The Genomic Era of Clinical Oncology: Integrated Genomic Analysis for Precision Cancer Care

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Abstract

Genomic alterations are important biological markers for cancer diagnosis and prognosis, disease classification, risk stratification, and treatment selection. Chromosomal microarray analysis (CMA) and next-generation sequencing (NGS) technologies are superb new tools for evaluating cancer genomes. These state-of-the-art technologies offer high-throughput, highly accurate, targeted and whole-genome evaluation of genomic alterations in tumor tissues. The application of CMA and NGS technologies in cancer research has generated a wealth of useful information about the landscape of genomic alterations in cancer and their implications in cancer care. As the knowledge base in cancer genomics and genome biology grows, the focus of research is now shifting toward the clinical applications of these technologies to improve patient care. Although not yet standard of care in cancer, there is an increasing interest among the cancer genomics communities in applying these new tech-

nologies to cancer diagnosis in the Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories. Many clinical laboratories have already started adopting these technologies for cancer genomic analysis. We anticipate that CMA and NGS will soon become the major diagnostic means for cancer genomic analysis to meet the increasing demands of precision cancer care.

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Cancer is a genomic disorder resulting from cellular accumulation of genomic alterations. These genomic alterations include: single-nucleotide variants (SNVs) leading to missense or nonsense amino acid change, or a splice site substitution affecting normal RNA splicing; small deletions, duplications, insertions, or a combination of deletion(s) and insertion(s) known as indels; copy number variants (CNVs), and structural rearrangements such as deletions, duplications, inversions, or translocations resulting in novel fusion genes. Many of these cancer-associated alterations can serve as biomarkers that can be used to assist cancer diagnosis and prognosis, stratify patient risk, predict sensitivity/resistance to a specific therapy, and monitor treatment response or residual

diseases. Several high-throughput molecular technologies have been developed to identify these alterations including chromosomal microarray analysis (CMA) and next-generation sequencing (NGS) technologies. Microarray-based technology has revolutionized modern cytogenetics, allowing interrogation of the human genome for CNVs with unprecedented resolution. This technique has accelerated our capability to discover and characterize critical genomic copy number alterations or CNVs that cause and/or promote tumorigenesis [Rusk and Kiermer, 2008; Li and Andersson, 2009; Armengol et al., 2010; Stephens et al., 2011; Shyr and Liu, 2013]. NGS technologies have transformed molecular genomic analysis by increasing the sequencing throughput and decreasing the cost by several orders of magnitude. Application of these state-of-the-art technologies in cancer research has revealed a wide spectrum of genomic alterations in cancer and uncovered a broad range of phenotypic expression of specific mutations and mutation signatures. These discoveries have raised new questions about our understanding of the complexity of the cancer genome and modified our view of the genomic contribution to human cancer [Alexandrov et al., 2013; Peterson et al., 2015]. Clinical application of these technologies marks the emergence of a new era of genomic medicine and is driving clinical oncology towards precision cancer care. This review provides an introduction to microarray and NGS technologies, including different microarray/NGS platforms, and the clinical utility of these modern technologies in precision oncology.

The Technologies

Chromosome Microarray Technologies

Two major microarray-based platforms are currently used in clinical cytogenetics: microarray-based comparative genomic hybridization (aCGH) and single-nucleotide polymorphism (SNP)-based arrays [Li and Andersson, 2009; Li et al., 2012]. aCGH, which directly measures genomic copy number differences between the patient DNA and a known normal reference DNA, allows the construction of a high-resolution map of genome-wide copy number alterations. Commonly used aCGH arrays contain thousands of in situ-synthesized oligonucleotide probes. These probes may either be enriched for specific disease-associated genes or genomic regions, or distributed relatively evenly across the whole genome. The flexibility of aCGH design allows researchers and clinical laboratories to create customized arrays for their research

and clinical diagnostic needs, such as cancer-specific aCGH arrays with enriched probe coverage for cancer genes and aCGH arrays designed to detect CNVs of particular NGS panels. SNP-based arrays probe thousands of SNPs and provide both copy number and genotype data; the latter can be used to study copy number neutral genomic alterations, such as uniparental disomy and loss of heterozygosity (LOH). Although SNP-based arrays have the advantage of revealing genotype information, they offer a poor representation of genomic regions with low SNP incidences (SNP deserts). Additionally, SNP-based platforms do not use an intra-experimental control; rather, they compare patient data to a pre-established laboratory standard. Though the underlying technology and the design of the probes are substantially different among different microarray platforms, studies have shown a high level of concordance between different platforms [Li et al., 2015]. As microarray-based technologies continue to improve, many new platforms are being developed. These new platforms provide a combination of high probe density and optimal probe distribution across the genome (including SNP deserts), allowing detection of CNVs, absence of heterozygosity, and LOH. A comparison of the 2 major microarray platforms and the newly developed platforms using combined technologies is summarized in Table 1.

