion-positive lung cancers, treatment with crizotinib is superior to chemotherapy. Activity against *ALK*-rearranged NSCLC has also been described with second-generation ALK inhibitors such as ceritinib and alectinib.[21] *ROS1* and *RET* fusions are each found in approximately 1% to 2% of unselected lung cancers, and responses to crizotinib and cabozantinib, respectively, have been described in early studies.[22,23]

Gene fusions share structural features that lend to their detection via a number of distinct assays. *ALK, ROS1,* and *RET* fusions retain the full-length tyrosine kinase domain that is fused to an upstream gene partner, which may provide coiled-coiled domains, resulting in ligand-independent activation and constitutive downstream pathway signaling. These rearrangements can be formed via pericentric or paracentric chromosomal inversions, or translocation between nonhomologous chromosomes. Breakpoints of the downstream gene tend to be relatively conserved; however, within a given gene fusion (eg, *EML4-ALK*), breakpoints of the upstream partner gene can vary, resulting in variable partner lengths.[24]

Gene amplification has likewise begun to emerge as a therapeutically relevant target in NSCLC. *MET* amplification can occur de novo in 1% of adenocarcinomas and 6% of squamous cell lung cancers and has been associated with an early report of response to crizotinib, which is also active against MET.[25-27] In squamous cell lung cancer, *FGFR1* gene amplification occurs in approximately 20% of patients, and trials of FGFR inhibitors for molecularly enriched cohorts of *FGFR1*-amplified squamous cell lung cancer are currently ongoing.[16,28] In contrast, genomic loss of *PTEN* is known to result in activation of the PIK3CA-mTOR pathway and drive tumor growth in both adenocarcinomas and squamous cell carcinomas of the lung.

Single-Gene Molecular Diagnostic Assays

The earliest approaches to molecular diagnostic testing were characterized by the use of a combination of assays that each interrogated genomic changes involving a specific gene. These tests included Sanger sequencing, immunohistochemistry, and fluorescence in situ hybridization.

Sanger sequencing. Direct DNA sequencing after polymerase-chain-reaction–based amplification was one of the earliest methods used to detect mutations in lung cancers such as those involving *KRAS* and *EGFR*. This method was pioneered in the late 1970s by Frederick Sanger, and is thus termed *Sanger sequencing*. The procedure involved a single-stranded DNA template and DNA extension from a bound primer using standard deoxynucleotides via DNA polymerase. DNA fragments were then subjected to capillary electrophoresis and detection of fluorochromes for automated sequence analysis. While Sanger sequencing was more widely used several years ago in the clinic, this technique has largely been replaced by multiplex or high-throughput assays that are described later in this article.

Immunohistochemistry (IHC). Several studies have investigated the use of mutation-specific antibodies for the immunohistochemical detection of known driver oncogenes. In *EGFR*-mutant NSCLC, two monoclonal antibodies that detect exon 19 deletions (clone 6B6; Cell Signaling Technology) and the *L858R* point mutation on exon 21 (clone 43B2; Cell Signaling Technology) have a reported sensitivity of between 70% to 100% and a specificity of almost 100%.[29-31] Many institutional algorithms have incorporated IHC testing for the rapid diagnosis of *EGFR* mutations in situations in which an answer is required within a few days, as in the case of patients who are highly symptomatic from their lung cancers. In addition, these antibodies have proven useful in situations where limited tissue precludes sequencing, such as in cytology or small biopsy samples, and in samples with decalcified tissue.[29]

While useful, mutation-specific IHC is limited by a number of factors. Currently available antibodies for *EGFR* exon 19 deletions are limited to the detection of a specific number of base pair deletions (eg, 15 base pairs) and are unable to detect the breadth of mutations that are seen in this molecularly heterogeneous population (eg, 9 or 12 base pairs).[32] IHC cannot be used in isolation and will invariably require more comprehensive genotyping to confirm the absence of a therapeutically relevant target. IHC for mutant EGFR proteins must also be contrasted with IHC for wild-type EGFR. Positive staining for the latter does not indicate the presence of an *EGFR* mutation and is not a biomarker for increased sensitivity to EGFR TKI use.[33] Testing for wild-type EGFR protein expression has been investigated, however, as a potential biomarker for EGFR monoclonal antibody use in NSCLC.[34]

