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INVESTIGATING THE APPLICATION OF NEXT-GENERATION SEQUENCING IN MOLECULAR DIAGNOSTICS: OPPORTUNITIES AND CHALLENGES

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Abstract:

Molecular biology and clinical diagnostics have been transformed by next-generation sequencing (NGS) technologies, which allow for the quick and affordable study of genetic data. In this overview, we go through the development of NGS platforms and their uses in oncology and constitutional disorders, among other areas. With NGS, we can now identify new genes linked to disease and develop individualized treatment plans, which has greatly improved our understanding of genetic diseases. NGS is essential to oncology because it helps to predict treatment outcomes, characterize genetic changes in cancer, and guide therapeutic choices. NGS has the potential to change clinical practice, but obstacles including complicated data analysis and payment problems prevent it from being widely used. However, there is hope that future developments in NGS technology and legal frameworks will help to overcome these obstacles and further incorporate NGS into standard patient treatment. All things considered, NGS is a paradigm shift in genomic analysis that is propelling precision medicine forward and changing the way that genetic illnesses and cancer are diagnosed and treated.

Key words: Next-generation sequencing, NGS, molecular diagnostics, constitutional disorders, oncology, precision medicine, genetic diseases, cancer genomics, personalized medicine, clinical applications.

Introduction:

Next-generation sequencing (NGS) is a technology that enables the simultaneous sequencing of millions of DNA or RNA sequences. It is sometimes referred to as massively parallel sequencing or high-throughput sequencing [1]. NGS has several advantages over traditional sequencing techniques, such as reduced cost, faster turnaround times for large sample volumes, better throughput with sample multiplexing, and higher sensitivity in finding low-frequency variations. Following Sanger sequencing, NGS constitutes a true technological

revolution in sequencing [1]. A whole human genome can now be sequenced in a matter of days for less than $1,000 thanks to the development of Next-Gene Sequencing (NGS). Sanger sequencing of the first human genome took several years and billions of dollars to accomplish [2]. NGS is becoming an integral component of precision medicine and has a wide range of applications in laboratory medicine. Infectious diseases, cancer, and constitutional disorders have all benefited greatly from the widespread application of this technology in diagnosis, prognosis, and therapy selection [3-5]. At the same time, NGS is producing a growing quantity of well selected clinical, genetic, and genomic data, which is propelling precision medicine forward [6]. In addition to approving a number of NGS-based tests and targeted medicines, the U.S. Food and Drug Administration (FDA) has published a set of recommendations for the creation, development, and validation of NGS testing [7-9]. Furthermore, in an effort to guarantee that NGS-based tests are covered, the Centers for Medicare & Medicaid Services (CMS) has been closely tracking the quick invention of NGS testing. The use of NGS in laboratory medicine for clinical purposes has increased as a result of all these developments. This review focuses on the latest advancements in next-generation sequencing (NGS) technology and their clinical utility in the diagnosis, prognosis, and treatment of genetic disorders, cancer, and infectious diseases.

Next Generation Technologies:

Throughout history, DNA sequencing technologies have held significant sway in both molecular biology and clinical arenas [10–13]. The inaugural sequencing platform, known as Sanger sequencing, emerged from the pioneering work of Fred Sanger in 1977 and has since served as a cornerstone in both research and clinical genetics [14–16]. However, the landscape of sequencing underwent a transformative shift three decades later with the rapid evolution of next-generation sequencing (NGS) technologies, leading to the emergence of second and third-generation sequencing platforms. Notably, the advent of NGS has ushered in marked reductions in sequencing turnaround time and cost, particularly since the milestone achievement of the first draft map of the human genome in 2001 [17–26]. In this section, we delve into the commonly employed next-generation sequencers, delineating their respective strengths and challenges. Different commercial entities have developed a number of second-generation sequencing technologies, all of which are typified by a workflow consisting of three main steps: (1) preparation of the template, which involves nucleic acid extraction; (2) preparation of the library, which involves clonal amplification; and (3) sequencing and alignment of short reads.

