Invest Clin 65(4): 476 - 494, 2024 https://doi.org/10.54817/IC.v65n4a09

High throughput sequencing technology and its clinical application in circulating tumor DNA detection in patients with tumors.

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Keywords: high throughput sequencing; tumor; circulating tumor DNA; tumor diagnosis; tumor treatment; tumor prognosis.

Abstract. The high-throughput sequencing (HTS) is now a highly favoured technology in the field of genome research. A distinctive feature of this sequencing method is its data-yielding capability, which is capable of generating more than 100 times than the first-generation Sanger sequencing platform. HTS technology has been widely adopted for its advantages, including high throughput, sensitivity, automaticity, information density and cost-effectiveness. Not only does it help in the treatment and diagnosis of multiple diseases, but it also provides new insights into the research in molecular biology of tumors. Moreover, circulating tumor DNA (ctDNA) tests based on HTS technology are increasingly extensively implemented for clinical purposes. In this review, we will focus on the significant achievements and performances of the HTS, and first-hand data from extensive experience will be summarized and analyzed to discuss the advantages and specifics associated with each sequencing system and further summarize the characteristics of their clinical applications.

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Tecnología de secuenciación de alto rendimiento y su aplicación clínica en la detección de ADN tumoral circulante en pacientes oncológicos.

Invest Clin 2024; 65 (4): 476 – 494

Palabras clave: secuenciación de alto rendimiento; tumores; ADN tumoral circulante, diagnóstico de tumores; tratamiento de tumores; pronóstico de tumores.

Resumen. La secuenciación de alto rendimiento (HTS) es una tecnología popular en el campo de la investigación genómica. Una característica distintiva de este método de secuenciación es su capacidad de generación de datos, que puede generar 100 veces más datos que la Plataforma de secuenciación Sanger de primera generación. La tecnología superconductora de alta temperatura es ampliamente utilizada debido a sus ventajas de alto rendimiento, alta sensibilidad, automatización, densidad de información y rentabilidad. No solo ayuda a tratar y diagnosticar múltiples enfermedades, sino que también proporciona nuevas ideas para la investigación de biología molecular tumoral. Además, la detección de ADN tumoral circulante (ctDNA) basada en la tecnología HTS se utiliza cada vez más ampliamente con fines clínicos. En esta revisión, nos centraremos en los principales logros y rendimiento de HTS, y resumiremos y analizaremos datos de primera mano de una amplia experiencia, discutiremos las ventajas y detalles específicos de cada sistema de secuenciación y resumiremos aún más las características de su aplicación clínica.

Received: 15-08-2024 Accepted: 17-10-2024

INTRODUCTION

The past decade has witnessed the introduction and broad application of HTS technologies. Not only can it perform chromosome mapping, but it can also conduct deep sequencing and whole genome sequencing analysis on blood, body fluids and excreta such as urine, feces, sputum, cerebrospinal fluid, sperm, saliva, vaginal secretions, milk and effusions [1-7]. This revolutionary technology facilitates diagnosis at the genetic level and is especially suitable for complex diseases that are highly heterogeneous and involve both genes and mutations, such as tumors[8]. ctDNA is an important biomarker, a circulating cell-free DNA (cfDNA) generated by the apoptosis, necrosis and secretion process of tumor cells, and it contains relatively complete genetic information about tumour cells [9]. Additionally, it contains the same mutations as the DNA in tumor cells, including insertions, deletions, rearrangements, copy number variations and methylations. Therefore, using HTS technology to test for ctDNA can provide crucial information on the diagnosis, treatment and prognosis of tumors [10].

In this review, we introduced the main features of the HTS, such as first-generation Sanger sequencing, second-generation HTS platforms (454 Life Sciences pyrosequencing, Illumina/Solexa technology and SOLiD ligase-mediated sequencing) and third-generation high throughput - next generation sequencing (HT-NGS) platforms (Ion Torrent technology, Single-molecule real-time (SMRT) sequencing and Nanopore sequencing technology). In addition, this article also

describes the application of ctDNA in the diagnosis, treatment and prognosis of tumors.

