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Clinical Lung Cancer Mutation Detection

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Abstract

As the promise of personalized medicine in the treatment of cancer begins to be realized, the diagnostic techniques needed to drive that revolution have continued to evolve. What started as optical imaging of banded chromosomes for karyotyping has progressed to DNA sequencing and now next-generation sequencing capable of producing billions of reads. There are currently a large number of techniques that are used in the clinical laboratory for assessing the presence of mutations in lung tumors, all with their own strengths and weaknesses. Here, we survey the technology that is available and take a closer look at next-generation sequencing. We discuss the instruments that are currently on the market and demonstrate the common workflow from patient to data. Additionally, the outside factors that influence the use of these technologies, from government regulation to insurance reimbursement, are presented.

Keywords: detection, lung cancer, mutations, next-generation sequencing

1. Introduction

Ultimately, cancer is a genetic disease of the DNA. Changes in chromosomal sequences result in altered gene expression, protein structure, and enzyme activity, leading to increased cell growth, motility, and the associated clinical symptoms. Every cancer is different, and understanding the mutations present in each case is crucial in choosing the proper treatment.

The field of molecular diagnostics, the detection of DNA abnormalities, has come a long way since 1902 when Theodor Boveri first noted through microscopic observations that cancer likely came from abnormal chromosomes [1]. From the discovery that DNA consists of nucleotides, to the ability to sequence those nucleotides and the technology to do it more and more efficiently, our knowledge of cancerous mutations and their role in treating the disease

has grown. We have progressed from manually generating one data point at a time to using automation to create billions of them in a matter of hours.

DNA abnormalities may be present in a number of forms. These range from the gain or loss of whole chromosomes to the substitution of one DNA nucleotide for another, leading to a change in the resulting protein. Between these extremes are chromosomal translocations, small or large deletions and insertions, amplifications, and inversions.

There are multiple generations of techniques currently being utilized in the clinical laboratory for the characterization of lung cancer. Each has its own inherent advantages and disadvantages that must be considered prior to ordering tests. The results of these diagnostics have profound impacts on the treatment of individual patients. They are also being used for research into better understanding the disease and have been responsible for the generation of the International Cancer Genome Consortium and The Cancer Genome Atlas [2]. Their impact on medicine will certainly only increase in the future.

2. Scope of mutation testing in lung cancer

The scope of mutation testing in lung cancer is determined by the mutation landscape described in these tumors. Considering which mutations need to be detected during diagnostic workup, two differentiations must be applied to mutations: driver mutations versus passenger mutations and therapeutically accessible *versus* inaccessible mutations. Driver mutations directly cause the development of cancer and allow it to grow and metastasize [3]. Other mutations that may either enhance the driver mutation or have no functional role in cancer progression are known as passenger mutations. As our knowledge of mutations is improving, the percentage of known driver mutations detected in lung cancer is increasing. The percentage of lung adenocarcinomas with no detected driver mutation dropped from 40% in 2013 to 24% in 2016 [4], increasing the number of known driver genes from 10 to 15 in the same time span. Some of the driver mutations are “actionable” in that they are therapeutically accessible and may be targeted in treatment. It is such mutations that are primary candidates for mutation screening assays. A nice summary of gene mutations, treatment options, and clinical trials in lung cancer is available from the mycancergenome.org website. Developing a lung cancer mutation assay with full coverage of the genes listed in the mycancergenome database would comprise a fairly comprehensive assay (**Table 1**). The absolute minimum of genes to be tested for mutations in lung cancer can be determined from the National Comprehensive Cancer Network (NCCN) Guidelines on Non-Small Cell Lung Cancer [5], which recommends at least EGFR mutation and ALK translocation testing.

The molecular characterization of lung cancer has focused primarily on those of the adenocarcinoma type for two main reasons. It is responsible for more deaths than any other form of cancer [6], and adenocarcinomas tend to be under the strong influence of identifiable driver mutations, making testing and treatment less nebulous than other cases. The three most common such mutations are of the epidermal growth factor receptor (EGFR), KRAS,

and anaplastic lymphoma kinase (ALK), with ERBB2 mutations also being quite common [7]. Less common mutations in lung cancer are reviewed in Ref. [8].

