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ORIGINAL ARTICLE

Next generation sequencing in cancer: opportunities and challenges for precision cancer medicine

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ABSTRACT

Over the past decade, testing the genes of patients and their specific cancer types has become standardized practice in medical oncology since somatic mutations, changes in gene expression and epigenetic modifications are all hallmarks of cancer. However, while cancer genetic assessment has been limited to single biomarkers to guide the use of therapies, improvements in nucleic acid sequencing technologies and implementation of different genome analysis tools have enabled clinicians to detect these genomic alterations and identify functional and disease-associated genomic variants. Next-generation sequencing (NGS) technologies have provided clues about therapeutic targets and genomic markers for novel clinical applications when standard therapy has failed. While Sanger sequencing, an accurate and sensitive approach, allows for the identification of potential novel variants, it is however limited by the single amplicon being interrogated. Similarly, quantitative and qualitative profiling of gene expression changes also represents a challenge for the cancer field. Both RT-PCR and microarrays are efficient approaches, but are limited to the genes present on the array or being assayed. This leaves vast swaths of the transcriptome, including non-coding RNAs and other features, unexplored. With the advent of the ability to collect and analyze genomic sequence data in a timely fashion and at an ever-decreasing cost, many of these limitations have been overcome and are being incorporated into cancer research and diagnostics giving patients and clinicians new hope for targeted and personalized treatment. Below we highlight the various applications of next-generation sequencing in precision cancer medicine.

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Applications of Next-generation sequencing

Identification of genomic variants that underlie susceptibility and causation of disease is of great interest. Next-generation sequencing (NGS), or massively parallel sequencing (MPS), is the 'catch-all' term used to describe a number of different modern sequencing technologies that allow sequencing of DNA and RNA much more quickly and cheaply than previously used Sanger sequencing [1,2].

NGS allows for an efficient method to identify and characterize both known and novel variants at any location in the genome. Currently, approaches to do this can be as broad as whole-genome (WGS), whole-exome (WES), whole transcriptome (RNA-seq) and targeted sequencing of specific regions of the genome [3]. Many factors have to be taken into account in deciding which approaches could be the most suitable: budget, sample quality and quantity, depth of coverage needed, etc [4]. A comparison of these different approaches is summarized in Table 1.

In most cases a reference genome is available and the DNA derived from a specific individual is tested for known mutations or scanned for variation in entire regions, exomes or smaller target regions.

The most comprehensive approach to interrogate the genome is through whole genome sequencing (WGS). In an unbiased way, the entire genome can be sequenced and analyzed for the presence of mutations (both somatic and germline), copy number variants (CVNs), and other chromosomal structural variants including translocations, transversions and inversions. Furthermore, this analysis is not limited to just the coding regions of the genome, but also the non-coding portions. As interest in these regions of the genome gains, and functional mutations have been identified, WGS enables the interrogation of them. Despite these advantages, the ability to generate enough sequence coverage throughout the whole genome in a cost-effective manner remains a challenge. The high heterogeneity of many tumor types will result in many variants being present at low frequency and their identification would require a high read depth that is often not achieved with WGS due to the cost of generating sequence. Furthermore, the often high tumor heterogeneity caused by extracellular matrix and other non-cancerous cells can dilute the signal of a driver or pathological mutation. Last but not least, the large amount of data generated becomes a bioinformatic challenge, as vast amounts of computational resources are needed to process and store the data [5].

In comparison to WGS, targeted sequencing (TS) approaches offer a more affordable and specific method for

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Table 1. Comparison of our Next Generation Sequencing approaches.