Next-Generation Sequencing Technology NGS Platforms

Multiple NGS sequencing platforms are commercially available, including Illumina HiSeq, MiSeq, and NextSeq systems (Illumina, San Diego, CA, USA), ThermoFisher PGM, Proton, Ion S5 systems (ThermoFisher, Waltham, MA, USA), and Pacific Biosciences PacBio RS II system (Pacific Biosciences, Menlo Park, CA, USA). Illumina platforms use reversible dye terminator sequencing-by-synthesis chemistry involving iterative cycles of single base incorporation, imaging, and cleavage of the terminator chemistry. Ion Torrent (ThermoFisher) systems use different Torrent chips that function as ultrasensitive pH meters capable of detecting hydrogen ions released when nucleotides are incorporated during DNA synthesis. PacBio systems use single molecule real-time sequencing technology that allows monitoring of the activity of a single DNA polymerase enzyme at a single molecule of single-stranded DNA template in real time during DNA sequencing. Illumina systems generate the highest sequencing output, Ion Torrent systems require the least amount of input DNA, and the PacBio system provides longest reads. Each sequencing technology has its inherent ad-

Table 1. Comparison of aCGH and SNP-based arrays

	aCGH array	SNP-based arrays	aCGH + SNP array	SNP-based array + copy number probes
Reference	intra-experimental	extra-experimental	intra-experimental	extra-experimental
Probes	BAC/PAC, oligos	oligos	oligos	oligos
SNP genotyping	no	yes	yes	yes
SNP desert coverage	yes	no	yes	yes
UPD detection	no	yes	yes	yes
LOH/AOH detection	no	yes	yes	yes

aCGH, array comparative genomic hybridization; AOH, absence of heterozygosity; LOH, loss of heterozygosity; oligos, oligonucleotide probes; UPD, uniparental disomy.

vantages and weaknesses; clinical laboratories should choose the platform that best fits their needs. Extensive reviews about the technical details of the major NGS platforms have been published elsewhere [Metzker, 2010; Chang et al., 2015; Luthra et al., 2015]. The most commonly used NGS platforms in clinical laboratories are Illumina MiSeq, HiSeq, and NextSeq 500 as well as ThermoFisher PGM and Proton.

Targeted Enrichment Methods

In whole-exome sequencing (WES) and gene panel sequencing, the region of interest (ROI) for sequencing needs to be captured and enriched. There are 2 major groups of technologies currently available for targeted enrichment: amplification-based technology and hybridization-based technology (Table 2).

Amplification-Based Enrichment Methods. Amplification-based enrichment methods include multiplex PCR and single-plex PCR approaches. Multiplex PCR-based targeted enrichment is well represented by Ion AmpliSeq technology (ThermoFisher). Ion AmpliSeq uses a proprietary ultra-high multiplex PCR technology to generate thousands of amplicons in a single PCR tube for massively parallel sequencing [Chang and Li, 2013]. Multiplex PCR-based targeted enrichment requires significantly less nucleotide input than hybridization-based enrichment does; however, interactions between different primers may lead to amplification of undesired fragments and decrease the specificity of PCR products [Turner et al., 2009; Haas et al., 2014]. Single-plex PCR-based enrichment approaches include Microdroplet PCR (RainDance Technologies Inc., Billerica, MA, USA) and the Access Array System (Fluidigm, San Francisco, CA, USA). Microdroplet PCR technology utilizes picoliter-sized droplets as individual reaction vessels to per-

form over one million unique PCR reactions per sample in less than one day [Tewhey et al., 2009]. The Access Array System performs several thousands of individual reactions in separate chambers inside a microfluidic chip [Mertes et al., 2011; Moonsamy et al., 2013]. Single-plex PCR-based enrichment approaches consume significantly more DNA or RNA but are particularly suitable for sequencing small numbers of genes in a large number of samples.

Hybridization-Based Enrichment Methods. In hybridization-based enrichment approaches, DNA templates are sheared using nebulization, sonication, or random enzymatic digestion techniques prior to hybridization [Mardis, 2013]. Among the fragmentation methods, enzymatic fragmentation shows the highest consistency but performed slightly worse than sonication and nebulization with regard to the identification of insertions/deletions [Knierim et al., 2011]. However, nebulization and sonication methods show little genomic bias compared to enzymatic fragmentation. After fragmentation, ROIs are captured by predesigned oligonucleotides (known as probes) complementary to the ROIs through hybridization. Hybridization may be performed using an array-based hybridization method, in which high-density oligonucleotide arrays containing probes complementary to the ROIs are used to capture DNA molecules of interest [Okou et al., 2007] or a solution-based method, in which biotinylated probes complementary to the ROIs are used to bind targets, which are then purified using streptavidin-labeled magnetic beads [Gnirke et al., 2009]. Although array-based hybridization methods show similar performance to solution-based hybridization methods, the latter is much more commonly used due to the simplicity of the technology and much lower DNA input [Mamanova et al., 2010].

Table 2. Comparison of commonly used targeted enrichment approaches of different platforms