Gene	Alteration	Frequency in NSCLC (%)	Available drugs
AKT1	Mutation	1	Drugs in clinical development
ALK	Rearrangement	3–7	Drugs approved in NSCLC
BRAF	Mutation	1–3	Drugs approved in other cancer
DDR2	Mutation	~4	Drugs approved in other cancer
EGFR	Mutation	10–35	Drugs approved in NSCLC
FGFR1	Amplification	20	Drugs in clinical development
ERBB2	Mutation	2–4	Drugs approved in other cancer
KRAS	Mutation	15–25	Drugs in clinical development
MEK1	Mutation	1	Drugs approved in other cancer
MET	Amplification	2–4	Drugs approved in NSCLC but for other molecular subtype
NRAS	Mutation	1	Drugs in clinical development
PIK3CA	Mutation	1–3	Drugs in clinical development
PTEN	Mutation	4–8	Drugs in clinical development
RET	Rearrangement	1	Drugs approved in other cancer
ROS1	Rearrangement	1	Drugs approved in NSCLC

Table 1. Frequency of mutations and availability of targeted therapies in NSCLC. Drug availability for the most frequent mutations detected in NSCLC is shown. Data are from mycancergenome.org.

EGFR-activating mutations represent a critical determinant for proper therapy selection in patients with lung cancer. There is a significant association between EGFR mutations, especially exon 19 deletions and exon 21 (L858R, L861), exon 18 (G719X, G719), and exon 20 (S768I) mutations, and sensitivity to EGFR inhibitors [9, 10]. A secondary mutation, T790M, is present in approximately 60% of tumors with acquired resistance to EGFR inhibitors [11] and confers resistance through steric interactions in the inhibitor binding site [12]. Primary resistance to EGFR-targeted therapy is associated with KRAS mutations. That is why some laboratories choose to add KRAS mutation testing to their assays, as concurrent EGFR and KRAS mutations occur in <1% of patients with lung cancer, and KRAS mutations are associated with intrinsic EGFR inhibitor resistance.

KRAS undergoes mutations resulting in single amino acid changes. In lung cancer, those mutations are most commonly present at the 12th amino acid position [13]. While KRAS mutations are the second most common mutation in lung cancer [7] and are especially prevalent in adenocarcinoma of the lung [13], their use as a prognostic marker or therapeutic guide in lung cancer has been limited. Studies have shown both a correlation between a KRAS mutation and a worse prognosis [14], but also no association with outcome [15]. ALK rearrangements

represent the fusion between ALK and various partner genes, including echinoderm microtubule-associated protein-like 4 (EML4) [16]. ALK fusions have been identified in a subset of patients with NSCLC and represent a unique subset of NSCLC patients for whom ALK inhibitors may represent a very effective therapeutic strategy. That is the reason why ALK translocation testing should be included in any basic lung cancer mutation panel. For the most part, ALK translocations and EGFR mutations are mutually exclusive.

The parameters of tests employed in mutation analysis should fulfill the criteria suggested by professional organizations like the College of American Pathologists, International Association for the Study of Lung Cancer, and the Association for Molecular Pathology that have recently offered guidance on the use of molecular pathology in the screening of lung cancer patients. Their recommendations focus largely on the importance of EGFR mutations and ALK rearrangements. While they encourage testing for these abnormalities in patients with early stage lung adenocarcinoma, leaving the decision to the physician and laboratory, they require testing in advanced lung adenocarcinoma. Additionally, these tests may be performed on lung tumors other than adenocarcinoma if there is reason to believe an oncogenic driver is likely to be found.

3. Current technologies to detect mutations

There are multiple techniques that are currently used to detect mutations in clinical tumor samples. The specific technique used for each case is dependent on a number of factors including fundamental requirements such as the type of analysis to be performed (structural/copy number changes *vs* sequence mutations), the length of sequence required, and the type of nucleic acid being sequenced (DNA or RNA), as well as practical considerations including amount and quality of the sample, turnaround time (TAT), and cost to carry out the analysis. While the ability exists to sequence a patient's entire genome, most clinical assays are more targeted, focusing on one specific nucleotide, a chosen gene, or a number of genes due to the cost and labor involved.