Overview of high-throughput sequencing platform

First-generation Sanger sequencing

In the mid-1970s, DNA sequencing saw a major technological innovation, the Sanger dideoxy synthesis method, proposed by Sanger. The advent of this method provided scientists with a new means of determining four different nucleotide bases in single-stranded DNA using radio-labelling [11,12]. Sanger sequencing technology, as a landmark development in the field of DNA sequencing, has dramatically advanced the process of genomics research. However, this technology has certain limitations in practical application, i.e., the amount of DNA that can be processed in each experiment is limited. Although this limitation made Sanger sequencing unable to meet the demand for high throughput in some cases, it laid the foundation for developing subsequent sequencing technologies. In pursuing higher throughput and more efficient sequencing technologies, scientists have made continuous efforts and eventually succeeded in developing the second, third and even higher throughput sequencing platforms [13-15]. Their high throughput and accuracy enable researchers to access genetic information more quickly and accurately, providing powerful support in areas such as disease diagnosis, personalized medicine and biotechnology.

Second-generation HTS platforms

The second-generation HTS uses a different analysis principle than the first-generation Sanger sequencing. The key technologies of the second-generation HTS platform include bridge sequencing and synthetic sequencing. These technologies have enabled the platform to have a wide range of applications in areas such as genomics research, variant detection and gene expression analysis. However, despite the significant advances in sequencing length and accuracy of the second-generation platforms, they still have some limitations, such as shorter read lengths and higher error rates.

454 Life Sciences pyrosequencing

A unique sequencing method has attracted much attention in exploring the early development of NGS technologies. In 2000, Jonathan Rothberg successfully developed the first commercially available NGS platform, which was innovative in that it used a unique mechanism to read the signals of individual nucleotides added to a DNA template. It utilizes the properties of luciferase, which generates light signals when new nucleotides are added to a DNA strand, and these signals are subsequently captured and converted into readable data[16]. This method combines pyrosequencing technology with single-molecule emulsion PCR; sequencing is done through a synthetic process in which four nucleotide bases are added one by one to a DNA template. Each time a new nucleotide is added, it triggers the production of a different coloured light, which is caused by the release of pyrophosphate from the microwells [12,17]. In general, pyrosequencing is centred on the concept of "sequencing by synthesis", which is in sharp contrast to the traditional Sanger sequencing method, which is done by detecting the release of pyrophosphate to determine whether a specific nucleotide has been added to the DNA strand. Pyrophosphate is released when a nucleotide is added to a growing DNA strand. These pyrophosphate releases are detected, and a signal is generated. By monitoring these signals, we can determine the bases on the DNA template strand at each position. The essential advantage of this method is that it does not require ddNTPs, which means that no terminating strand synthesis is required during sequencing, thus increasing the accuracy and efficiency of sequencing[14,18]. During the nucleotide doping, more than one nucleotide may be doped into the same position. This situation leads to the

formation of a homopolymer because the nucleotide used lacks molecules capable of preventing further doping. This fully doped homopolymer will form in one cycle [19].

With the rapid development of NGS technologies, various sequencing platforms are emerging. Roche discontinued support of the platform in 2016, which resulted in the platform being phased out, and other more efficient and accurate sequencing platforms are gradually replacing it as technology advances. This change reflects the rapidly evolving field of sequencing technology and the importance of continually updated platforms and technologies.

Illumina/Solexa technology

In 2006, Solexa introduced an innovative sequencing technology that employs a reversible terminator strategy to enhance adapter-linked DNA fragments through bridge amplification. This method allows the bases on the template strand to be read employing a nucleotide-by-nucleotide process that involves successive nucleotide doping, a cleaning step, imaging, and a subsequent cleavage step [14,15,20,21]. The extraction and segmentation of DNA is the primary step aimed at breaking down complex DNA molecules into smaller, manageable fragments. This process usually involves using specific enzymes to cut the DNA, resulting in fragments of a certain length. Next, these DNA fragments need to be ligated to specific adapter sequences to facilitate subsequent sequencing steps. Adapter sequences are short pieces of DNA or RNA sequences that bind to the ends of the DNA fragments and provide an interface for connection to the sequencing machine. Once the adapter sequences have been successfully ligated to the DNA fragments, these conjugates are transferred to a flow cell. The flow cell is a unique device that precisely positions the DNA fragments on the cell surface. The DNA fragments are copied in large numbers through clonal amplification, forming clonal "clusters". These clusters consist of many identical single-stranded DNA fragments that are physically tightly packed to facilitate high-throughput sequencing by sequencing machines [17,19,22].