	Company	Platforms	Enrichment approach	Available kits/panels	Nucleotide input	Duration time	FFPE compatibility
Targeted DNA sequencing	ThermoFisher Scientific	Ion AmpliSeq	multiplex PCR	Cancer HotSpot Panel v2; Comprehensive Cancer Panel; Inherited Disease Panel; Sample ID Panel	5 ng–1 µg	2.5–6 h	yes
	Illumina	Nextera; TruSight; TruSeq	hybridization or multiplex PCR	TruSight Cancer; TruSight Tumor; TruSeq Custom Amplicon v1.5; TruSeq Amplicon	10 ng–2 µg	1.5 h–2 d	yes
	Agilent	SureSelect; Haloplex	hybridization	XT Reagent Kits; QXT Reagent Kits; XT2 Reagent Kits; HaloPlexHS Kits; HaloPlex Prepack Reagents	50 ng–3 µg	6 h–2 d	yes
	ArcherDX	VariantPlex	multiplex PCR	p53 Panel; Solid Tumor Panel; CTL Panel	25 ng–200 ng	6 h	yes
	RainDance	ThunderStorm; ThunderBolts	single-plex PCR	ThunderBolts Cancer Panel; Thunderbolts Myeloid Panel	10 ng	9 h	yes
	Fluidigm	Access Array	single-plex PCR	BRCA1/BRCA2/TP53 Target-Specific Panel; EGFR/MET Target-Specific Panel	50 ng	5 h	N/A
Targeted RNA sequencing	ThermoFisher Scientific	Ion AmpliSeq	multiplex PCR	RNA Cancer Panel; RNA Fusion Lung Cancer Research Panel; RNA Apoptosis Panel	500 pg–5 ng	4–5 h	yes
	ArcherDX	FUSIONPlex	multiplex PCR	Oncology Research Panel; Lung Thyroid Panel; Solid Tumor Panel; ALK, RET, ROS1 Panel; Heme Panel; Sarcoma Panel; NTRK Panel; FGFR Panel; CTL Panel	25 ng–200 ng	8 h	yes
	Illumina	TruSeq RNA	hybridization	Apoptosis Panel; Apoptosis Panel; Cardiotoxicity Panel; Cell Cycle Panel; p53 Panel; Wnt Panel; Stem Cell Panel; Cytochrome p450 Panel; Hedgehog Panel	50 ng	1.5 d	yes
	Agilent	SureSelect RNA	hybridization	Strand Specific RNA Library; XT RNA Reagent Kit; RNA Capture	>50 ng	N/A	yes

d, day; h, hour; N/A, not applicable.

Clinical Application of CMA and NGS Technologies

The Role of CMA in Cancer Genomics

Compared to the conventional cytogenetic analysis, the major advantages of microarray methodologies include: assessing the whole genome for CNVs at much higher resolution, being independent of cell division allowing copy number analysis on frozen and formalin-fixed, paraffin-embedded tissues, and providing genotype information for the detection of LOH [Gijsbers and Ruivenkamp, 2011; Kudesia et al., 2014; Li et al., 2015].

Many clinical laboratories have implemented CMA as an adjunct test for cancer diagnosis and have demonstrated the clinical utility of CMA in cancer patient management.

Clinical Application of CMA in Cancer Diagnosis and Prognosis

One of the major advantages of microarray technologies is high-resolution data which enables the discovery of CNVs characteristic for different types or even subtypes of cancers. The CNV data can be essential for cancer diagnosis and prognosis. For example, deletions of the

lymphoid transcription factor gene *IKZF1* (encoding Ikaros) are a hallmark of both BCR-ABL1-positive acute lymphoblastic leukemia (ALL) and Ph-like ALL, both of which are associated with poor outcomes in pediatric patients [Den Boer et al., 2009; Mullighan et al., 2009]. These deletions often involve only the *IKZF1* gene and can only be detected by CMA.

In addition to deletions or duplications of genes, CNVs may lead to novel fusion genes associated with specific types of cancer. For example, a small interstitial deletion in the pseudoautosomal region 1 of chromosomes X and Y juxtaposes the first noncoding exon of *P2RY8* to the coding region of *CRLF2* and results in a novel *P2RY8-CRLF2* fusion and *CRLF2* overexpression [Mullighan et al., 2009]. Importantly, elevated *CRLF2* expression correlates with poor outcomes in high-risk B-ALL patients and identifies 24–60% of patients with Ph-like ALL [Roberts et al., 2014]. Notably, CMA can detect this cryptic deletion and provide genomic information critical for patient management. Similarly, in brain tumors, the most common genetic alteration identified in low-grade gliomas is an approximately 2-Mb tandem duplication at 7q34 resulting in the *KIAA1549-BRAF* fusion [Roth et al., 2014]. Due to the size and the nature of the aberration, this fusion cannot be detected by conventional cytogenetics or FISH. However, it can be easily identified by CMA, providing genomic evidence of low-grade glioma without performing additional RNA-based testing (which is not always feasible).

CMA can provide genome-wide CNV profiles characteristic to specific tumors. Medulloblastoma represents an example of such application. Medulloblastoma, the most common pediatric malignant primary brain tumor, is a group of heterogeneous diseases. Based on the CNV profiles generated by CMA, these tumors can be classified into 4 subgroups termed WNT (wingless-type mouse mammary tumor virus), SHH (sonic hedgehog), group 3, and group 4 [Taylor et al., 2012]. While partial or complete monosomy 6 is characteristic of the WNT subgroup, loss of 9q along with gain of 3q and loss of 10q are frequently associated with the SHH subgroup. Loss of 17p with or without concomitant 17q gain was most frequently found in group 3 and group 4; however, the 2 groups differ in the frequency of these abnormalities with *MYC* amplification unique for group 3 [Taylor et al., 2012]. The significance of this CMA-based classification is important because the prognosis of each group is different. Studies have shown that WNT tumors had by far the best outcome with a 5- and 10-year overall survival of 95% in children ($n = 39$) and a 5-year overall survival of 100% in

adults ($n = 5$). The worst outcome in all age categories was seen for patients with group 3 tumors with 5- and 10-year overall survival of 45% and 39%, respectively in infants and 58% and 50% in children [Kool et al., 2012; Ramaswamy et al., 2016].