3.1. Analysis of gross chromosomal changes

3.1.1. Cytogenetics

Still widely used today, karyotyping was the first clinical mutation assay and was responsible for the identification of the Philadelphia chromosome in 1960, which is the result of a translocation and is the defining aberration in chronic myeloid leukemia. When stained, generally with Giemsa stain, chromosomes develop a characteristic appearance of alternating light and dark "bands." This allows for the documentation of gross changes in chromosome number or structure. Structural changes able to be identified include large insertions or deletions, sequence inversions, and translocations. The technique has been considerably improved over the years, including the ability to digitally photograph the chromosomes; however, the preparatory work-up is still very labor intensive, and the analysis requires highly trained individuals. This limits the number of cells that can be analyzed for each sample, increasing the risk of false-negative results in samples that are highly heterogeneous. Additionally,

as cells must be cultured to generate the necessary metaphase spreads, genotyping is mostly limited to rapidly dividing hematological malignancies and is only used sparingly in solid tumors such as lung cancer.

3.1.2. Fluorescence *in situ* hybridization

Rather than using a generic dye, fluorescence *in situ* hybridization (FISH) uses single-stranded, fluorophore-labeled DNA probes that hybridize to complementary regions in the genome and can be visualized as fluorescent spots on a metaphase spread or in an interphase nucleus. Due to the paired nature of human chromosomes, a FISH probe would be expected to identify two instances of the target sequence in each cell. Any numeric deviance from that indicates a loss or amplification of that locus (**Figure 1A**). Abnormal distances between probes targeting neighboring regions on the same chromosome can also indicate a translocation or an inversion has occurred.

Figure 1. Techniques for measuring gross changes in chromosomes. (A) Fluorescent *in situ* hybridization (FISH) probes are applied to metaphase spreads or interphase cells. Complementary locations on the target chromosomes are identified as fluorescent spots due to the binding of the probe. Anything other than one locus on each of the sister chromosomes, represented by one fluorescent spot, is abnormal. This is shown at the chromosomal (top panel) and cellular (bottom panel) levels. (B) Array-comparative genomic hybridization binds fluorescently dyed and fragmented genomic DNA to immobilized probes. As tumor and normal DNA are dyed different colors, when both are applied to the array, it will be visualized as yellow if there are equal quantities of DNA from each source. Overrepresentation of green or red indicates overrepresentation of the corresponding DNA source. The fluorescence is then plotted as a graph of copy number *versus* chromosome position. (C) The single-strand conformation polymorphism utilizes the fact that wild-type (WT) and mutant (M) DNA strands will adopt different conformations due to differing intramolecular forces. This will lead them to migrate at different rates during polyacrylamide gel electrophoresis. If a sample of tumor DNA contains a band that migrates at a different rate than a control sample, it is indicative of a mutation. A "normal" band is also expected from the tumor sample due to the likely heterogeneity of the sample. (D) As mutations will alter the forces holding complementary strands of DNA together, a melting curve analysis measures the fluorescence emitted by DNA with increasing temperature. Since the DNA dye only fluoresces when bound to double-stranded DNA, as the DNA denatures the fluorescence decreases.

In lung cancer, FISH is the current FDA-approved gold standard method for detecting translocations involving the ALK gene. ALK translocations were the second driver mutation described in NSCLC [16]. ALK partners in the translocation can vary, but the most frequently involved is the EML4 gene. The FISH test based on the Vysis ALK Break-Apart FISH Probe by Abbott detects the translocation of ALK, but does not identify the fusion partner. This test consists of two probes that bind on either side of the common 2p23 break point. In the absence of a translocation, the probes will be seen as adjacent or overlapping. After a translocation, the probes will be visualized with distinct separation. The test results are not always clear cut, and there is a large gray zone area of inconclusive results which can be influenced by subjective judgment. The test is thus highly dependent on optimal sample quality and experienced medical technologist staff.

With appropriate setup, FISH can be used for detection of other less frequent translocations or copy number changes in lung cancer. The technique has a fast TAT and is widely used. The largest drawback of FISH is throughput. Generally, one sample/set of probes is used per slide, making analysis of multiple samples/targets labor intensive.

3.1.3. Comparative genomic hybridization

The sensitivity of karyotyping and FISH is limited by the magnification of the microscope since the results are visualized by the human eye. Comparative genomic hybridization (CGH) is an extension of karyotyping that utilizes fluorescent dyes and microscopy to improve the ability to detect smaller changes in chromosome structure. In this method, chromosomal material from the tumor is dyed with a fluorescent dye, such as red, while chromosomal material from a normal sample is labeled with a different fluorophore, such as green. The two samples are combined, denatured, and allowed to hybridize to a reference metaphase spread in classical CGH. Differential fluorescence (i.e., greater red than green) indicates more or less of that chromosomal segment in the tumor.

