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Strategies and Clinical Applications of Next Generation Sequencing

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Abstract

DNA sequencing is one of the great valuable techniques in molecular biology, which can be used to detect the sequence of nucleotides in a DNA fragment. The high-throughput sequencing known as Next Generation Sequencing (NGS) revolutionized genomic research and molecular biology; therefore, the whole human genome can be sequenced with a low cost in several days. NGS technology is similar to the traditional method, Sanger, which detects small DNA fragments by emitted signals at the time of synthesis of each fragment (from the DNA template), but the difference is that NGS can determine the massive simultaneous sequencing in a few days with high accuracy and the results are directly detected without the need for electrophoresis. In fact, NGS technology combines a variety of steps such as sample preparation, fragmentation of the sample of the studied genome, attachment of adapter to the ends of the fragments, imaging, and data analyses. In recent years, NGS technology continuously expanded the range of applications in different fields by reducing costs, increasing rates, and improving the quality of the data. The current review provided the potential applications of the NGS technology by emphasizing the diagnosis of the genetic diseases, identification of several types of cancers, prenatal screening, epigenetic modifications, personalized medicine, and identification of pathogens.

INTRODUCTION

In the early 1970s, DNA sequencing was evolved from the Maxam-Gilbert and the Sanger-Coulson methods. The Maxam-Gilbert approach was based on the radioactive labeling at the end of DNA fragments targeted to specific chemical cleavage followed by gel electrophoresis to separate the reaction products and subsequently visualized by film autoradiography. The Sanger-Coulson used a specific primer to begin the amplification at a particular position along the DNA template, and also employed the dideoxy terminator for DNA molecule that caused base-specific termination of synthesized DNA with the various labels for each nucleotide and finally by the capillary electrophoresis (CE) separated the chain termination products [1-3] . The Sanger DNA sequencing can accurately read 700-900 bp and is therefore appropriate for a particular gene sequencing. The Sanger dideoxy termination was selected as the pivotal procedure for DNA sequencing due to less chemicals and higher efficiency [4].

Despite the widespread application of Sanger sequencing in the laboratories, this technique is no longer responsive to the needs of the researchers due to some limitations such as low throughput, low speed, high cost, and timing. In order to

overcome the obstacles and improve the quality of information, a cost effective, high throughput, quick, and high accuracy technique was required that was also capable of sequencing with a little bit of genomic sample, which led to the discovery of a new technology, or a new generation of sequencing in 2005 referred to as next generation sequencing (NGS) [4, 5]. NGS, also known as massively parallel sequencing is a kind of DNA sequencing technology that produces short sequences by immobilizing millions of amplified DNA fragments on an array to determine sequence in a single run [6, 7]. This progressive technology revolutionized genomic research, biomedicine, and molecular biology, compared with the traditional sequencing methods [8].

NGS is faster than the Sanger dideoxy sequencing since the chemical reaction and signal detection are merged with each other and minimize the need for the fragment-cloning methods used with the Sanger sequencing. Among them, Illumina/ Solexa, Roche/LS454, ABI/SOLiD, and Helicos are the main technologies [9]. NGS is an efficient and promising tool to capture a large amount of genomic information and now widely applied in clinics in many fields such as molecular diagnostics, identification of polymorphisms and their association with Mendelian and complex genetic diseases, identification of several types of cancers, understanding RNA structure, transcriptomics, pathogen detection, prenatal screening, epigenetic modifications, personalized medicine, etc. [10, 11]. The current review represented the potentialities and clinical applications of NGS technology and the power of this novel genomic tool.

Overview of NGS Technology

NGS workflow includes four major steps: sample preparation, library construction, sequencing, and data analysis [12]. The process begins with dsDNA, RNA, and smaller portions of the genome as a starting material extracted from a sample to provide a template [13, 14]. Typically, the main step in sample preparation is fragmentation and size selection of the target sequences performed by chemical, enzymatic, and physical methods. In general, the physical and enzymatic manners are performed by sonication and using the endonucleases or transposases, respectively [15]. Subsequently, a certain size range of adapters is ligated to both ends of DNA fragments to construct the library, and the suitable library size is determined according to the length of insert size and the sequencing application [15]. Following adapter ligation, these libraries are targeted for sequencing directly or pre-amplified on the flowcell or the bead surface prior to sequencing to generate clusters based on the protocol and sequencing platform [15, 16]. Subsequently, the generated data should be imagined and analyzed by specific software to align with a reference genome or classified as a de novo assembly in the absence of reference genome [17]. Sequencing for de novo assembly is often performed when the microorganisms are uncharacterized or the purpose is to find out the genomic content and functional potential of the organism under exploration [9]. Therefore, improvements in bioinformatic tools as well as sequencing technology probably boost the success rate of sequencing.