Another prime example that elucidates the role of CMA in cancer diagnosis is renal cell carcinoma (RCC). There are 4 common histologic subtypes of adult RCC: clear cell RCC, papillary RCC, chromophobe RCC, and oncocytoma. Each subtype is characterized by a specific CNV pattern. Almost all clear cell RCCs show a deletion of 3p including the *VHL* gene. Papillary RCCs typically display gains of 7 and 17 and a loss of Y. Hypodiploidy with a loss of multiple chromosomes including 1, 2, 6, 10, 13, 17, 21, and the X or Y is characteristic of chromophobe RCCs. Benign oncocytomas usually have a normal genetic profile but may show a loss of 1p and/or Y chromosome. CMA has demonstrated significant clinical utility for the diagnosis of RCCs, especially in morphologically challenging cases [Li et al., 2015]. CMA results are so important in these cases, that discrepant microarray results have prompted further morphological review with ancillary studies, resulting in an amendment of the final diagnosis in approximately 29% of the cases [Hamilton et al., 2015].

In addition to providing CNV data, microarrays with SNP probes offer genotype information which allows for the detection of LOH. In certain cancers, this genotype information is critical in disease differentiation and treatment selection. In the cases of ALL, the high-hyperdiploid subtype accounts for 20–30% of childhood ALL and is usually associated with a good prognosis. Patients with high-hyperdiploid ALL have clones of 51–68 chromosomes, though rarely identical, with extra copies of chromosomes 4, 6, 10, 14, 18, and 20. In contrast, hypodiploid ALL is a rare subtype of ALL which includes near-haploid ALL with 24–31 chromosomes, low-hypodiploid ALL with 32–39 chromosomes, and high-hypodiploid ALL with 40–43 chromosomes [Raimondi, 2012]. Unlike hyperdiploid ALL, hypodiploid ALL is associated with poor clinical outcome. The hypodiploid genome may undergo endoduplication resulting in a hyperdiploid karyotype – this is known as masked hypodiploid ALL. Unfortunately, the prognosis of masked hypodiploid ALL remains poor, which necessitates a method of identifying masked hypodiploid ALL in order to determine the therapeutic strategy for these patients. The genotype pattern afforded by microarrays with SNP probes can unequivocally distinguish masked hypodiploid ALL from true high-hyperdiploid ALL and thereby provide genomic evidence for treatment selection [Carroll et al., 2009].

Fig. 1. **A** aCGH shows 2 regions of genomic amplifications on chromosome 2p including the *MYCN* gene (arrow) in a patient with high-risk neuroblastoma. The copy number of the *MYCN* gene is estimated based on the $\log_2$ ratio of the probes in the amplified region. **B** A normal control.

In cancer cytogenetics, geneticists often encounter chromosomal material of uncertain origin that cannot be identified by its banding pattern, including marker chromosomes (free-standing chromosomal material of unknown origin), add chromosomes (chromosomal material of unknown origin attached to a recognizable chromosome), extrachromosomal double minutes (dmin; indicative of gene amplification), and intrachromosomal homogeneously staining regions (hsr; also indicative of gene amplification). Identification of the origin of these unknown materials may provide vital information for cancer diagnosis and prognosis. CMA not only reveals the origin and the content of the unknown material, but also estimates the copy number of the genes amplified (Fig. 1). *MYCN* amplification is a common phenomenon in neuroblastoma and often presents as dmin or hsr. *MYCN* gene amplification is the strongest independent adverse prognostic factor in neuroblastoma and is associated with rapid disease progression in patients of all ages and stages [Thompson et al., 2016]. Thus, it is critical to determine if *MYCN* is amplified using CMA for patient risk stratification. In addition to identifying *MYCN* amplification, CMA can simultaneously identify other CNVs critical for neuroblastoma risk stratification, such as the ploidy of the genome, presence or absence of 11q deletion and/or 17q duplication. Consensus has been reached that near-triploid tumors with only numerical gains are low or intermediate risk with excellent outcomes, while tumors with near-diploid/tetraploid and/or displaying

structural chromosome aberrations such as *MYCN* amplification, loss of 11p or gain of 17q are associated with high-risk and aggressive behavior [Pinto et al., 2015].

Another genomic alteration phenomenon that can only be visualized by CMA is chromoanagenesis represented by 2 one-step catastrophic events, chromothripsis and chromoanasythesis, in which complex genomic rearrangements are thought to be derived from a single catastrophic event rather than by several incremental steps [Salaverria et al., 2015; Forero-Castro et al., 2016; Ortega et al., 2016]. Poor disease outcome has been associated with patients with chromoanagenesis-associated cancers [Molenaar et al., 2012].

Clinical Application of CMA in Cancer Treatment

While CMA has produced a wealth of useful information for cancer diagnosis and prognosis, CMA may also provide genomic information useful for formulating cancer treatment strategies. CMA can assist cancer risk stratification avoiding over- or undertreatment during the course of disease. Certain CNVs may serve as biomarkers for specific targeted therapies.

Significant improvements in the treatment of tumors have been achieved by risk-directed therapy. Clinically relevant CNVs can be used to stratify patients into different disease risk groups for risk-directed therapy. As an example, intrachromosomal amplification of chromosome 21 (iAMP21), almost always involving the *RUNX1* gene, defines a distinct cytogenetic subgroup of childhood B-cell precursor ALL. The definition of iAMP21 is the presence of 3 or more additional copies of *RUNX1* alleles on one chromosome 21. Patients with iAMP21 ALL have a high relapse rate when treated as standard risk. However, recent studies have shown that intensified therapy can significantly reduce the risk of relapse in iAMP21 patients [Heerema et al., 2013; Harrison, 2015]. SNP-based arrays can distinguish the duplication of 2 homologous alleles from triplication of one allele when FISH demonstrates the presence of 4 copies of *RUNX1*, and therefore, identifying iAMP21 ALL in time for intensified therapy (Fig. 2).