This technique has now evolved into an array-based assay. Array-CGH utilizes the same principles, except the labeled input DNA is fragmented and hybridized to an array consisting of small probes rather than a metaphase spread. Where FISH hybridizes fluorescent probes to immobilized chromosomes, array-CGH hybridizes fluorescent genomic DNA to immobilized probes. If the target locus is neither under- or over-represented in the tumor sample, there will be equal numbers of normal and tumor fragments binding to the designated location on the array, and the equal mixture of red and green fluorescence will yield a yellow light. An imbalance of the target DNA in the tumor will lead to a shift in the observed fluorescence to red or green (**Figure 1B**).

While a novel technique, array-CGH has a number of limitations that have prevented it from widespread use in cancer diagnostics. The fundamental principles of the assay allow it to only detect quantitative differences in the amount of the target sequence. Chromosomal abnormalities that involve only the rearrangement of DNA, such as translocations and inversions, cannot be detected. Additionally, the presence of non-tumor cells in the tumor sample will dilute the mutation signal with normal background making the method less sensitive. For this reason, array-CGH is commonly used in oncologic hematology for chronic lymphocytic

leukemia (CLL) [17], where a highly pure tumor sample can be obtained, but is rarely used in solid tumors such as lung cancer, which are generally very heterogeneous. Array-CGH is slowly being phased out of the cancer field entirely due to the emergence of next-generation sequencing (NGS), which is discussed in-depth below. The long TAT of NGS currently requires some samples to be analyzed with array-CGH instead, but that will surely improve as the technology evolves. Outside of oncology, array-CGH is routinely used for postnatal diagnostics where a homogeneous sample is readily available [18].

3.2. Analysis of sequence mutations

3.2.1. Sequence mutation screening

Prior to analyzing samples for specific mutations, it is possible to screen samples for the presence of mutations using a qualitative assay yielding a yes or no answer. This is particularly useful when carrying out a large sequencing study as it allows the targeted use of more labor intensive and expensive techniques for only those samples most likely to contain the targeted mutation. These methods can be efficiently scaled to high throughput assays and are based on the chemical and physical principles of DNA structure.

The conformation of single-stranded DNA is a direct function of its nucleotide sequence; therefore, mutant and wild-type DNA adopt different conformations that result in differing migration rates in polyacrylamide gel electrophoresis which can be detected using the single-strand conformation polymorphism technique (**Figure 1C**). Similarly, denaturing high pressure liquid chromatography hybridizes potentially mutant DNA to wild-type DNA. Subsequent ion pair, reverse phase chromatography, can then detect heterodimers as additional peaks due to differences in retention time [19]. Another common technique is melting curve analysis. Changes in sequence will alter the forces holding the double-stranded DNA together, resulting in differences in the temperature required to denature various loci [20]. By staining DNA with intercalating dyes that fluoresce only when bound to double-stranded DNA, the loss of this signal can be monitored with increasing temperature, generating a graph of fluorescence *versus* temperature (**Figure 1D**). While unique fingerprints are sometimes seen for specific mutations, analysis generally consists of simply looking for the presence of any differences compared to the wild-type DNA. If they are identified, other methods may be used to find the exact mutation present.

3.2.2. Allele-specific PCR

Techniques for analyzing single nucleotide mutations can be divided into those that give a direct yes/no readout as to the presence of a specific mutation and those that provide the exact DNA sequence, allowing the user to examine it for various mutations. Allele-specific PCR is an example of the first type of assay. The original allele-specific PCR utilized the fact that Taq polymerase is inefficient when there is a mismatch at the 3' end of a primer hybridized to the target DNA. Therefore, if the primer is designed such that the final nucleotide will line up exactly with the genomic nucleotide in question, and its sequence is complimentary to the suspected mutation, a much larger quantity of PCR product will be yielded by a sample that contains the target mutation than by a sample that does not (**Figure 2A**). Output can be quan-

titated in a number of ways including electrophoresis or a follow-up round of qPCR. Due to the exponential nature of PCR, this method is very sensitive, allowing for the detection of mutations in a sample diluted by a large number of normal cells.