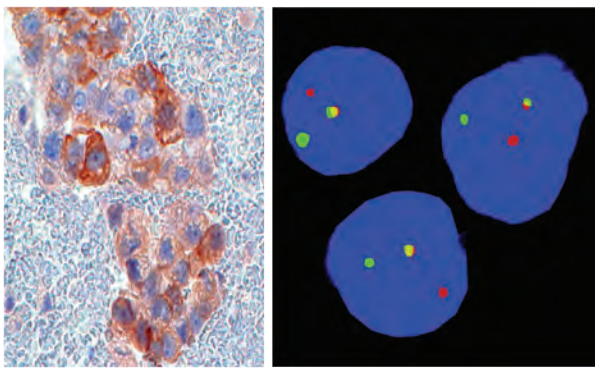
As with *EGFR*-mutant lung cancers, IHC provides the capacity to rapidly diagnose *ALK* or *ROS1* fusion-positive lung cancer in the clinic.[35] In contrast to mutant-specific EGFR antibodies, the antibodies used in this space are designed to detect wild-type ALK and ROS1 on the premise that tumors harboring recurrent

rearrangements involving *ALK* or *ROS1* will have high levels of expression of the corresponding proteins in the majority of cases.

IHC with ALK clone 5A4 (Abcam) has demonstrated a sensitivity and specificity of 90% and 100%, respectively, when compared with the ALK Break Apart FISH Rearrangement Probe Kit (**Figure 2**).[36,37] Similarly, a monoclonal antibody D5F3 (Cell Signaling Technology) has demonstrated near-complete correlation with ALK FISH testing.[26] Other studies have reported concordance of rates of 97% to 98% between ALK IHC and ALK FISH testing.[38,39] IHC can be performed fairly quickly on tumor biopsies or surgical samples, and many institutions have incorporated at least ALK IHC as an initial method for screening for *ALK* fusions prior to FISH testing.

In a study investigating ROS1 IHC with the D4D6 antibody in *ROS1*-rearranged lung cancer, ROS1 protein expression in tumor cells was 100% sensitive and 92% specific for *ROS1* rearrangements by FISH testing.[40] In contrast, screening for *RET* fusions with IHC has not been successful.

FIGURE 2. Testing for ALK Fusions in Lung Adenocarcinoma



Screening for *ALK* rearrangements can be performed via several diagnostic assays. On the left, positive immunohistochemical staining for *ALK* using the anti-*ALK* 5A4 antibody is shown. On the right, a positive fluorescence in situ break-apart test is shown with split red and green signifying the presence of an *ALK* rearrangement. (Courtesy of Maureen Zakowski, MD, and Lu Wang, MD, PhD, Department of Pathology, Memorial Sloan Kettering Cancer Center)

Reverse transcriptase-polymerase chain reaction (RT-PCR). This method allows for the detection of gene fusions from an analysis of RNA extracted from a patient's tumor.[35,41,42] Primers are designed to hybridize with chimeric transcripts, and the RNA sequence is reverse transcribed to DNA that is amplified via PCR. Advantages of this method include a rapid processing time and a low volume of cells needed. However, RT-PCR is highly specific for particular fusion genes and will not detect alternate partners. Other caveats to using this method include high levels of technical skill that are required to carry out the test, and the need for high-quality RNA. While RT-PCR is an effective research tool that is often used for the initial identification or screening of recurrent gene fusions, these factors pose a challenge to this method's use in routine practice, and testing for recurrent gene fusions in the clinic has largely been performed via FISH, described next.[41]

Fluorescence in situ hybridization (FISH). FISH is the most widely used assay in the clinic for the detection of gene fusions in lung cancer. As such, the FDA-approved companion diagnostic test for the detection of *ALK* fusions for crizotinib use in *ALK*-rearranged lung cancer is a FISH assay (Vysis LSI ALK Break Apart Rearrangement Probe Kit; Abbott Molecular). The test involves the use of break-apart probes, labeling the fusion breakpoint with an orange fluorochrome on the 3' (telomeric) end, and a green fluorochrome on the 5' (centromeric) end. In the nonrearranged state, these probes lie close to each other on the chromosome and appear as a fused signal. In contrast, the presence of a gene rearrangement, chromosomal inversion, or translocation results in split signals or an isolated split pattern at the 3' or 5' ends (**Figure 2**).[27] The paradigm for the detection of *ROS1*[43] and *RET*[44] fusions via FISH is similar, and assay validation for these genes is currently ongoing.