Although relatively rare, there are a few CNVs that are targetable either directly or indirectly with United States Food and Drug Administration (FDA) approved drugs or investigational therapies in the context of specific cancer types. These actionable or potentially actionable genomic alterations often include gene amplifications containing well-characterized oncogenes such as *MYCN*, *ERBB2*, *ALK*, etc. or deletions including certain tumor suppressor genes [Meric-Bernstam et al., 2015]. Identification of these CNVs, together with additional genomic informa-

Fig. 2. SNP array displays an iAMP21 in a patient with ALL. **A** FISH with ETV6 (green) and RUNX1 (red) probes shows 4 copies of the *RUNX1* gene on chromosome 21. **B** SNP array B-allele frequency plot of chromosome 21q demonstrates one normal allele that is either A or B genotype and an abnormal allele that is triplicated leading to either AAA or BBB genotype. **C** SNP array log *R* ratio plot of the same region shows copy number variations of chromosome 21 including the regions of chromothripsis.

tion revealed by other high-throughput technologies, has created an opportunity for delivering genomically informed personalized therapy. Clinical trials based on tumor CNV profiles have accelerated the emergence of novel therapeutic strategies. *ERBB2* (also known as *HER-2/neu*) amplification has been observed in 15–20% of patients with breast cancer and is associated with poor clinical outcome [Slamon et al., 1987]. The clinical course of *HER-2/neu*-positive disease was fundamentally altered upon the release of the FDA approved first-generation anti-*HER-2/neu* antibody, trastuzumab, for metastatic breast cancer in 1998, and the natural history of this breast cancer subtype was forever changed with the adoption of combining trastuzumab with chemotherapy in 2005 [Perez et al., 2011]. Chromosome 17p deletion in patients with chronic lymphocytic leukemia (CLL) confers very poor prognosis when treated with standard chemo-immunotherapy. Venetoclax, an oral small-molecule BCL2 inhibitor, can induce CLL cell apoptosis. Previous studies of Venetoclax have shown an overall response to this drug in patients with relapsed or refractory CLL. In a recent phase II clinical trial aiming to assess the efficacy and safety of venetoclax monotherapy in patients with relapsed or refractory 17p deletion CLL, the results showed that venetoclax monotherapy is active and well tolerated in these patients [Stilgenbauer et al., 2016], which led to the FDA approval of venetoclax for CLL patients with 17p deletion (<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm495253.htm>, accessed June 28, 2016).

Discovery of Conditions Associated with Germline CNVs

It is important to realize that not all the CNVs identified in the tumor tissues are somatic changes related to the cancer. Clinical laboratories usually do not report all germline CNVs; however, CNVs involving tumor-related genes should be detailed in the report as they may predispose patients to certain cancers. A study performed on a cohort of individuals with different tumor-predisposing conditions by multiple assays including array analysis revealed that microdeletions involving 12 cancer genes, mostly tumor suppressors, accounted for 14% of the identified germline mutations [Smith et al., 2016]. This rate might be higher in pediatric patients with hereditary cancer syndromes. Therefore, CNVs detected in tumor tissue but suspected to be of germline origin based on their nature (genes involved and the level of mosaicism) should be confirmed by testing germline tissue if the family history, tumor type, or patient's medical history suggests [Schiffman, 2012] in conjunction with appropriate genetic counseling.

The Role of Next-Generation Sequencing in Cancer Genomics

NGS is a high-throughput genomic technique that has many advantages over traditional sequencing technologies, such as Sanger sequencing [Luthra et al., 2015]. NGS allows the sequencing of multiple genes and many genomic regions in parallel and provides genomic mutation

signatures of many genes associated with specific cancers. Furthermore, NGS is quite adept at detecting SNVs, indels, CNVs, LOH, and structural rearrangements, facilitating integrated genomic analysis. Additionally, NGS-based tests require only small amounts of input DNA/RNA and can tolerate low-quality DNA/RNA quite well. Therefore, these tests can be easily performed on different types of tissues including formalin-fixed, paraffin-embedded tissue. These features lead to significant time and cost savings with a lower tumor tissue requirement when compared to multiple parallel, stepwise or tiered testing algorithms [Luthra et al., 2015]. These features are critical in clinical oncology, where the material for testing is often limited and the turnaround time of testing is frequently constrained to just a few days. Unlike Sanger sequencing, NGS can sequence genomic ROI hundreds or thousands of times enabling the detection of low-level mosaicism. The detection limit is usually 5% or less in clinical samples and can be 1% or lower for follow-up samples for minimal residual disease. The high sensitivity of NGS allows the detection of subclones within a population of tumor cells; this is particularly important in patients who carry small clones of cells with mutations resistant to certain therapies.