Figure 2. Allele-specific PCR (A) In the original assay, a primer with a 3' end that aligns perfectly with the nucleotide in question is designed such that it is complementary to the mutant allele and has a mismatch with the wild-type allele. During PCR, elongation will only occur if the mutant allele is present. (B) The TaqMan approach to allele-specific PCR utilizes a probe that will bind only to the mutant allele. The probe contains both fluorescent (F) and quencher (Q) epitopes and does not fluoresce when it is intact. A mutually complementary primer is used to initiate elongation upstream of the probe in both wild-type and mutant alleles. If the elongation encounters the probe, it will release the fluorescent moiety, emitting a detectable signal.

Since its introduction, this method has been simplified, allowing the user to quantitate during the initial PCR step. This is typified by the TaqMan probe system from ThermoFisher Scientific. In this technique, a probe that is complimentary to a mutant, but not wild-type, allele contains a fluorochrome reporter attached to its 5' end and a quencher moiety on the 3' end that prevents a fluorescent signal from the reporter being detected. An unlabeled primer upstream of the targeted sequence is used to initiate PCR. As the DNA polymerase elongates the strand starting from the primer, it degrades the oligonucleotide backbone of the TaqMan probe. The fluorochrome reporter emits fluorescence which can now be detected due to this spatial separation from the quencher [21] (**Figure 2B**). This method is highly efficient as it is one step, can easily be done in a 386-well format with multiple probes and samples, and can be automated, resulting in a fast TAT. The assay itself requires only a qPCR instrument, and the reagents are relatively inexpensive. This ease and low cost have resulted in many labs creating their own laboratory-developed tests using this technology, and there are a number of FDA-approved kits available for purchase. As the TaqMan probe assay requires a large amount of genomic DNA per reaction, the number of different probes tested or other diagnostic procedures possible for the sample may be limited.

Having the lowest limit of detection and highest specificity, the Therascreen system from Qiagen combines both of the above methods. A mutation-specific primer is used to amplify the mutated region, and then a Scorpion probe, containing fluorescent and quencher moieties, again binds to this same site. As elongation occurs, the epitopes are separated, and fluorescence occurs. FDA-approved Therascreen kits are available for both EGFR and KRAS genes. However, these assays look only at a limited number of mutations. The EGFR assay can detect 5 point mutations, exon 19 deletions, and exon 20 insertions; the KRAS kit can detect 7 point mutations. The largest flaw of these allele-specific PCR assays is their propensity for false-negatives due to large mutations. The primers and probes are designed to detect single nucleotide mutations and anything greater than that will cause a mismatch and prevent hybridization. A 1% rate of false negatives has been reported for the FDA-approved Therascreen KRAS assay [22]. These assays are promoted as companion assays for therapeutics, such as the BRAF assay from Roche, which is the companion diagnostic assay for therapy with BRAF inhibitor vemurafenib. The test only detects the c.1799T>A p.V600E mutation, though up to 20% of BRAF mutations are non-V600E mutations [23].

3.2.3. Sanger sequencing

The first widely used sequencing method, Sanger sequencing employs PCR in the presence of unlabeled nucleotides and labeled (i.e., radio- or fluorescent labels) chain terminating nucleotides [24]. Since each form of nucleotide (A, T, C, G) is differentially labeled, incorporation of chain terminating nucleotides stops the elongation step and labels the product, indicating the identity of the final nucleotide added (**Figure 3A**). The sample is then subjected to electrophoresis, either gel or capillary, which separates the amplicons by size, allowing the sequence to be read from smallest to largest simply by the label present.

3.2.4. Pyrosequencing

Where Sanger sequencing uses chain termination followed by electrophoresis, pyrosequencing uses a real-time process of sequencing by synthesis [25]. Here, the four possible nucleotides are added and removed sequentially. When the proper nucleotide is added, elongation occurs, releasing pyrophosphate that is converted to light through bioluminescence (**Figure 3B**). The template DNA is immobilized through binding to a biotinylated primer bound to sepharose beads. The technique provides semiquantitative data in regard to prevalence of the mutant allele in the sample [26]. As each template strand is elongated to completion, smaller sample quantities are needed, generally only requiring 10 ng of DNA. For a number of reasons, read lengths obtained with pyrosequencing are not as long as those from Sanger sequencing, generally being well below one hundred bases [27]. This prevents its use for sequencing large-scale mutations. Due to the fact that the technique requires a pre-PCR step and manual analysis of the results, it is time intensive. However, the method is routinely used clinically, both as laboratory-developed tests [28] and commercial kits [29] for analysis of a number of forms of cancer, including lung cancer. Its much lower limit of detection of 5–10% sets it apart from Sanger sequencing and makes it an optimal assay to confirm NGS variants occurring at low variant allele frequency.