Challenges in the use of FISH testing include the technical complexity required to carry out and interpret the test, as well as specific details regarding preparing and storing tissues.[45] However, unlike RT-PCR, FISH affords the identification of fusions with variant partners. It is also worth noting that variations on the classic FISH assay as described are currently in development (eg, four-probe assays that can interrogate more than one gene rearrangement, and multicolor assays with probes for specific upstream partners).

FISH testing is likewise the most widely used method to determine changes in gene copy number in the clinic. For *MET* amplification, for example, probes are designed against *MET* (red) which lies on chromosome 7, and the centromere of chromosome 7 (CEP7, green).[46,47] Normal tissues will have an average of two *MET* signals and two control probe or *CEP7* signals. *MET* copy number can increase in the face of high polysomy, where copies of the entire chromosome 7 are increased and the ratio of *MET/CEP7* is not elevated. However, in order to say that *MET* is likely truly amplified, copies of only the specific region on chromosome 7 containing *MET* must be increased, and the *MET/CEP7* ratio must be elevated.[46]

While the cutoff for a positive *MET/CEP7* ratio defining therapeutically relevant *MET* amplification is currently being validated, a recent report noted that responses to the MET inhibitor crizotinib were only noted in patients whose ratio exceeded 2.2. In addition, barring small patient numbers, a higher response rate was noted in patients whose ratio equaled or exceeded 5.0.[27] Data in this field will continue to emerge as results from ongoing trials of MET inhibitors in *MET*-amplified lung cancer and FGFR inhibitors in *FGFR1*-amplified lung cancer are reported.

Multiplex Testing for Gene Mutations

As illustrated by the previous section on single-gene diagnostic

assays for driver alterations in lung cancer, previous molecular diagnostic testing paradigms were characterized by a one-gene, one-test strategy. This approach was initially tenable in the face of a few genes that required interrogation (eg, *EGFR, KRAS*, and *ALK*). As the number of actionable genomic alterations in lung cancer grew impressively since the discovery of *EGFR* mutations in 2004, platforms quickly migrated toward assays that were able to test for changes in multiple genes in a single test. As such, many institutional lung adenocarcinoma algorithms adopted both mutational hotspot testing and multiplex sizing assays for *EGFR, KRAS, NRAS, BRAF, PIK3CA, PTEN,* and *AKT*. These multiplex tests have been performed in combination with standard FISH assays for *ALK, ROS1, RET,* and *MET*. Approaches such as this have been highly successful in providing comprehensive genotyping for lung adenocarcinomas.[48]

Multiplex hotspot mutational testing. Multiplex PCR is defined as the simultaneous amplification of at least two DNA or cDNA targets in a single reaction vessel. The cobas EGFR Mutation Test (Roche; Basel, Switzerland), the current FDA-approved companion diagnostic test for the presence of an activating EGFR receptor, is a multiplex real-time PCR-based diagnostic test that identifies 41 mutations across exons 18, 19, 20, and 21 of the *EGFR* gene.

In contrast, the SNaPshot assay (Applied Biosystems) and Sequenom assays (Sequenom) are examples of multiplex tests that can sequence a large number of relevant mutations in several hotspots (recurrently mutated regions) of oncogenes or tumor suppressor genes. The SNaPshot platform sequences through a multiplex-PCR system, followed by individual base extension reactions that detect at least 50 mutation sites in up to 14 individual cancer genes, where up to 10 single nucleotide polymorphisms may be tested by one base extension. Testing is carried out on formalin-fixed paraffin embedded (FFPE) tissue, with a 2- to 3-week processing time.[49] This method has an approximate 10% improvement in sensitivity compared with single-gene tests. The Sequenom platform tests for up to 238 or more somatic mutations across 19 different genes commonly associated with cancer, and can be done on FFPE tissue, fresh-frozen tissue, or cell lines that contain a minimum of 10% mutation-positive tumor cells. This assay can be tailored to include a panel specific to each cancer type (eg, a lung adenocarcinoma panel might include 91 mutations in 8 relevant genes such as *EGFR, ERBB2, KRAS, NRAS, BRAF, PIK3CA, PTEN,* and *AKT*). This test utilizes an array-based method that starts with purified PCR reactions, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for rapid multiplexed nucleic acid analysis.[50,51]