There are multiple types of NGS tests: whole-genome sequencing (WGS), WES, transcriptome sequencing, and targeted gene panels. WGS and WES provide the broadest view of the cancer genome, and transcriptome sequencing is an unbiased examination of both coding and multiple forms of noncoding RNA for sequence variations and gene expression of the entire transcriptome. These tests are perfect for cancer research and discovery; however, they have limited clinical utility at this point of time due to the high cost and the difficulty in interpreting the vast amount of data within the clinical context of the patient's disease. Alternatively, targeted gene panels, which are designed to contain anywhere from approximately 3 to 100s of genes, are clinically feasible. The advantage of panel tests is that they can be designed for specific tumor categories (i.e., hematologic malignancies or solid tumors) and can contain hotspot mutations and/or whole gene coding regions. The disadvantage of targeted panels is that only variants in the ROI will be detected. Therefore, mutations, CNVs, and genotype data gleaned from targeted sequencing is limited to those areas covered by the assay. However, targeted panel testing has emerged to be the test of choice due to its fast turnaround time, low cost, and ease of data interpretation.

Additionally, a variation on the targeted NGS test can be used to sequence reverse-transcribed RNA (cDNA)

from tumor cells to detect fusion genes in an open-ended way [Zheng et al., 2014]. Sequencing from one end of a fusion transcript with a known fusion partner gene towards the other partner will result in sequencing reads from the unknown partner that can be aligned to the reference sequence to determine its origin. This allows for detection of promiscuous fusion partners to known genes (such as *EWSR1* and *KMT2A*, previously *MLL*). It also results in the discovery of new fusion partners, but the breakpoints in the known gene are limited to those exons covered by the assay design [Zheng et al., 2014; Beadling et al., 2016].

NGS Tumor Genome Profiling for Clinical Diagnosis and Prognostication

Since the discovery of t(9;22) in chronic myelogenous leukemia, it has been known that certain tumors contain recurrent genomic aberrations. Many of these genomic aberrations have been incorporated into the tumor classification guidelines set by the World Health Organization (WHO) and International Agency for Research on Cancer (IARC) via the WHO/IARC Classification of Tumors project along with histopathologic and immunohistochemical features (<http://whobluebooks.iarc.fr/>, accessed June 18, 2016).

One of the major advantages of NGS is its high throughput, sequencing multiple genes and identifying a variety of genomic alterations such as SNVs, indels, and CNVs at the same time. Various tumor types have been shown to express characteristic genome profiles or mutation signatures. Often, these mutation signatures cannot be distinguished by histomorphology alone, requiring genomic profiling for diagnosis, classification, and prognostication. For example, the International Society of Neuropathology Haarlem Brain Tumor Consensus Guidelines recommends the use of an "integrated" diagnosis that combines histomorphology and molecular criteria [Louis et al., 2014]. They give examples of particular tumors, such as gliomas, where molecular tests for the classification may include *IDH* mutations, *EGFR* gene amplification, *ATRX* expression, and 1p/19q deletion. These guidelines are similar to and modeled after the WHO classification of hematologic neoplasms, which includes genetic categories for narrowly defined disease entities [Swerdlow et al., 2008].

In particular for hematologic malignancies, multiplex NGS panels that allow for high efficiency, low cost, and short turnaround time are changing the landscape of diagnosis and prognosis. Being able to test for multiple gene variants with one sample both at diagnosis and over the

course of the patient's disease is helping to establish evidence-based diagnosis, monitor disease evolution, and shape prognosis and treatment strategies. One large category of leukemia that has benefited from NGS sequencing is intermediate (standard) risk cytogenetically normal acute myelogenous leukemia (CN-AML), which comprises approximately 50% of newly diagnosed AML. By analyzing a number of cancer gene mutations in these intermediate-risk tumors, patients can be further divided into those with better prognosis, delaying hematopoietic stem cell transplant (HSCT), and those with worse prognosis, necessitating earlier HSCT [Falini and Martelli, 2015]. The 3 most important prognostic markers in CN-AML at this time are *NPM1* mutations, *FLT3*-internal tandem duplication (ITD), and biallelic *CEBPA* mutations. *NPM1* mutations are very specific for AML, occurring in about 60% of de novo cases and are driver mutations with distinct gene expression and micro-RNA profiles [Falini and Martelli, 2015]. These mutations are stable over time and at relapse, making them desirable targets for minimal residual disease evaluation [Ivey et al., 2016]. *FLT3*-ITD occurs in about 30% of CN-AML and most commonly ranges in size from 15 to 300 bp. The ITD is sometimes challenging to detect by NGS due to its size and difficulty with alignment, but specific algorithms can increase its detection [Spencer et al., 2013]. Similar to *NPM1*-mutated CN-AML, *CEBPA* double mutations in CN-AML are associated with a distinct gene expression profile; however, biallelic *CEBPA* mutations are less common, comprising about 5% of CN-AML cases [Falini and Martelli, 2015]. *NPM1*-mutated CN-AML and biallelic-mutated *CEBPA* CN-AML both in the absence of *FLT3*-ITD mutation are considered favorable genetic risk categories, likely with survival similar to that of core-binding factor leukemia [Döhner et al., 2010; Falini and Martelli, 2015]. In contrast, the *FLT3*-ITD mutation confers a worse prognosis, routing patients towards earlier transplant and *FLT3* inhibitor therapy [Hu et al., 2014]. In addition to the mutations discussed here, CN-AMLs can harbor a number of other mutations in tumor suppressor genes, DNA-methylation genes, myeloid transcription factors, spliceosome-complex genes, and chromatin-modifiers, all of which are readily detectable by clinically validated NGS panels [Falini and Martelli, 2015]. As multi-gene prognostic models become available, NGS testing of CN-AML will become an increasingly efficient way to categorize, classify, and prognosticate disease [Grossmann et al., 2012; Patel et al., 2012].