The first massively parallel sequencing platform to be made commercially available was Roche 454 sequencing, which was debuted in 2005. Roche 454 sequencing, which uses pyrosequencing technology, uses the detection of pyrophosphate (PPi) release as a sign of particular base incorporation. After attaching fragmented DNA to beads using ligated adaptors, the fragments are amplified using emulsion PCR in an emulsion droplet. These beads are then put into PicoTiterPlate (PTP) wells, each containing multiple copies of the same DNA template. As each nucleotide is added one after the other into the PTP wells, pyrophosphate is released during DNA synthesis and is then transformed into ATP. The transformation of luciferin into

oxyluciferin is then catalyzed by luciferase, producing visible light that a coupled-charge device (CCD) camera can record. The interpretation of these light signals is crucial to the sequencing process' accuracy; misreads or missing signals, especially in homopolymer sequencing, can result in base mistakes as well as insertions or deletions [27–30]. Despite being introduced in 2008 and capable of producing 700 Mb of sequence data per run with read lengths up to 1,000 bases in about 20 hours, the Roche 454 sequencer genome sequencing (GS)-FLX's adoption began to decline in 2016 because of its prohibitively high costs in comparison to other high-throughput NGS platforms like the Illumina and Ion Torrent systems.

Unlike techniques based on fluorescence or chemiluminescence, Ion Torrent uses hydrogen ion detection technology for sequencing. This technology detects the release of protons during nucleotide integration into strands during synthesis. This procedure involves tying fragmented DNA to 3-micron-diameter beads with particular adaptor sequences, then amplifying the clone using emulsion PCR. Following the loading of the beads into microwells, the sensing layer of the microwell detects the pH shift brought on by proton release during synthesis, translating the chemical signal into a digital one [32]. Targeted sequencing or smaller genomes were catered to with the 2010 debut of the Ion Torrent Personal Genome Machine (PGM) sequencer, which touted an output of up to 2 GB per run with quick run durations (2–7 hours) [32, 33]. Later versions, including the Proton sequencer that was unveiled in 2012, provided higher throughput at comparable rates, making it possible to sequence human genomes as well as exomes [34, 35]. Launched in 2015, the Ion GeneStudio S5 series sequencers further simplified reagents and instrument cartridges, allowing for quicker run times and simpler preparation. Unlike the CCD cameras and laser scanners used in other sequencing methods, the Ion Torrent platform offers a faster, more straightforward, and more affordable method. However, sequencing errors such as artifact insertions/deletions (indels) linked to homopolymeric stretches and repeats can still occur using Ion sequencers [35].

In the field of NGS, Illumina platforms have been the most popular option [37–43]. Using a bridge PCR technique, Illumina's methodology entails annealing both ends of fragmented DNA to two fixed adapters that are mounted on the solid surface of the flow-cell for clonal amplification and sequencing via reversible termination technology. Following bridge amplification, clusters containing clonal DNA fragments are encouraged to develop. The ddATP, ddGTP, ddCTP, and ddTTP reversible terminator (RT) nucleotides are all carrying cleavable fluorescent dye and are protected at the 3′-OH group. During synthesis, these modified RT nucleotides are integrated into the expanding DNA strands. Upon inclusion, they release fluorescent signals, which are subsequently photographed and recorded with a CCD camera. By requiring the insertion of a single base at a time and requiring the removal of a terminator before another base can be added, this design successfully mitigates homopolymer sequencing errors [44, 45].

The MiSeq tabletop sequencer, which was first available in 2011, is one of Illumina's most popular products. With a data output spanning from 540 Mb to 15 Gb, it is appropriate for

sequencing bacterial genomes and small gene panels [46]. On the other hand, the 2012 release of the production-scale HiSeq2500 demonstrated the potential to sequence a whole genome in about a day. Both systems use the four-channel sequencing by synthesis (SBS) method, in which distinct pictures are used to identify each nucleotide. On the other hand, the 2014 NextSeq 500 has a two-channel SBS system, meaning that it only needs two pictures to determine all four base calls. This invention lowers cycle counts and imaging capture times, which lowers sequencing costs and turnaround times. By using billions of pre-formatted nanowell grids at set locations, the HiSeq X Ten, HiSeq 3000, and HiSeq 4000 systems revolutionized data output in 2015 and produced significantly better data yields than MiSeq and HiSeq2500 [45]. A major step forward was the following announcement of NovaSeq in 2017, which aimed to bring the price of sequencing a human genome down to $100. Two-channel chemistry, bigger flow-cells with more nanowells, and a quicker imaging capture mechanism are all used by NovaSeq. NovaSeq gives clients the freedom to choose from a variety of flow cell types that meet a range of sequencing needs. It can produce up to 6 Tb of sequence data and 20 billion reads in around two days. All things considered, Illumina platforms are unmatched in the clinical and scientific arenas because of their remarkable accuracy, affordability, and high throughput capacities.