Most NGS technologies use sequencing by synthesis approach [18]. In other words, to sequence DNA fragments, they should be bounded to an array, then labeled nucleotides have to be added by DNA polymerase and accordingly, high-resolution camera detects the signal from nucleotides; then after, the sequence at each spot can be interpreted by a specific computer program in order to read the DNA sequence [18]. There are various platforms or sequencers that differ in their throughput capabilities, read length, time per run and accuracy [18]; and therefore, researchers can choose one of them based on their needs.

Quality Control (QC) of NGS Technology

Since NGS workflows are complex and there is a lack of quality control programs in sequencing experiments, it is quite pivotal to set up and improve the procedures for standardization as well as quality documentation. Besides, verification and validation of sequenced data to obtain the reliable and reproducible data are crucial points [19]. Furthermore, quality control programs in NGS include several check points, which should be examined after each step such as sample preparation and library construction, before and after the sequencing run [11]. These can differ depending on the selected approach and sequencers, but generally comprise measurement of sample quality, quantity, purity, and integrity to ensure that samples with the appropriate quality maintained along the workflow [20]. Following the sample quality control, there is another QC check point in the library preparation in

NGS workflow in order to guarantee the size, purity, concentration and efficiency of ligated adaptors according to the platform requirements [21]. The last stage of QC prior to the sequencing run and size selection of amplified fragments is performed in order to remove all contaminants such as unligated adapters and biases in base composition [19, 22]. Finally, after sequencing run, it is necessary to verify and validate the obtained results using an alternative technology such as pyrosequencing [21].

Moreover, there are various kinds of software tools that can focus on quality features of NGS data [19, 20, 23]. One of the most common software to evaluate the sequenced data with a known reference genome is FastQC and also in the absence of reference genome, some pre-processing tools exist that perform in the k-mer space to assess the quality parameters such as over-represented sequences and sequencing errors to remove the bad quality reads and trimming [23-25].

Limitations of NGS Technology

In addition to the advantages of NGS technology mentioned in the introduction section, the most important challenge that can be addressed is the data analysis that requires advanced computers, specialized software, fast data processing, large data storage capabilities, and individuals experienced with NGS data analysis approaches especially regarding bioinformatics and troubleshooting to analyze and clinically interpret the data [26].

Strategies of NGS Technology

Genomics experiments are mainly interpretative and success of all experimental designs is very important both for researchers and clinicians; therefore, choosing a sequencing strategy that matches with the goal of project should be prioritized. The technology of NGS follows one of the two general strategies: whole-genome sequencing (WGS) and targeted sequencing [27, 28]. The first approach evaluates the entire genome and contains both gene-coding and non-coding regions [29]. Targeted sequencing utilizes target-specific primers for Polymerase Chain Reaction (PCR) amplification and selectively amplifies genomic areas of interest [9].

Potential-targeted strategy for NGS technology is exome sequencing, which focuses on the coding regions of the genome and can also involve either the whole exome sequencing (WES) or a panel of genes [29]. One of the main challenges for clinicians is choosing between region specific sequencing and WES, according to their applications and characteristics. Therefore, WES seems to be more cost-effective than region specific sequencing, but a region specific method has a much higher coverage of all the specific genes using the complementary approaches containing the Sanger sequencing or long-range PCR to obtain more confident results [30, 31].

Whole transcriptome sequencing or RNA-Seq is another kind of sequencing that reveals the presence and quantity of RNA in a biological sample and can evaluate the gene expression and alternative splice variants [32, 33].

Clinical Applications of NGS Technology

There are many potential applications of NGS in clinical practice due to its high-throughput and cost-effectiveness in comparison with traditional Sanger sequencing that some of its applications were summarized in the current review.

Detection of Gene Variations

Since most of diseases have a genetic basis, identification of genetic variants related to such diseases is very important and is performed by genome-wide association studies (GWAS), but detection of rare and structural variants is not possible with the genotyping arrays and needs a new technology with the potential capability of WGS [34]. Although there is a wide spectrum of DNA variations in a human genome such as insertions/deletions (indels), substitutions, and arrangements (inversions and translocations), which some of them like substitutions and small indels cannot be detected by routine sequencing methods, researchers, with the advent of NGS technology, can capture a range of novel mutations and disease causing genes by investigation of the full genome or exome without bias [35]. For instance, by applying the WES, the causal variants of Miller syndrome, as the first rare Mendelian disorder, were identified [36]. Although WES encompasses the protein-coding regions of the genome, they may not be completely covered due to the presence of the high GC content and repetitive sequences, which are poorly sequenced [35, 36].