Furthermore, NGS can identify CNVs and LOH within the same sequencing assay. For example, the Heme

Panel offered at the Children's Hospital of Philadelphia can detect *IKZF1* and *CDKN2A* deletions and *JAK2* mutations in a single assay for ALL patients (<https://apps.chop.edu/service/laboratories/olsd.cfm/olsd.cfm/laboratories/B261DC04-D1F0-B40D-47B7F4AD1DF1CA73>, accessed June 28, 2016), which reduces the need of running CMA on every single ALL patient and decreases the health care cost.

Fusion genes are the results of chromosomal rearrangements such as deletions, duplications, insertions, and translocations. Cancer-associated fusions are often cancer specific and significant in cancer diagnosis. Though some cancer-specific fusions can be detected by cytogenetics, such as the *RUNX1-RUNX1T1* fusion in AML caused by the translocation t(8;21)(q22;q22), many fusions are the results of cryptic deletions or duplications beyond the resolution of cytogenetics. A great example is the *KIAA1549-BRAF* fusion in low-grade glioma; not only is it not detected by conventional cytogenetics, it cannot be reliably identified by FISH either due to the close vicinity of the 2 genes or the duplication nature that leads to the fusion. Well-designed RT-PCR tests can detect known fusions, but they require the prior knowledge of both fusion partners and the breakpoints. Although CMA can detect submicroscopic deletions and duplications, it does not provide the exact breakpoints and the fusion sequence which are important for designing personalized RT-qPCR assays to monitor treatment response and minimal residual disease. Many genes may have multiple translocation partners, such as t(2;13)(q35;q14) or t(1;13)(p36;q14) in alveolar rhabdomyosarcoma and *KMT2A*-associated fusions in a variety of leukemias. Many known cancer translocations also have prognostic and therapeutic significance [Sorensen et al., 2002; Parker and Zhang, 2013]. Using reverse-transcribed RNA and advanced NGS techniques, most, if not all, of known or novel fusions can be detected by whole-transcriptome sequencing also known as RNA-seq or by a single multiplexed, unbiased assay using fresh or formalin-fixed tissue (Fig. 3) [Soda et al., 2007; Zheng et al., 2014; Beadling et al., 2016].

NGS Tumor Genome Profiling for Targeted Therapy

Tumors have a number of abnormal pathways that vary even within the same histologic tumor type. Testing tumors by NGS to determine which dysregulated pathway(s) can be targeted by specific drug therapy can improve patient outcomes and decrease cost. Preliminary data have shown that therapy decisions based on the result of NGS assays leads to increased median survival by

Fig. 3. A novel *NOTCH1-ROS1* fusion detected by the NGS analysis in a patient with angiosarcoma. The red arrow line indicates the location where the 2 genes fuse together.

nearly 1 year in lung cancer patients [Kris et al., 2014]. Furthermore, treating a patient with an accurate drug will prevent unwanted side effects from therapies that are unlikely to succeed [Gagan and Van Allen, 2015]. Finally, testing for tumor-associated mutations in a multiplex format, such as an NGS panel assay, is highly favorable because one can identify downstream pathway mutations that may confer resistance to a targeted therapy, such as concomitant *NRAS* and *BRAF* mutations leading to vemurafenib resistance in melanoma [Nazarian et al., 2010].

One of the best-studied solid tumors with recurrent targetable genomic changes is non-small cell lung cancer, in particular lung adenocarcinoma. The standard of care for patients with these tumors is to perform molecular testing for mutations in the *EGFR* gene to assess sensitivity to FDA-approved EGFR inhibitors [Dietel et al., 2015; Plönes et al., 2016]. This could be accomplished with low-throughput “hotspot” assays; however, a high-throughput NGS approach that can detect *EGFR* sensitizing and resistance mutations as well as mutations in other genes, such as *KRAS*, which may alter patient response to EGFR inhibitors, providing further clinical advantages. Additionally, detection of chromosomal rearrangements in *ALK* and *ROS1* genes as well as overexpression/increased copy number of the *MET* gene is critical because patients with these genomic alterations have shown significant response to ALK inhibitors [Keedy et al., 2011; Korpanty et

al., 2014; Plönes et al., 2016]. Overall, specific targeting of tumors with alterations in these genes leads to better patient outcomes and increased survival, especially in those patients with metastatic disease [Keedy et al., 2011; Korpanty et al., 2014]. All of these aberrations can be directly tested by NGS methods using a combination of DNA- and RNA-based panels for SNVs, indels, fusions, and CNVs. However, at this time only certain methods and products have been FDA approved as companion diagnostics for testing and certain results obtained by NGS methods may require orthogonal testing confirmation for treatment in the US [Dietel et al., 2015; Plönes et al., 2016].

In addition to single-gene targets, NGS multiplex panel testing can identify dysregulated pathways within a cancer that can be targeted by various agents. For example, myeloid malignancies typically have 5 classes of genetic aberrations: signaling pathway components, transcription factors, epigenetic regulators, tumor suppressors, and spliceosome components [Murati et al., 2012]. While individual gene variants may not be entirely reproducible between certain tumor types, many classes of tumors carry certain genetic profiles or signatures that are recurrent and can predict response to pathway inhibitors. Certain classes of mutations are seen together and vary between the myeloid malignancies in both children and adults [Murati et al., 2012; de Rooij et al., 2015]. NGS pan-

els that examine multiple genes with one sample in a multiplex format are hugely important to elucidate these complex pathways for an individual patient.