Whole Genome Sequencing (WGS)	**Whole Exome Sequencing (WES)**
• Comprehensiveness	• Interrogates protein-coding regions
• Detection of all the variants present in a genome	• Cost-effective
• Detection of structural and CNVs	• Gene discovery
• Hypothesis-free and gene discovery	• Required high quality
• Lowest depth of coverage	• Hard to identify structural variations
• Highest data complexity	• Increasing coverage depth over WGS
• Required high quality	
Transcriptome sequencing or RNA-seq	**Targeted Sequencing (TS)**
• Detection of variants present in the	• Regions of interest
• trascriptome and fusion genesRNA editing	• Highest depth of coverage
• Differential expression	• Hypothesis-driven
• Hard to identify variants in low expression transcript	• More manageable data storage and analysis
• Required high quality RNA	• Optimized also for low quality DNA (e.g. FFPE)

identifying genomic variants. With targeted approaches, as much as whole exome, only a select few genes or the entire coding regions, respectively, are sequenced. While with these approaches, the ability to interrogate the entire genome is lost and much of the information will be missed, there are certain advantages to them over WGS. First, as only a small region of the genome is being sequenced, a much higher read depth can be achieved for a fraction of the cost compared to WGS. This increased coverage enables identification of low frequency mutations that otherwise would be missed with WGS. Second, a comprehensive approach will sequence many regions of the genome that are not needed for disease diagnostics or therapeutics. In fact, only a handful of genes have repeatedly been associated with cancers. Therefore, targeted cancer gene panels ranging from as little as a dozen to several hundred genes where mutations are more likely to occur in specific regions or hotspot, have been readily incorporated into a clinical setting for disease diagnostics [6,7].

NGS platforms and technologies

DNA sequencing methods generally work by stopping the process of copying the template strand using dideoxynucleotides, reversible terminators or natural nucleotides [2]. Strategies for massively and ultrafast sequencing include sequencing-by-hybridization, nanopore sequencing and sequencing-by-synthesis (SBS). Most commercially available platforms use the SBS technology where the sequence of a template is inferred by stepwise primer elongation. The process involves immobilizing the sequencing templates and primers on a solid support followed by base primer extension and termination. The color of the fluorophore carried by the extended base is recognized and then the fluorescent tag and the 3-OH blocking group are removed and the steps are repeated. The whole process has been popularized as a second-generation sequencing technology on the Illumina platform. Several optical systems can be utilized to monitor synthesis of the complementary strand. In most cases four different fluorescently labeled nucleotides are involved, but recently Illumina introduced a three fluorescently labeled nucleotide system for the NextSeq sequencer. In this case a 2-channel system is required to detect the red (C) and green (T) signal, while the incorporation of A is detected in both channels as yellow and no signal is interpreted as G incorporation [8].

Non-optical detection systems are available including semiconductor-based sequencing commercialized by Fisher Scientific (Waltham, MA). In the Ion Torrent, also an SBS-based sequencer, reactions take place in a semiconductor chip, which detects the hydrogen ions produced during DNA polymerization. Following clonal amplification, the DNA library fragment is poured sequentially with each nucleoside triphosphate and incorporated into the new strand by complementary to the nucleotide on the target strand. Each time a nucleotide is successfully added, a hydrogen ion is released, and is detected by the sequencer's pH sensor. The Ion torrent sequencing is the first commercial platform to use non-fluorescence and camera scanning making it a cost-effective tool compared to other methods. Its main disadvantage is the difficulty to decode repetitive sequences, such as, for example, a homopolymer repeat of length 6 which may generate a pH change indistinguishable from a homopolymer of length 8 [9].

All SBS sequencing systems rely on DNA amplification and therefore are unable to perform single-molecule sequencing. Helicos Biosciences previously reported a single-molecule technique; however, its dependence on reversible terminators limited it to the analysis of short DNA fragments [10].

Pacific Biosciences (Menlo Park, CA) solved the single-molecule challenge by developing the zero-mode waveguide (ZMW) array, a nanostructured device that reduces the detection chamber volume to the zeptoliter (10^{-21} L) range representing an improvement of three orders of magnitude over confocal fluorescence microscopy. At this resolution volume/resolution, an estimated single molecule in the detection layer provides a very low signal-to-noise ratio. To perform parallel sequencing, inside each ZMW, an active DNA polymerase with one molecule of single-stranded DNA template is immobilized on the bottom surface through which light can penetrate and create a visualization chamber that allows monitoring in real-time monitoring of the activity of the DNA polymerase activity as it traverses a single molecule. To allow uninterrupted monitoring of nucleotide incorporation, four discrete fluorescent dyes on a synthetic nucleotide's terminal phosphate group rather than on the base, are employed in the sequencing reaction [11–13].