Third Generation Sequencing:

Second-generation sequencing technologies have made great strides in the NGS field, but a number of problems still exist, including PCR artifacts, alignment problems related to repetitive sections or pseudogenes, and short sequence reads that result in gaps in the sequence. Third-generation sequencing methods, based on single molecule sequencing, have been developed to address these constraints [47–49]. These platforms, which are represented by Oxford Nanopore Technologies, Oxford, UK, and Pacific Biosciences, PacBio, single molecule real-time (SMRT) sequencing technologies, offer potential solutions [50]. When compared to second-generation sequencing methods, PacBio SMRT technology stands out for its potential to produce substantially longer reads without the need for amplification. The second-generation technologies' library preparation procedure is similar to this one, but using adapters that have a hairpin structure to help circularize double-stranded DNA fragments into SMRTbell templates. On a chip with millions of zero mode waveguides (ZMWs), where DNA polymerase and template molecules are immobilized, sequencing takes place in real time. Fluorescently labeled deoxyribonucleotide triphosphates are used by DNA polymerase to extend the complementary strand of the template during sequencing. Fluorescence signals are captured and recorded in real-time by the CCD camera [48, 50, 51]. PacBio RS has developed since its launch in 2011, with later iterations showcasing greater average read durations. The Sequel system, which was introduced in 2015, provides even longer read lengths and more data output [53, 54].

Oxford Nanopore, on the other hand, uses nanopores to measure changes in current as molecules pass through, enabling direct sequencing of proteins, RNA, and DNA without the requirement for fluorescent tagging or amplification. This method has a high sequencing error rate, mostly from indels, despite having benefits including quick turnaround times and no GC

bias [48]. All things considered, longer sequence reads are made possible by third-generation sequencing technology, which makes it easier to characterize structural alterations in genomes and fill in gaps in reference assemblies. High mistake rates are still a major problem, though. One way to solve these problems would be to use a hybrid sequencing strategy that combines second- and third-generation technology [55–59].

Applications of Second-Generations Sequencing:

The field of study into the genetic foundations of constitutional illnesses has undergone a radical change with the introduction of next-generation sequencing (NGS). With this technique, comprehensive genomic information may be produced effectively, affordably, and in a timely manner. Comparative studies between NGS and other DNA testing techniques have shown the diagnostic usefulness and cost-effectiveness of NGS. For example, high-resolution chromosomal microarray analysis (CMA) produces a 15–20% diagnostic yield for similar illnesses, while older approaches such as G banding, which detects chromosomal abnormalities, offer approximately 3% diagnostic rate for unexplained constitutional disorders. On the other hand, NGS-based methods such as whole-exome sequencing (WES) and whole-genome sequencing (WGS) provide diagnostic yields for Mendelian disorders of 25% and 27%, respectively [61–62].