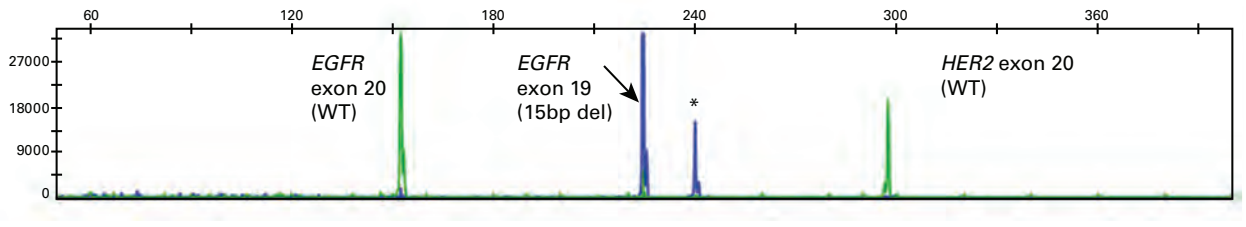
Multiplex sizing assays. Similar to hotspot mutation testing, multiplex sizing assays are designed to simultaneously detect insertions or deletions in multiple relevant cancer genes (ie, *EGFR* and *ERBB2*).[52] For *EGFR*, sizing assays for exon 19 or 20 insertions or deletions are based on length analysis of fluorescently labeled PCR products **(Figure 3)**. Sizing assays can be performed with as little as 1ng of DNA, are highly sensitive, and demonstrate 100% concordance with direct sequencing results in some series.[53]

Next-Generation or Massively Parallel High-Throughput Sequencing

Next-generation sequencing (NGS) refers to an assessment of the genome at different levels of modification. It encompasses targeted exome, whole-exome, whole-genome, whole-transcriptome, and whole-epigenome analyses.[54] To date, the most clinically relevant of these methods is targeted exome sequencing, which can interrogate several hundreds of therapeutically relevant cancer-related genes in a single test. Whereas whole-exome or whole-genome efforts provide broader scope, a targeted exome approach intentionally distributes sequencing reads to specific genomic locations, allowing for higher sequencing depth of coverage and ensuring accurate detection of sequence variants at these loci.[55]

The methodology by which NGS is carried out can vary significantly. In general, a DNA library is prepared from a patient's tumor sample. DNA is then amplified via PCR and the templates sequenced in a massively parallel fashion in a single run.[56] Depth of "coverage" refers to the average number of sequencing reads that align to each base within the sample DNA. This is adjustable, and indicates the degree of certainty with which a base change can be detected.[50] Different machines carry out these processes, including the Ion Torrent (Life Technologies)

FIGURE 3. Electropherogram of an EGFR-Mutant Lung Adenocarcinoma



A representative case of a patient's tumor harboring the most common *EGFR* exon 19 deletion. ABI tracing of a sizing assay shows a heterozygous 15bp deletion (arrow). The asterisk (*) marks the wild-type peak. This case was concurrently tested for indels in exon 20 of *EGFR* and *HER2 (ERBB2)* using a multiplex assay. The latter returned negative and illustrates the mutually exclusive nature of these mutations. (Courtesy of Maria Arcila, MD, Department of Pathology, Memorial Sloan Kettering Cancer Center)

TABLE. Available Molecular Diagnostic Platforms for NSCLC in the Clinic

Selected Therapeutically Relevant Genomic Alterations in NSCLC	Sanger Sequencing	Immunohisto-chemistry	Fluorescence In Situ Hybridization	Multiplex Hotspot Mutation Testing	Multiplex Sizing Assays	Next-Generation Sequencing
Point Mutations *EGFR* *KRAS* *ERBB2 (HER2)* *MAP2K1 (MEK)* *BRAF* *PIK3CA* *AKT*	✓	✓ (*EGFR* L858R)		✓		✓
Insertions or Deletions *EGFR* *ERBB2 (HER2)*	✓	✓ (*EGFR* exon 19 deletion)			✓	✓
Rearrangements *ALK* *ROS1* *RET* *NTRK*		✓ (for *ALK* and *ROS1* amplification, requires FISH confirmation)	✓			✓
Amplification *MET* **Loss** *PTEN*		✓ (*MET* amplification requires FISH confirmation)	✓			✓
Non-Recurrent Genomic Alterations Involving the above genes and other potentially relevant oncogenes and tumor suppressor genes						✓