A non-SBS, non-optical technology, single molecule sequencing referred to as nanopore sequencing works by feeding a small molecule through a membrane-spanning protein channel in a buffered ion solution. When a voltage is

Table 2. A summary of widely used next-generation sequencers and their technologies.

Company	Sequencer	Library amplification	Carrier	Sequencing technology	Detection method	Comments
Illumina Inc	MiniSeq MiSeq Hiseq NextSeq	Bridge PCR	Flow cell	Reversible terminator SBS	Optical detection of fluorescence from dye-labeled nucleotides	75–600 bp Capability: WES, WGS, target seq, WTS
Life Technologies	Ion PGM Ion Proton	emPCR	Ion Chip	Semiconductor-based SBS	Transistor-based detection of H^+ shift after nucleotide incorporation	200 and 400 bp Capability: WES, WTS, target seq
Qiagen	GeneReader	NA	Flowcell	SBS	Fluorescence detection	12 gene cancer test: Actionable Insight Tumor Panel
Pacific Biosciences	RS II Sequel	NA	SMRT cell	Single Molecule Real Time sequencing	Real-time fluorescence detection	up to 40,000 bp Capability: WES, WGS, WTS, target seq, *de novo* genome assembly
Oxford Nanopore	MinION PromethION GridION	NA	Protein nanopore	ssDNA nanopore-based	Change in electrical Field	Up to 10,000 bp Capability: WES, WGS, WTS, target seq, *de novo* genome assembly
Complete Genomics – BGI	Revelocity Sequencing System	Combinatorial Probe-Anchor Ligation (cPAL)	DNA nano-ball (DNB array)	Ligation-based cPAL sequencing	fluorescence detection	Restricted to WGS WES

applied across the membrane, an ionic current is induced through the protein nanopore. As a molecule passes through the pore, one base at a time, it produces a measurable disturbance in the ion current providing information to decipher the sequence of the molecule. The technology is scalable and has been commercialized on a device, the size of a USB memory stick, the MinION, by Oxford nanopore (Oxford, UK) to distinguish between nucleotide bases [14]. The Oxford Nanopore's 'strand sequencing' advantages are that it can read much longer strands of DNA than other sequencing methods, requires unamplified DNA, removing the need for PCR and less sample. In principle these features make it possible for a doctor to read a patient's DNA directly from a blood sample [15]. A summary of widely used NGS sequencers and their technologies is shown in Table 2.

There are four core steps in the standard workflow associated with NGS: (1) sample preparation; (2) amplification; (3) sequencing; and (4) data analysis. In most cases, after nucleic acid extraction, DNA is fragmented to generate smaller strands by physical methods like acoustic shearing or by enzymatic methods. Based on the NGS-platform, the strands are ligated to double-stranded pieces of synthetic DNA referred to as adapters, enabling the sequence to become bound to a complementary counterpart for library construction by clonal bridge amplification or emulsion PCR. Platform-specific adapters allow multiplex samples to be clonally amplified being spatially arranged or separated. Numerous kits for making sequencing libraries from DNA and RNA are available commercially from a variety of vendors. Library size is determined by the desired insert size while optimal insert size is determined by the limitations of the NGS instrumentation and by the specific sequencing application such as WGS, WES, coding and non-coding RNA sequencing and target gene resequencing. Finally, a bioinformatics pipeline is required to process and analyze all the collected information and to detect the genetic variant by sequence alignment, quality assessment, variant calling and variant association.