Depending on the gene makeup, targeted NGS gene panels with a focus on particular disease subsets show varying diagnostic yields. While the diagnostic yield for congenital glycosylation diseases may be as low as 14.8% in certain panels, it can reach as high as 53% in others for illnesses such as prenatal skeletal dysplasia. Interestingly, the limited detection range of these panels makes them unsuitable for use in unusual presentations or in the search for new genes [63–64]. On the other hand, clinical ES includes about 22,000 genes that code for proteins, which increases the possibility of finding harmful variations. With diagnosis rates ranging from 24% to 52%, ES has been especially helpful for patients with diseases that had not been previously identified [78–80]. Moreover, whole-genome sequencing (WGS) provides a thorough examination of complete genomes, outperforming ES and CMA combined in terms of diagnostic power. Research has indicated that WGS detected genetic variations that are clinically significant in 34% of cases, which is a noteworthy rise when compared to alternative techniques [84–85]. For newborns suffering from acute diseases, rapid whole genome sequencing (rGS) has been shown to be an invaluable resource, enabling prompt diagnosis and precise treatment planning. This method's clinical value has been highlighted by its 43% diagnostic yield as well as significant decreases in morbidity and death [86–87]. NGS technologies are not always as expensive as other testing methods, but economic analyses constantly point to their superiority and cost-effectiveness [88]. In the end, NGS has a clinical influence that goes beyond diagnosis to affect treatment and medical management choices. More than half of patients with diagnoses have had their medical care impacted by NGS data, which have also influenced family counseling and cancer surveillance programs. These results highlight how NGS can revolutionize precision medicine and patient care [89–90].

Applications in Oncology:

Precision medicine has turned its attention to cancer, which is typified by genetic aberrations. Next-generation sequencing (NGS) is essential for detecting these abnormalities. Single nucleotide variants (SNVs), tiny insertions/deletions (indels), copy number variations (CNVs), and fusion genes are among the genomic abnormalities that can be found in both hematologic and solid malignancies using next-generation sequencing (NGS) [91–93]. Even with the growing availability of whole genome, exome, and transcriptome sequencing, targeted gene sequencing is still the method of choice in clinical oncology labs. This methodology guarantees the best possible sequencing quality, economy, and speed of analysis, which qualifies it for routine cancer detection. Small NGS panels with fewer than 50 genes are frequently used in clinical settings for particular cancer types like breast cancer or acute myeloid leukemia (AML). On the other hand, bigger NGS panels are useful in commercial laboratories and academic hospitals because they provide thorough coverage for a wide range of cancer types. For hematologic malignancies and solid tumors, specially created NGS panels have been established at organizations such as the Children's Hospital of Philadelphia (CHOP), making it easier to find SNVs/indels, CNVs, and fusions [91, 94]. NGS panel testing showed a significant therapeutic benefit across a variety of cancer types, including leukemia/lymphomas, central nervous system (CNS) malignancies, and non-CNS solid tumors, in a study encompassing 367 pediatric cancer samples [91].

The FDA and CDC are two organizations that have regulatory authority over the use of NGS in clinical laboratories. The validation of NGS panels, pipeline validation in bioinformatics, and sequence variant reporting and interpretation in cancer have all been given standards and guidelines [96–98]. These rules support the broad use of NGS-based testing in oncology diagnostics by ensuring its consistency and dependability. In conclusion, NGS has become a key component of precision oncology, providing a thorough and effective method for locating genetic changes in cancer. Tight regulatory guidelines regulating its utilization in clinical laboratories could improve cancer diagnosis and provide individualized treatment plans.

The US FDA's approval of multiple next-generation sequencing (NGS) panels has had a major impact on the landscape of cancer diagnoses. These panels provide thorough genomic profiling of malignancies, facilitating the discovery of relevant genetic changes and directing focused treatment approaches. The Thermo Fisher Oncomine Dx Target Test, which obtained FDA approval in June 2017, is one prominent FDA-approved NGS panel. Developed as a companion diagnostic (CDx) test, it assesses variations in 23 genes linked to NSCLC, including important indicators like ROS1 fusions, EGFR L858R, BRAF V600E, and EGFR exon 19 deletions. Approved reimbursement decisions by major commercial health insurers and CMS have made it easier for national reference laboratories to implement. Two other NGS panels, the Foundation One CDx (F1CDx) and the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) panel, were approved by the FDA in November 2017. While F1CDx examines variants in 324 genes together with microsatellite instability

(MSI) and tumor mutational burden (TMB), MSK-IMPACT discovers genetic variants in 468 genes. Additionally, F1CDx was given approval to be used as a CDx for olaparib in advanced ovarian cancer with a BRCA mutation.