A selection of currently available molecular diagnostic platforms are shown in relation to the genomic alterations these tests are poised to detect. For multiplex platforms and next-generation sequencing, the genomic alterations that are interrogated by these assays can often be customized based on histology and clinical need.

and Illumina (Illumina, Inc).

Unlike multiplex hotspot mutational testing and sizing assays, NGS does not require knowledge of recurrent genomic changes in NSCLC. NGS has the potential to sequence the entire length of target genes for changes that might occur in both hotspot and non-hotspot regions. Interrogating non-hotspot areas is particularly relevant to both tumor suppressor genes and oncogenes where mutations might be found scattered across the length of the gene. NGS also has the capacity to uncover gene fusions by the inclusion of introns from recurrently rearranged cancer genes, and copy number changes such as gene amplification or loss, by referencing a known standard or matched normal nontumor DNA. At the end of the day, a well-designed NGS platform offers the clinician and patient a single test that is able to capture: (1) base substitutions/point mutations; (2) insertions and deletions; (3) gene rearrangements; and (4) amplification or loss in several hundred genes **(Table)**.

In order to investigate the utility of NGS in the clinic, we performed NGS on lung adenocarcinomas from never- or former light smokers whose tumors tested negative for known genomic alterations via an institutional algorithm (Sequenom multiplex hotspot testing and sizing assays for *EGFR, KRAS, ERBB2, BRAF, MAP2K1, PIK3CA, PTEN,* and *AKT*, and FISH assays for *ALK, ROS1,* and *RET*).[57] NGS uncovered an actionable genomic alteration with a targeted agent based on National Comprehensive Cancer Network NSCLC guidelines in 36%

of patients. These included mutations in *EGFR, BRAF,* and *ERBB2,* and rearrangements involving *ALK, RET*, and *ROS1* that previous non-NGS testing did not pick up. Several of these patients went on to receive targeted therapy (ie, crizotinib for *ALK* and *ROS1* rearrangements, and cabozantinib for *RET* rearrangement) and responded to treatment. NGS also identified a targeted agent available in a clinical trial in an additional 32% of patients. In addition, the series illustrated that a significant amount of tumor tissue was consumed by standard "piecemeal" non-NGS testing, with the majority of patients (84%) requiring two or more biopsies to complete both non-NGS and NGS testing. Results from this study suggested that compared with non-NGS testing with multiple assays, NGS may represent a more efficient approach to the molecular profiling of lung cancers.

It is important to note that NGS does not come without its challenges. The added breadth, coverage, and data complexity have resulted in the need to develop more robust bioinformatic tools.[58] Furthermore, determining the relevance of each of the genomic alterations uncovered via NGS can pose a daunting hurdle for clinicians. Guidance will ultimately need to be provided to clinicians on various fronts to help distinguish drivers from passenger alterations. Commercial NGS companies have already begun to include information in clinical reports on potential targeted therapeutics for specific genomic alterations. In addition, novel web-based portals such as www.mycancergenome.com have been developed to provide a resource for clinicians, patients, and caregivers for the interpretation of molecular diagnostic results.[59] Lastly, the increasing use of NGS will have implications for companion diagnostic approval. Historically, companion diagnostic approval by the FDA has followed a one-gene, one-test paradigm, and drug development will very quickly need to factor in the utility of NGS.

Conclusion

Non-small cell lung cancer is a genomically complex disease that is characterized by the presence of therapeutically relevant genomic alterations in the majority of tumors that undergo comprehensive molecular testing. While a number of single-gene and multiplex assays are used to profile NSCLC with continued success, increasing tumor requirements and limited coverage are growing concerns. NGS holds immense promise for the future of diagnostic testing in NSCLC, given the breadth and depth of coverage that this single test offers. Recognizing the strengths and limitations of such an approach, our recommendation is to consider NGS on a validated platform should this be available to the clinician.

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