For targeted genomic assays, several methods are available for capture and sequencing of specific genes and regions relevant to the tumor type. They can be subdivided in two main classes: (1) enrichment by amplification; and (2) enrichment by hybridization [16]. The former involves several locus-specific primer pairs and a multiplexed amplification reaction. Small amounts of DNA are required to achieve deep coverage of specific regions of interest. This type of panel typically interrogates discontinuous regions around hotspot mutations and not the entire coding sequence of the gene of interest. Therefore, this method is most suitable in testing FFPE samples and is not recommended for detection of structural alterations (CNVs and translocations). Enrichment by hybridization or hybridization capture requires more input and higher quality DNA. Therefore, it is most suitable for fresh or frozen samples where synthetic DNA or RNA probes, specific to the genomic area of interest, bind the complementary sequence on the genomic DNA, followed by target capture and amplification. This method can detect copy number gain and loss and selected structural rearrangements [16]. Several probe-captured based technologies are available (TruSeq and Nextera, Illumina, San Diego, CA; Sureselect and Haloplex, Agilent technologies, Santa Clara, CA). These methods are performed on a bench-top or production sequencer like Illumina or Ion Torrent.

Several studies have already tested and compared different enrichment technologies, and could be used as a guide in the selection of the most appropriate procedure. Comparisons have been made between a few technologies and data were analyzed using a variety of parameters including the percentage of targeted bases covered by probes and by sequence reads, e.g. design coverage and sensitivity, respectively, and the number of reads mapping to the targeted sequence, e.g. specificity. Uniformity and reproducibility or overall variation in per-base coverage over the targeted region and

variation between replicates, respectively, were also taken into account [17,18].

Cancer panel-gene targeted sequencing: challenges and outcomes

The use of NGS in cancer screening has generated a large catalog of potential somatic tumor mutations that cover different exons in a large number of genes, available for both solid and liquid tumors. An 'actionable' genomic mutation is defined when 'it is potentially targetable with a US Food and Drug Administration (FDA)-approved drug or other agent that can be used in early clinical trials' [19]. Serial single-gene testing could be time consuming especially for FFPE tumor biopsies; therefore, hot-spot based assays or cancer panels covering actionable variations that could affect patient's treatment choice are widely used to identify single validated variants [20,21]. Therefore, through a comprehensive genomic analysis, patients may benefit from genotype-directed therapy or genotype-matched clinical trials.

The decision about how and which genes to sequence requires consideration of the number of cases, cost, accessibility to matched normal tissue and turn-around-time. One option is to outsource the entire process to a commercial reference laboratory. A wide variety of commercially pre-designed panels are available and are listed in Table 3 with the reference laboratory performing cancer targeted resequencing [4,16]. Most also can be customized with the addition of relevant genetic variants and non-coding sequence information such as promoters and/or regulatory regions. Furthermore, to provide more flexibility and allow screening of specific genes relevant only to a specific tumor type, most companies have made either large or small cancer-specific panels where the customer can select not only genes, but also which area of those genes have to be analyzed. However, setting up an in-house panel allows the laboratory the flexibility of adding new biomarkers and variants as soon as new evidence or clinical trials are available. In addition, the laboratory has access to all data (quality, frequencies, etc.), custom pipelines for bioinformatic analysis and results may be integrated with associated clinical information. Finally, once the assay is validated it can be used on other samples or optimized for other types of specimens.

Tumor heterogeneity

An emerging field of interest is the analysis of tumor heterogeneity, cell clonality and effects on therapy response. The possibility of easily accessing sample tumor cells over time allowed for the identification of a wide range of driver mutations in many NGS studies and also facilitated the investigation of tumor frequency over time [22]. Analysis of cancer genomes of solid tumors presents several challenges. Tumor biopsies are often arduous to obtain because of quantity and quality. They usually contain a mixture of tumor cells and non-tumor cells, and some of the tumors are highly heterogeneous [23]. Therefore, to achieve desirable detection sensitivity, a high coverage of sequencing may be required (500–1000X). To address this, custom-targeted NGS panels have been developed, especially for large numbers of samples where the optimal choice is to genotype the primary tumor, matched non-tumor tissue, and, if available, recurrent metastatic sites. One example is the MSK-IMPACT, a hybridization capture-based, next-generation sequencing assay for interrogating somatic alterations in 341 oncogenes and tumor suppressors in formalin-fixed, paraffin-embedded (FFPE) tumor specimens [24]. Patient-matched tumor and normal samples were sequenced and then sensitivity, specificity, and reproducibility of the MSK-IMPACT panel assessed through the validations on 284 known positive tumor samples with predefined point mutations and insertions/deletions variant detected at 500× coverage depth. Detection limit for low frequency variants in hotspot mutation and non-hotspot mutation was 2% and 5%, respectively [24].