Clinical Trial Enrollment

As therapies rapidly evolve and change, NGS tumor testing for SNVs, indels, CNVs, and gene fusions will reveal new potential therapeutic targets in patients who are failing standard therapy. Genomic alteration data on a patient's primary and/or metastatic tumor generated by NGS may qualify patients to enroll in active clinical trials or be treated "off-label or off-protocol" [Meric-Bernstam et al., 2015]. Since genomic testing is revolutionizing the field of cancer therapy, the traditional trial design is also undergoing changes. Umbrella trials examine tumors of one particular morphology/type, assigning patients to various treatment arms based on genetic mutations, effectively testing a "precision medicine" approach to cancer treatment. Bucket or basket trials are another strategy where patients are assigned treatment based on a genetic mutation, regardless of tumor type [Gagan and Van Allen, 2015; Meric-Bernstam et al., 2015]. The National Cancer Institute's Molecular Analysis for Therapy Choice Program (MATCH) is one example of a bucket/basket trial design [Abrams et al., 2014; Redig and Jänne, 2015]. Additionally, other trial designs are being explored; "n-of-1 trials" measure endpoints such as objective response or increased time to failure in patients undergoing individualized treatment plans. Overall, NGS data on tumor samples allows patients to enroll in trials in a timely fashion to help advance cancer care.

Detection of Underlying Germline Variants

In addition to somatic changes, NGS can also detect or suspect underlying germline mutations. Tumors harboring known mutations in mismatch repair genes, *BRCA1*, *BRCA2*, *TP53*, and others, may suggest an underlying genetic cancer predisposition syndrome. Follow-up germline testing can confirm a suspected germline variant. This is of critical importance because patients with germline mutations may be predisposed to and can be screened for additional malignancies based on their mutation. Screening guidelines are being established for multiple cancer predisposition syndromes for mutation carriers, but in many syndromes such as Li-Fraumeni, early detection of cancer leads to improved outcomes [McBride et al., 2014]. In addition to future cancer risk to the patient and their family, germline mutations may also direct therapy. For example, mutations in the *BRCA1* and *BRCA2* genes may confer tumor sensitivity to PARP in-

hibitors in multiple cancers, including ovarian and prostate [Dietel et al., 2015; Robinson et al., 2015]. Thus, cancer predisposition genes are vital to include on cancer panels as they may provide therapeutic and prognostic information. Targeted panels may also include genes in drug metabolism. Including genes such as *TMPT* and *NUDT15* on a hematological cancer panel can provide crucial genotype information that clinicians may use to alter thiopurine dosing, reducing medication toxicity [Burnett et al., 2014; Yang et al., 2015; Roy et al., 2016].

The Limitations of CMA and NGS

As with any technology, CMA has its advantages and limitations. CMA cannot detect genetic events that do not lead to copy number change, such as balanced rearrangements, point mutations, or alteration beyond its resolution such as deletions and duplications less than one or a few kilobase in size. Polyploidy may be detected by SNP-based microarray analysis but may be difficult to appreciate by aCGH array. Low-level mosaicism is another challenge for this technology. The sensitivity of CMA for detecting mosaicism can be influenced by the platform, sample type, copy number state, DNA quality, data quality, and the size of imbalance. In addition, array analysis cannot detect CNVs in regions without probe coverage.

While massive parallel sequencing of numerous short DNA fragments has made NGS an unprecedentedly powerful high-throughput tool for genomic analysis, it also leads to some limitations [Luthra et al., 2015]. Indels greater than approximately 120 bp will often be missed, such as certain *FLT3*-ITDs. GC-rich regions are challenging to capture for sequencing and are likely underrepresented in the final sequence output. Tumor heterogeneity is well established, and the mutation signature of a subclone may vary within the tumor and at different metastatic sites. For this reason, small needle biopsies, while technically feasible to sequence, may not represent the heterogeneity of the entire tumor cell population. Most importantly, NGS provides extremely complex data that requires expertise at all levels of processing from wet bench through bioinformatics pipelines, to variant analysis and reporting [Luthra et al., 2015]. This limits the clinical use of this technology to highly trained molecular geneticists and pathologists experienced in rigorous assay validation and performance testing [Dietel et al., 2015; Meric-Bernstam et al., 2015]. Finally, due to the complex nature of the results, molecular tumor boards are highly recommended to assist clinicians in the interpretation of the genomic results, especially in tumor types where genetic information is not well established.

Concluding Remarks

As the knowledge of cancer genomics and genome biology accumulates, CMA and NGS technologies are beginning to revolutionize the practice of oncology. Although not yet standard of care, there is an increasing interest in the cancer genomics community in applying these new technologies to cancer diagnosis and monitoring in the Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories. In fact, many clinical laboratories have already adopted these technologies for cancer genome analysis in order to inform all levels of oncologic care, from diagnosis and prognosis to directing

treatment strategies. The advantages are many, including whole-genome analysis for hundreds of mutations with relatively low nucleic acid input without the requirement for living cells or culture. We anticipate that CMA and NGS will soon become the major diagnostic means and standard of care for cancer genomic analysis to meet the increasing demands of precision cancer care.

Disclosure Statement

All authors have no disclosures.

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