Oncology

Cancers are induced by a broad spectrum of genomic alterations including point mutations, deletions, insertions, copy number alterations, and structural

variations, which can be somatic or inheritable. Therefore, genomics and transcriptomics (RNA) data of cancer cells and structures with new DNA sequencing technologies (NGS) coupled with powerful bioinformatic tools provide opportunities to understand pathogenesis, diagnosis, management, treatment of disease, and improvement of the personalized treatment strategies [36, 37]. These technologies have the potential ability to identify the novel mutations and alterations in the cancer genome through whole-genome and whole-exome methods in order to distinguish the somatic and germ line variants by comparing these changes with those of normal samples [38]. In addition to the referred NGS technologies, the whole-transcriptome (RNA-Seq) and ChIP-Seq are utilized to detect the RNA editing, alternative splicing, fusion transcripts, and epigenetic alterations to gain an accurate and deep understanding of the cancer transcriptome and genome [39]. Many NGS-based researches are conducted to investigate the cancer progression, metastasis, tumor complexity, heterogeneity, fusion, and tumor evolution. Also, remarkable improvements are made for lung, breast, ovarian, liver, colorectal cancers, and leukemia [40-43]. For example, using the WGS in a patient with acute myeloid leukemia, an unfamiliar insertional fusion was detected that generated a classic bcr3 PML-RARA fusion gene and the findings changed the patient's treatment schedule [44]. Clinicians can design patient-specific probes that use DNA in the patient's blood serum to observe his/her improvement and check for any signs of relapse [45, 46]. Although clinicians use the tumor biopsy as a gold standard for molecular diagnostic analysis, collection of fresh biopsies poses troubles for patients and researchers attempting to improve approaches to sequence other sources of tumor cells containing circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA) detectable in plasma [47]. NGS analysis of cell-free tumor DNA suggests a strong procedure to identify the potential mutations in cancer and match patients with suitable targeted therapies. Furthermore, NGS analysis of this method allows visualization of the tumor evolution over time and treatment [47]. Therefore, NGS technologies are widely used in the clinics for cancer prognosis and diagnosis. The researchers hope to help the clinicians by discovering more biomarkers and developing targeted therapy to find the best personalized treatment [39]. It should be kept in mind that due to the variety in the cancer genomes and phenotypes, interpretation of the NGS data also require more analysis in combination with multi-omics data and clinicopathological data in a larger sample size to achieve comprehensive and efficient results [48].

Breast Cancer

Nowadays, the next generation sequencing is widely used in gene research and plays an important role in various cancers including breast, ovary, prostate, lung, pancreatic, liver, etc. Breast cancer is a disease of multifactorial inheritance, originating from the mutations of the normal cells. In recent years, researchers gained great improvements in breast cancer, especially using NGS. The NGS in breast cancer research is mostly used in three features: genome DNA sequence analysis (i.e. the WGS, exon sequencing, targeting gene sequencing), RNA transcription sequencing (i.e. the whole transcriptome analysis, small RNA sequencing, non-coding RNA analysis), and epigenetic sequencing [49]. Researchers reported several mutations or deletions of many genes related to breast cancer such as TP53, PTEN, RUNX1, CCND3, and PTPN22 [50].

D'Argenio et al., employed the NGS to detect BRCA1 and BRCA2 mutations and reported that this method was more sensitive than the traditional Sanger sequencing [51]. In another study by Ma et al., a novel mutation of BRCA2: c.8946_8947delAG (p.D2983FfsX34) was identified in a Chinese female by NGS [52] . Some studies revealed that specific miRNA abnormalities associated with specific types of breast cancer such as miR-10b, miR-9, miR-31, miR-126, and miR-335 are connected with breast cancer metastasis [53, 54].

Thompson et al., and Kiiski et al., demonstrated in two separate studies by whole-genome and exon sequencing that the rare mutations of FANCC, BLM, and FANCM genes were the potential susceptibility alleles of breast cancer [55, 56]. Pronina IV et al., using NGS, found a strong association between hypermethylation of MIR-127 and MIR-125b-1, and breast cancer progression [57].

About 5%-10% of breast cancers are hereditary. Genetic testing such as Sanger-based sequencing is used for hereditary cancers. But this traditional approach for genetic testing of hereditary cancers is time consuming and has low throughput and high cost [58]. Although BRCA1 and BRCA2 are the most identified hereditary cancer genes, only an estimated 5%-10% of breast cancers appear in

individuals with inherited mutations in these genes in families [59, 60]. Currently, hereditary cancer testing is suggested by a variety of specialists both for affected and unaffected individuals [61, 62].