The main concerns about clinical genomics and the sequencing data generated is how they currently impact therapy decisions, whether they can lower the costs of health care, improve survival rate and/or improve quality of life. The number of publications reporting clinical utility of comprehensive genomic profiling in cancer patients is still

Table 3. Commercially available pre-designed panels and representative reference laboratories performing cancer-targeted resequencing.

Vendor	Assay name	No. of genes
Illumina, Inc (San Diego, CA)	TruSight Cancer	94
	TruSight Tumor 15	15
	TruSight Myeloid	54
	TruSeq Amplicon Cancer Panel	48
Thermo Fisher Scientific (Waltham, MA)	Ion AmpliSeq Cancer Hotspot Panel v2	50
	Ion Ampliseq Comprehensive Cancer Panel	400
Qiagen (Hilden, Germany)	Actionable Inside Tumor Panel	12
Agilent Technologies (Wilmington, DE)	ClearSeq Comprehensive	151
	ClearSeq Cancer	47
	ClearSeq AML	20
	Cancer Research Panel	47
Foundation Medicine®, Inc (Cambridge, MA)	Foundation One	315
University of Washington (Seattle, WA)	UW-Oncoplex	234
ParadigmDX (Phoenix, AZ)	PCDx	114
ARUP Lab (Salt Lake City, UT)	Solid Tumor Mutation Pannel	48
Caris Life Science (Irving, TX)	MI Profiles	46
Knight Diagnostic Lab (Portland, OR)	GeneTrails Cancer Gene Panel	38
PathGroup (Brentwood, TN)	SmartGenomics	35
Life Technologies (Carlsbad, CA)	Pervenio Lung NGS assay	25

limited. In June 2015 the American Association Cancer Research Precision Medicine Series conference took place to discuss the integration of NGS into clinical practice. One study highlighted potential benefits using precision medicine approaches [25]. A 100 gene-targeted panel was evaluated for identification of molecular targets in metastatic patients of diverse cancer types. The cohort of 72 cancer patients was subdivided into two groups; 36 patients received standard care and the other 36 were treated using a precision medicine approach. Progression-free survival (PFS) and total treatment costs were taken into account to establish if the precision medicine approach would improve PSF of the metastatic cancer patients without increasing healthcare costs. The precision medicine group showed an increase in PSF with comparable total costs [26]. In a second study, a 48 NGS gene panel was employed to analyze 1893 cancer patients with different histologies in a retrospective study. Although 13% could not be analyzed due to low DNA quality or lack of sufficient FFPE material, 80% of all other tested samples showed at least one mutation. Unfortunately, only 5% of these patients followed a genotype-matched clinical trial, and did show a better response, emphasizing the need to improve on design of these kinds of trials [27].

Tumor heterogeneity, drug resistance and liquid biopsy

The intra-tumoral heterogeneity and clonal evolution under treatment selection pressure are two causes for tumor recurrences. Cancer cells in a slowly-proliferating or even 'dormant status' may continue to accumulate genetic and epigenetic alterations that may conceal metastases. Acquisition of resistance to therapy could be due to tumor heterogeneity or influenced by an appropriate reaction to treatment regimen.

NGS studies should be designed to detect heterogeneity and the dynamic status of the tumor. Unfortunately, solid biopsies do not fit this need, due to sample collection challenges including accessibility, especially during the course of the disease. Most often the procedure for sampling is invasive and even if available, the region obtained may not be representative of overall tumor heterogeneity.