Figure 3. Sequencing technologies. (A) Sanger sequencing consists of PCR of template DNA in the presence of standard and fluorescently labeled chain terminating nucleotides. If a chain terminating nucleotide is incorporated into the strand, it is permanently labeled with a tag that identifies the final nucleotide added. Following PCR, fragments are separated by size, and the sequence is read from smallest to largest strand. (B) Pyrosequencing utilizes a sequencing-by-synthesis approach during PCR. Nucleotides (A, T, C, G) are added sequentially and then removed. When the correct nucleotide is added, elongation occurs, and pyrophosphate is released, resulting in a luciferase reaction and the emission of light.

3.2.5. Next-generation sequencing

If one focuses on detection of only small mutations in a limited number of genes, single-analyte assays will deliver reliable and informative results. However, running mutation assays aiming to capture mutations in all 15 driver mutation-prone genes requires a multianalyte assay, which is why the current direction of the sequencing field is to multiplex. While different technologies that would all be considered next-generation sequencing (NGS) utilize very different sequencing methods, the similarity is that they are able to sequence incredibly large numbers of DNA strands at the same time. This massively parallel sequencing is capable of providing millions or billions of short reads. This ability lends NGS to a wide range of applications including untargeted sequencing of entire genomes, exomes, or transcriptomes, as well as targeted sequencing of large numbers of cancer mutation locations at the same time. The NGS instrument market space is currently occupied by four competitors utilizing slightly different approaches. By far, the largest player is currently Illumina, who offers a wide range of instruments ranging, among others, from the miniSeq to the HiSeq X Ten, the latter of which is capable of producing up to six billion reads. A small range instrument, the MiSeqDx, is FDA approved for *in vitro* diagnostic use. These instruments use chemistry that is a million-wise multiplexed variant of Sanger sequencing with clonal amplification and sequencing by synthesis chemistry. The color of the signal detected when a nucleotide is added indicates the identity of the base added and thus the sequence. Currently, the only other company with a significant market share is ThermoFisher with their Ion Torrent instruments. Similar to traditional pyrosequencing, the Ion Torrent technology sequentially adds and removes

nucleotides looking for elongation. Instead of converting pyrophosphate to fluorescence, Ion Torrent measures small-scale changes in pH caused by the release of H⁺ during the polymerization of nucleotides [30]. Similarly, their Ion PGM™ Dx System is FDA approved. While both Pacific Biosciences and Qiagen have offerings for NGS, they are not currently widely used. The Pacific Biosciences instruments suffer from low throughput and high cost, while Qiagen only recently entered the market.

3.2.5.1. *Clinical NGS workflow*

Most NGS runs are conducted on either genomic DNA or mRNA. While formalin-fixed and paraffin-embedded tumor blocks contain suitable quality genomic DNA, the quality of mRNA obtained from these samples will be variable and yield unsatisfactory results in up to 15% of cases. Fresh tissue is required to ensure high-quality mRNA for RNASeq experiments. Prior to beginning the workup, the tumor cell content of the sample must be determined. This requires an experienced and knowledgeable set of eyes. Between 5 and 20% tumor cell content is generally sufficient for NGS [31]. After isolation, DNA is sheared to produce uniform fragments which then undergo multiplex PCR or are hybridized to immobilized probes. The labor required for NGS is dependent on the level of automation available in the lab carrying out the sequencing. Illumina requires 1–2 days of workup, while Ion Torrent requires up to 3 days, although the Ion Torrent instrument has shorter run times. This means that individual labs need to identify their rate-limiting step in order to choose the instrument that will provide the highest throughput and shortest TAT.