Multiple studies demonstrated that multi-gene testing identifies more individuals with hereditary breast cancer than testing for BRCA1/2 alone. The individuals with a suspected hereditary breast cancer, previously reported negative for BRCA1/2, were tested for additional genes and the results were positive in 2.9%–11.4% of the cases. In spite of the benefits of multi-gene testing, some argue that it is better to implement new DNA testing technology. Therefore, multi-gene testing allows for increased detection of hereditary cancer syndromes by utilizing the advantages of NGS technology [59]. Advances in NGS technology made it possible to test multiple genes simultaneously. Jalkh et al., studied 45 Lebanese patients with a family history of breast cancer using WES technique followed the Sanger sequencing validation. The results showed that 19 pathogenic mutations were found in 13 different genes such as ABCC12, APC, ATM, BRCA1, BRCA2, CDH1,ERCC6, MSH2, POLH, PRF1,SLX4, STK11, and TP53 [62]. Walsh et al., by the application of NGS technology, detected 21 genes associated with hereditary breast and ovarian cancers including BRCA1 and BRCA2, with inherited mutations [63]. Therefore, application of NGS in genetic testing for hereditary cancer syndromes is the first and closest step for its transition into clinical phase [64].

Epigenetic

Epigenetics is the science that studies the heritable modifications in gene expression that do not contain the DNA sequence [65]. There are two groups of epigenetic modifications: DNA methylation and post-translational modifications of histone. Recently, microRNA (miRNA) gene expression regulation was classified as epigenetic modifications [66]. Epigenomics implies the complete study of these alterations across the whole genome. Epigenetic mechanisms have major role in the growth of cells and normal development [67, 68]. Aberrant epigenetic changes can be contemplated as one of the causative factors in cancer [69]. Analysis of the epigenetic modifications is a key factor to understand the heterogeneity and complexity of human that despite possession of identical genome, various cell types express their genes in different ways (epigenome) [70]. Therefore, it is highly important to investigate the profile of epigenome in a cell to be used as epigenetic biomarker for prognosis, diagnosis, and therapeutic applications [71, 72]. DNA methylation analysis is the most frequent application of NGS in the field of epigenetics. NGS technologies using methylated DNA immunoprecipitation (meDIP) and bisulphite methods can represent the properties of the methylated DNA to increase the understanding of specific cell-type expression patterns that cannot be explained at the genetic level [73]. Chromatin immunoprecipitation followed by NGS (ChIP-Seq) are applied to study the location of transcription factors, the transcription factor binding, and histone modifications, which comprises acetylation, methylation, phosphorylation, etc., at the whole-genome level [71].

Currently, by the combination of methylation array approach with massive DNA methylation analysis and RNA expression profiles, a large number of genes and miRNAs are identified under epigenetic regulation in many tumors such as colorectal, renal, prostate, and non-small cell lung cancer (NSCLC) [70, 74-76]. One of the earliest incentives to investigate the epigenetic modifications through NGS in clinical samples is achieving the extra information that can be gained from the pharmaco-epigenomics; it means that the presence of methylation at particular genes in certain cancers is associated with the clinical reaction to treatment that is extremely important to access the most effective treatment in the clinical epigenetics field [70].

Prenatal Diagnosis

Prenatal diagnosis contains features relating to the health of both the fetus and the parents [77]. Current methods including the combined test and invasive procedures (amniocentesis and chorionic villus sampling (CVS) used to screen the fetus for chromosomal abnormalities pose a risk to mother and fetus [78]. In addition to the risk, the rate of abortion related to CVS and amniocentesis is 1.0% to 2.0% [79] and the false positive rate of combined test is 5.0%-9.0% [80], and sometimes mothers with healthy fetuses may be selected for unnecessary invasive diagnostic tests that lead to spontaneous abortion [81]. Therefore, replacing the current invasive tests with Non Invasive Prenatal Diagnosis (NIPD) test reduces the risk and increases the detection rate for the three most prevalent aneuploidies; Down syndrome (trisomy

21), Edward syndrome (trisomy 18), and Patau syndrome (trisomy 13) [78] . The NIPT is based on the finding of cell-free fetal DNA (cffDNA) in maternal plasma detectable as early as four weeks gestation [47, 82], making NIPT accessible earlier in pregnancy in comparison with invasive methods [83]. The cffDNA analysis with the NGS technology can be performed on a blood sample taken from the pregnant mother, that is cffDNA is sequenced and the reads are mapped to each chromosome and then counted, and it can be calculated whether a chromosome is over- or underrepresented [84, 85]. The main advantages of NGS for prenatal diagnosis are that NGS can be used to analyze non-invasive samples and has the capacity to recognize micro chromosomal abnormalities [86].