Circulating tumor cells (CTCs) and circulating cell-free tumor DNA (cfDNA)

Circulating tumor cells (CTCs) and circulating cell-free tumor DNA (cfDNA) can be a source of cancer material, representative of disease status and compatible with repetitive, non-invasive sampling [28,29]. Use of cfDNA and CTCs enable testing patients over time and may overcome the need for serial solid biopsies.

The cfDNA are released into the bloodstream from tumor tissue and from lysis of CTCs. For this reason cfDNA and CTCs must be considered as two separate entities [30]. Several challenges are associated with cfDNA analysis since it represents fragmented DNA (160–180 bp) [31], is usually present at low concentrations and can be mixed with non-tumor DNA. The pre-analytical steps can have significant impact on analysis and the final yield. In addition, high sensitivities are often required to detect variation expected at a frequency of <0.1%. One of the best methods to achieve this level of sensitivity is the digital PCR (dPCR) droplet system, especially when a limited number of loci need to be evaluated [32]. However, characterization of rare variants is often a daunting task to track down by NGS because rare variants may be difficult to distinguish from sequencing errors. Template tagging methods such as Safe-Sequencing System, or Safe-SeqS [33] and tagged-amplicon deep sequencing, or TAm-Seq [34], have been developed to distinguish errors from real variants to allow comprehensive cancer genomic profiles [34]. The most immediate clinical application is in identification of genomic alterations to guide selection of target therapies and to monitor in real time during disease progression and therapy.

CTCs are, like the cfDNA, extensively studied for their potential clinical utility. They represent a rare population of cells in the background of 10^6–10^7 nucleated blood cells and they require a critical enrichment step before their detection and characterization. Although they were first described by T. R. Ashworth in 1869 [35] technologies have only recently been developed to address enrichment challenges in terms of capture efficiency/recovery rate, blood sample capacity, cell viability and purity. The main strategy to enrich CTCs can be based on biological features (like cell surface marker expression) or on physical properties (like size, density or deformability). The only currently FDA-approved technology is CellSearch® (Veridex, Rarithan, NJ), an EpCam-based capture method followed by immuno-fluorescent staining using epithelial markers such as cytokeratin 8, 18, 19 and leucocyte-specific marker CD45. However, this technology cannot detect CTCs that may have undergone an epithelial to mesenchymal transition (EMT). This relevant subpopulation may exhibit molecular and functional stem-like features often correlated to chemo-resistance. The value of CTC detection using the CellSearch® was demonstrated in metastatic breast, prostate and colon cancer patients [27,36,37]. In addition to the effort to characterize and detect CTCs, recently several studies have demonstrated mutational profiles of CTCs in various cancer types. In one study, CTC-enriched fractions using CellSearch® from six metastatic colorectal cancer patients were analyzed on a 68 cancer panel NGS array, and all mutations found were confirmed as subclonal mutations in primary tumors and/or metastases [38]. Moreover, several new techniques to isolate and analyze single cells are allowing single CTC mutational analysis to better investigate and understand the correlation between tumor heterogeneity and drug resistance. DEParray™ was used to isolate single CTCs from the CellSearch CTCs-enriched fraction, followed by single whole genome/transcriptome amplification [39] and targeted or comprehensive NGS analysis [40]. CTC-enriched cells from the CellSearch system and cfDNA were investigated in 48 patients with ER + positive metastatic breast cancer receiving systemic therapy [41]. This investigation involved 23 target genes with hot spot mutations in the following genes: ESR1, PIK3CA, TP53, FGFR1 and FGFR2. NGS detected three

Table 4. NGS assay parameters recommended to validate molecular diagnostic test.