3.2.5.2. *Targeted sequencing*

While NGS has great potential, its cost and massive data generation currently limit its clinical use to targeted sequencing of mutation hotspots rather than genome, exome, or transcriptome sequencing. Disease-specific gene panels are routinely used. The genes are chosen based on their immediate impact on patient care, either due to their use in determining prognosis or in selecting the proper treatment regimen. Sequencing of genes beyond the minimum is determined on a case-by-case basis according to the capability and throughput capacity of the laboratory as well as insurance coverage of the patient. Library preparation kits for NGS generally fall into two categories: those that use PCR amplification or hybridization probes. The first class is typified by Illumina's TruSeq Amplicon Cancer Panel, the Ion AmpliSeq panel from Ion Torrent, and HaloPlex kits from Agilent. The second group contains kits available from sequencing instrument manufactures and also from Agilent, Roche, and Integrated DNA Technologies, along with other third parties. Direct hybridization gives more reliable quantitative data since the possibility of bias introduced by the pre-PCR step is eliminated. These reagents can include probes for intronic regions rearranged in translocations which are detectable in this way [32], though larger rearrangements may lead to poor hybridization and false negatives [33] or misleading copy number data. The internal tandem duplications in the *FLT3* gene in acute myeloid leukemia [34] are an excellent example of mutations that require a special reagent and bioinformatics approach. Generally, with adequate expertise, wet lab and bioinformatics support labs are capable of developing their own lab-developed assays

as evidenced by cancer panels developed by Foundation Medicine (Foundation One Assay) [35], Memorial Sloan Kettering (Integrated Mutation Profiling of Actionable Cancer Targets) [36, 37], and many others.

3.2.5.3. *Whole exome sequencing*

While assays for sequencing the entire exome are available, they are not widely used in the clinical setting. As each run is capable of producing a set number of reads, sequencing a greater number of loci reduces the number of reads for each locus. Mutations in a highly heterogeneous sample may not be detected. Exome sequencing may identify rare or previously unidentified mutations, though clinicians would likely not have the knowledge on how to use that information to better care for their patient, so it would have no impact on patient care. Therefore, large-scale exome sequencing has found a better home in the research laboratory than the clinical lab setting.

3.2.5.4. *Bioinformatics*

Perhaps the largest hurdle to the adoption of NGS is data analysis. When an instrument is providing billions of reads, it can be a challenge not only to store the data, but also to utilize them. Instrument manufacturers have greatly improved their software offerings, making it easier to view the data. However, the challenge still remains of how to use the data. With so much information at your fingertips, how do you best utilize it to better care for the patient? Without a meaningful approach, substantial time may be devoted to analyzing irrelevant data. The significance of identified mutations must be denoted, and their impact on treatment must be delineated. A number of companies have begun offering services that would annotate a mutation report with clinically relevant content, including N-of-One, Genome Oncology, and PireanDx. Once the report is generated, commonly outdated electronic medical records systems often make the sharing of the results difficult. Due to file incompatibilities, highly complex documents are all too often printed, scanned, and uploaded as PDF documents. This has prompted data to be stored in third-party systems that greatly enhance the user experience [37].

3.2.5.5. *Reimbursement and government regulation*

In addition to the difficult science, the advancement of NGS in the United States has been hampered by reduced reimbursement rates for molecular pathology testing. Reimbursement is now only available for the test itself, not the interpretation of the results. Additionally, the testing classification has been greatly simplified, failing to differentiate between similar tests that may have very disparate costs while not being redundant [38]. Diagnostic procedures that are conducted in clinical laboratories are regulated by the federal Clinical Laboratory Improvement Amendments (CLIA) that were established in the 1970s. These regulations are administered by federal agencies and by the states, which require laboratories to be properly licensed. Additionally, the College of American Pathologists [39], Association for Molecular Pathology [40], and the American College of Medical Genetics [41] all help to draft

technique-specific regulations and offer certification programs of their own and require proficiency testing.

To date, most NGS tests have been developed by individual laboratories, over which the Food and Drug Administration (FDA) has no explicit regulatory oversight. The FDA has warned that NGS technologies, as with older technologies, will be subject to FDA regulation if they advance toward marketable *in vitro* diagnostic status, over which the FDA exerts considerable control. This is generally established on a case-by-case basis. Regulation by all of these bodies is complicated by the fact that NGS is continually evolving and improving. Guidelines must either be routinely revisited or generalized such that their interpretation may change to match the current technology. A fine line must be walked in order to protect the patients for whom the assays are being used, while at the same time not stifling the development of a technology that promises to usher in the era of personalized medicine.