Approved in November 2019, the NantHealth Omics Core test uses whole exome sequencing (WES) to report somatic changes and TMB in 468 cancer-relevant genes. Launched in October 2018, Illumina's TruSight Oncology 500 (TSO 500) aims to detect 523 SNV and indel variants and 55 fusion and splice variants genes; FDA approval as a CDx is still pending. Furthermore, the FDA designated the MI Transcriptome CDx from Caris Life Sciences as a breakthrough device for the detection of FGFR biomarkers, including gene fusions in solid tumors. This device uses RNA from formalin-fixed paraffin-embedded tumor tissue. These FDA-approved NGS panels are a major step forward in cancer diagnosis, providing physicians with useful information on the genetics of tumors and helping to tailor treatment plans for a wide range of cancer patients.

Challenges and Future Opportunities:

Genomic medicine has advanced significantly as a result of the use of next-generation sequencing (NGS) technology in clinical diagnostic laboratories. These technologies do, however, still have some issues that prevent them from being widely used. Even if they are efficient, widely used NGS technologies have limitations including producing short reads and depending on clonal PCR amplification for signal detection. New technologies, such as those from Oxford Nanopore and Pacific Biosystems, promise benefits like single-molecule sequencing and long reads that may require less starting material. However, at the moment, their high mistake rates keep them from taking precedence. Because whole genome sequencing (GS) may give complete coverage without requiring an initial enrichment step, it is expected to play a significant role in laboratory medicine. However, there are still major obstacles to GS implementation in the form of data storage and analysis issues, notably with regard to structural variant analysis and variant interpretation. The intricacy of cancer heterogeneity presents difficulties for oncologists in terms of interpretation, variation discovery, sampling, and therapy choices. Novel strategies like liquid biopsy and single cell sequencing have potential to solve this problem. Moreover, germline mutations make somatic cancer diagnosis more challenging, emphasizing the necessity of sequencing matched cancer and normal tissues from the same patient. Adopting NGS tests in clinical settings necessitates a significant investment of resources, including data storage, bioinformatics assistance, and test validation, all in compliance with established protocols. For smaller laboratories, these requirements may be prohibitively expensive. Furthermore, the use of clinical NGS tests is now restricted by their high cost, particularly when it comes to big panel testing, exome sequencing, and genome sequencing for cancer diagnoses. Another major obstacle to the broad use of NGS in laboratory medicine is insurance coverage. Although the Centers for Medicare & Medicaid Services (CMS) has extended coverage for next-generation sequencing (NGS) tests in specific scenarios, there are

still obstacles in guaranteeing all-encompassing reimbursement guidelines that promote wider use of these sophisticated genomic diagnostic instruments.

Conclusion:

In conclusion, the evolution of next-generation sequencing (NGS) technologies has revolutionized various fields, from molecular biology to clinical diagnostics, profoundly impacting our understanding and management of genetic disorders, cancers, and other diseases. From the first-generation Sanger sequencing to the advent of massively parallel sequencing platforms, NGS has enabled researchers and clinicians to decipher complex genomic landscapes with unprecedented speed, accuracy, and cost-effectiveness. In the realm of constitutional disorders, NGS has emerged as a game-changer, offering higher diagnostic yields compared to traditional methods like G banding and chromosomal microarray analysis. The shift from forward to reverse phenotyping has enhanced our ability to identify novel disease-associated genes and tailor precision treatments, showcasing the power of NGS in unlocking the genetic basis of rare diseases. Similarly, in oncology, NGS has become indispensable for characterizing the genomic alterations driving cancer initiation, progression, and response to therapy. FDA-approved NGS panels like the Oncomine Dx Target Test and FoundationOne CDx have paved the way for personalized cancer treatment strategies, guiding clinicians in selecting targeted therapies based on patients' genetic profiles. Despite its transformative potential, challenges persist in the widespread adoption of NGS in clinical practice. Limitations such as short reads, high error rates, and resource-intensive requirements for test validation and data analysis pose hurdles to implementation. Moreover, issues related to insurance coverage and reimbursement policies need to be addressed to ensure equitable access to NGS-based diagnostics. Looking ahead, ongoing advancements in NGS technologies, coupled with efforts to streamline data analysis pipelines and enhance regulatory frameworks, hold promise for overcoming these challenges. As NGS continues to evolve, its integration into routine clinical care is poised to drive further innovations, ultimately improving patient outcomes, and advancing the era of precision medicine.

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