Depth of coverage	The minimum sequencing coverage of target region under standard assay conditions, for each type of variant, should be established in order to achieve the required level of mutation detection.
Limit of detection	The lower limit of mutation detection under standard assay conditions for different variants (SNVs, CNVs, insertion, deletion, etc.).
Accuracy	The degree of concordance between NGS's sequences obtained with the assay and the reference sequence.
Precision	The degree of result reproducibility by the assay across users and runs.
Specificity	The probability of the assay to not detect sequence variants where none are present among samples validation set (false-positive rate).
Sensitivity	Capability of the assay to detect true sequence variants among sample validation set (false-negative rate).
Reproducibility	The capability of the assay to give consistent results within-run.
Reportable range	Range of values over which the test is evaluated valid.
Reference range	When a test has specific reference for designed population or study group.

activating mutations in ESR1, three hotspot mutations in PIK3CA and three in TP53 in baseline cfDNA. Only the ESR1 p.D538G mutation was found in one matched circulating tumor cell sample. This can be explained by the fact that only CTC pools were analyzed in which the predominant component is wild type, while the mutation was detected in cfDNA at only 1% [41]. In another study, a single CTC study on ERS1-activating mutations and their correlation with endocrine therapy resistance showed feasibility and potential clinical utility. ESR1 mutational status has been successfully performed on 72 single cells isolated from metastatic breast cancer patients, and mutations were reported in patients with an acquired endocrine-based therapy resistance [42]. The development of single-cell sequencing is making CTCs a potential target to assess prognosis, monitor response therapy and for rational selection of the best therapy. All these studies suggest that blood-based biomarkers can reveal uniquely the dynamics of primary tumor formation or metastases. CTCs and ct-DNA each can contribute differently to detection of somatic variation at a frequency of 1% or lower, which in most cases is impossible to differentiate from noise using a solid biopsy specimen.

Clinical validation and ethical implications

The integration of these NGS technologies in any clinical sequencing workflow requires analytic and clinical validation, stringent regulations and guidelines established by the agencies governing clinical laboratories. Clinical laboratories are subject to legal obligations designed to ensure that tests meet parameters for trueness, precision, sensitivity and specificity. Organizations like the College of American Pathologists (CAP) and the Association for Molecular Pathology (AMP) have defined assay parameters for low-throughput assays and have defined assay parameters (Table 4) for NGS sequencing implementation in diagnostic settings [43,44].

One strategy to validate cancer NGS assays is to test several tumor specimens with known somatic variants (insertion-deletion, CNVs, SNVs, etc.) and detect that aberration using another possible sequencing platform. Human cell lines positive for somatic mutation or germ-line polymorphisms with already characterized changes also can be used [8]. The reference sample utilized for validation should mimic features of the clinical samples for which the NGS assay is being validated. The quality and quantity of the starting material should be comparable to the clinical sample used. Most cancer specimens are usually FFPE samples in which nucleic acids are compromised, especially in quality. Therefore, protocols must be optimized to deliver deep-sequencing coverage for these kinds of specimens. Several synthetic standards are available and can be used for NGS validation. Theoretically, a synthetic standard or cell lines can be used for each gene and variant, especially for which one has a low allele frequency [8]. Cross-validation on different NGS platforms is also suggested for a new NGS clinical assay [8,45].

Matched germ-line DNA is typically used as a normal control to distinguish inherited variants and to increase analytical sensitivity. There are ethical, social and legal implications in this kind of approach. The analysis can incidentally reveal germ-line susceptibility to cancer or other diseases (incidental findings) with significant consequences for the patient and family members. Therefore, ethical considerations are critical and genetic counseling should be available to properly educate the patients as well as relatives if required, about risk and benefit of each genetic test and informed consent should be signed. Guidelines about how to manage incidental findings in the clinical setting have been released by the American College of Medical Genetics and Genomics (ACMG) and the American Society of Clinical Oncology (ASCO) [8,46,47].

Conclusions

Since January 2015 the 'Precision Medicine' initiative has represented an attempt at disease prevention and treatment that utilizes an individual's variation in genes in order to select the most appropriate therapeutic strategy for each patient on the basis of individual variability. These approaches offer much promise, but much work is still needed to ensure sensitivity, specificity, timeliness, cost-effectiveness and validity of such testing [48]. Furthermore, clear actionable clinical treatment strategies must be defined so that rationale for a precision medicine approach is clear.

Disclosure statement

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