4. Emerging technologies

4.1. Single-analyte vs multianalyte assays

While NGS will likely become the dominant technique for evaluating mutations in cancer moving forward, the other methods discussed here are hardly headed toward imminent obsolescence. Clinical labs must focus not only on the future, but also providing the best diagnostics for current patients. Today's limitations of NGS, including substantial labor requirements and a long TAT, mean that single-analyte methods are still widely used. While NGS may be able to provide a greater quantity of data and increased sample throughput, it has not been found to be qualitatively better than other technologies including allele-specific PCR and Sanger sequencing, among others, with all providing around 96% agreement [42, 43].

4.2. Liquid biopsy

Direct testing of tumor biopsies is preferred; however, circulating tumor DNA may be used to test for mutations after a lung adenocarcinoma diagnosis through direct biopsy has been established. For solid tumors, mutation detection has traditionally required a direct biopsy. In recent years, research has been conducted on the presence of circulating tumor cells, excreted miRNAs, and even tumor genomic DNA floating freely in the bloodstream. The ease of obtaining a blood sample makes sequencing these samples an appealing future technique not only for tumor characterization, but also screening and evaluation of therapeutic efficacy during treatment. The ratio of mutant DNA to normal DNA or tumor to normal cells collected is often very low and seems to be partially dependent on the tumor type, being detectable in greater than 75% of patients with breast, colorectal, and hepatocellular cancers, among others, but in less than half of patients with cancer of the brain, kidney, or prostate [44, 45]. Circulating tumor cells were detected in 78% of small-cell lung cancer patients [46], and both circulating DNA [47] and tumor cells [48] have been successfully used to screen for EGFR mutations in lung cancer. These technologies have not progressed to the point of widespread clinical use and are currently at the research stage. They are more thoroughly reviewed in Ref. [49].

4.3. Future sequencing technologies

Even with NGS technologies still not up to full speed, even better techniques are already being developed. A number of these sequencing instruments are able to decipher the sequence of single, existing DNA strands directly, rather than being based on PCR synthesis [50]. Perhaps showing the most promise, Oxford nanopore technologies has developed a new instrument, the MinION. By passing individual strands through nanopores containing ionic currents, the instrument is able to detect minute changes in current that differ depending on which nucleotide is passing by. The device is small enough to easily hold in the palm of the hand. It is capable of 60 kb read lengths, with 16,000 reads per run [51, 52]. While the portability and capabilities offer amazing promise, the accuracy is currently not sufficient for clinical use. It is gaining significant traction in the research space, however [53].

5. Conclusions

Clinical mutation testing in lung cancer is driven by the available therapies. Currently, there is only a limited armoire of targeted therapy options available which can be employed. If there is not a drug targeting a specific mutated gene available, the fact that the gene shows a mutation is not very meaningful. EGFR-targeted therapeutics may not be used without the presence of EGFR mutations. If the tumor continues to progress after treatment with an EGFR inhibitor, the presence of a resistance-conferring EGFR T790M mutation must be determined prior to giving a third-generation EGFR inhibitor to overcome the resistance. Similarly, ALK inhibitors are only to be given to patients showing ALK rearrangements; however, if treatment fails, there is no need to re-evaluate mutational status. ROS1 inhibitors receive a similar recommendation, while BRAF, RET, ERBB2, KRAS, and MET screenings are only recommended if included as a larger panel or if EGFR, ALK, and ROS1 tests come back negative.

Physicians and laboratories currently have a large number of techniques at their disposal for the interrogation of mutations present in lung cancer. The tests to be performed can be tailored to each patient based on a number of factors including type and number of data points needed, TAT, cost, and availability of quality biopsy material. The absolutely minimal mutation workup includes detection of SNVs and indels in EGFR by Sanger sequencing, pyrosequencing, allele-specific PCR or any other method, and detection of ALK translocations by FISH. However, NGS has the potential to replace many, if not all, of these techniques, but must first continue to improve. Its use in the clinic is currently limited by the fact that it is still quite expensive and has a relatively long TAT, and the results require special skills and reference databases in order to be fully utilized. In the future, it is the hope that every lung cancer patient may have their entire tumor genome sequenced cheaply and efficiently, allowing an in-depth understanding of all mutations driving the tumor and providing information to tailor the best possible treatment for that patient.

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