Personalized Medicine

Traditional medical model to detect and treat disorders is highly expensive for patients and healthcare system. Therefore, it is necessary to apply an innovative approach such as NGS technology to accelerate the early detection of disorders [87]. Personalized medicine (PM) is an approach to medical diagnosis, treatment, and risk assessment based on an individual's genetic in order to improve health care for the individual and predict which medical treatments are proper for the patient [88, 89]. Application of the PM can decline financial and time expenditures, and increase the quality of life in patients [90]. PM separates individuals into subpopulations that show different responses to a therapeutic agent for their specific disease. For example, Herceptin is a useful drug for patients with breast cancer and elevated expression of HER2. However, some patients with increased HER2 are resistant to Herceptin due to mutations to the HER2 gene. Therefore, molecular identification of patients with breast cancer allows for the optimal application of Herceptin through stratification of the patients [91].

To date, many studies applied NGS methods for personalized treatment of cancer. For example, NGS is used to treat pancreatic cancer [64]. It is also used to detect epidermal growth factor receptor (EGFR) deletions in NSCLC [92]. There is general agreement that NGS should be the standard method when several genes should be tested in the same patient. For instance, patients with estrogen receptor-positive breast cancer should be tested for mutations in PIK3CA, ESR1, AKT1, and ERBB2. It seems that NGS possibly becomes the standard method to diagnose genomic alterations in breast cancer [47].

Clinical Microbiology

NGS is applied in medical microbiology as a powerful tool for molecular case finding, outbreak management, bacterial typing, determination of biological properties such as the presence of virulence factors, fast recognition of bacteria through the 16S rRNA region that requires bacterial isolates, antimicrobial agent resistance and metagenomics approaches that may be applied directly on the sample [93, 94]. NGS technology, using the WGS approach, is performed to detect highly-virulent bacteria; for example, Shiga toxin-producing Escherichia coli that is liable for great outbreaks [95]. Another application of NGS technology in medical microbiology is molecular case finding performed within a few hours. Some cases are reported in Denmark, Germany, and the Netherlands in hospitalized patients to screen the mcr-1 gene in Enterobacteriaceae isolates, which is in charge of colistin resistance [96-98].

NGS is also helpful to identify novel resistance genes such as antibiotic resistance genes in bacteria [99]. NGS, using the culture-independent methods, allows researchers to sequence a number of pathogens directly from biological samples [100]. This approach is referred to as metagenomics and can detect all micro-organisms in a clinical sample without the prior need for culturing [101]. There are some reports about the metagenomics strategy that was favorably performed to sequence the eukaryote Plasmodium falciparum from a blood cell-depleted sample and the bioterrorism agent Francisella tularensis from abscess pus [102, 103].

NGS is an ideal tool in epidemiological typing since the typing of bacterial strains is crucial to study the transmission pathways and identify the single genomic alterations between two isolates [104]. Identification of the toxins known to cause severe diseases such as toxic shock syndrome caused by Streptococcus spp. is an important field in clinical microbiology performed by WGS [105]. Therefore, NGS technologies are helpful in various fields in the clinical microbiology.

Future Directions

In the near future, NGS technologies are very helpful for clinical purposes. The fast and high throughput sequencing method is considered as a good diagnostic and prognostic tool, which helps

clinicians determine specific features in each patient, and opens the road towards personalized medicine. But similar to other new technologies, there are still many technical, analytical, and ethical issues that need further processing.

CONCLUSIONS

NGS technologies represent a revolutionary tool for numerous applications and can produce multiple repeats in a single run. These technologies are increasingly used in various fields such as genome and transcriptome sequencing, polymorphism detection, mutation mapping, DNA methylation, histone modifications, alternative splicing identification, small RNA profiling, DNA-protein interactions, protein-protein interactions, sequencing of the mitochondrial genome, personal genomics, and diagnosis and treatment of common diseases due to their speed, cost-effectiveness, and high-throughput nature. DNA sequencing technologies are used as a clinical diagnostic tool and it takes the support systems at least a decade to develop and validate data analysis and interpretation for clinical diagnostic use.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ETHICS APPROVAL

The current study was approved by the Ethics Committee of Saman Tajhiz Noor Laboratory Diagnostic Network Company.

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