During each sequencing cycle, four fluorescently labelled nucleotides compete for the opportunity to bind to the template strand. This is a competitive process in which only nucleotides complementary to the corresponding nucleotide on the template strand can be successfully doped. Once a nucleotide has been doped, the laser detects a signal due to fluorescent labelling, identifying which nucleotide has been doped into the template strand. After identification, the next step is to remove the blocking group and fluorescent marker from the doped nucleotide. This step is in preparation for the next sequencing cycle, making the template strand available again for new nucleotides to be doped. During this process, the nucleotide sequence on the template strand is gradually built up, with each sequencing cycle adding a point of information for the final determination of the entire DNA sequence. The efficiency of this sequencing strategy lies in its ability to quickly and accurately determine the DNA sequence by detecting fluorescent signals and complementary nucleotide incorporation. As the sequencing cycle is repeated, sequence information of the entire genome is gradually revealed [21-23].

Illumina sequencing has become an indispensable tool in modern genomics research. This technology supports a wide range of sequencing protocols, covering areas ranging from comprehensive sequencing at the genome level to more specific exon sequencing, targeted sequencing, and macrogenomics for studying microbial communities. It is also widely used for methods such as RNA sequencing, chromatin immunoprecipitation sequencing (CHIP-seq) and methylome analysis [17].

However, despite the Illumina sequencing platform's market-leading position due to its high output capacity and broad applicability, this short-read technology still has limitations in certain areas. In particular, in

many applications in genomics, short read lengths limit resolution and accuracy. This means that when high precision is required to resolve genome structure or identify low-frequency variants, short-read technologies may not provide sufficient information [13]. Therefore, for these specific research needs, it may be necessary to use a combination of other sequencing technologies, such as long reads or single molecule sequencing, to obtain higher-resolution sequence data. Despite these limitations, Illumina sequencing technology continues to be essential in advancing genomics and biomedical research.

SOLiD ligase-mediated sequencing

In 2007, Applied Biosystems introduced a new sequencing platform, Supported Oligonucleotide Ligation Detection (SOLiD). This platform is similar to other sequencing technologies in that it detects the fluorescence intensity of dye-labelled molecules to determine the sequence of DNA fragments. However, the SOLiD platform employs a unique sequencing technique known as DNA ligase-based sequencing [12,24]. A distinguishing feature of this technique is that it generates relatively short read lengths, typically 35 base pairs [25,26]. Nonetheless, the SOLiD platform was a significant breakthrough at the time, providing new tools for genomics research.

The technology involves cutting the template DNA into small fragments and ligating them to a known junction sequence. This process ensures that the DNA fragments can be efficiently captured and sequenced. Next, these junction-connected DNA fragments are transferred to a particular type of beads, which are subsequently immobilized on a glass surface. These fragments can be clonally amplified on the beads using emulsion PCR, resulting in many identical DNA fragments. During the sequencing stage, the sequence of each DNA fragment is determined by a two-base colour coding method. This method relies on different dye pairs to identify and record each nucleotide in the DNA sequence. The SOLiD sequencing platform is particularly adept at detecting single nucleotide polymorphisms (SNPs), which can be detected with an astonishing 99.85% accuracy. This high level of accuracy makes SOLiD a powerful tool for genomics research, especially for SNPs detection. With this kind of precise sequencing, researchers can better understand genetic variations and their relationship with diseases, providing a scientific basis for personalized medicine and disease diagnosis [12,27,28].

Like other NGS systems, SOLiD's computational infrastructure is more costly and less convenient to operate. Nonetheless, SOLiD technology has been widely used in several fields, including but not limited to whole genome resequencing, transcriptomics research, targeted resequencing, and epigenomics analysis. Currently, these three leading second-generation high-throughput sequencing platforms are available on the market, as shown in Fig. 1. The commercialization of these platforms provides powerful tools for researchers and promotes research progress in related fields. Meanwhile, with the continuous development of science and technology, more sequencing platforms are under development and are expected to join this competitive market in the future. This will help further promote the development of genomics research and provide more possibilities for biomedical research.

Third-generation HTS platforms

Third-generation sequencing technology is gradually changing our understanding of genomics. Compared with the previous two generations of platforms, the advantage of third-generation sequencing is that it provides longer read lengths and higher accuracy. The third generation of HTS platforms uses single-molecule sequencing technology, capable of simultaneously sequencing millions to billions of DNA molecules. The core of this technology is represented by the Ion Torrent technology, Single Molecule Real-Time Sequencing and Nanopore sequencing technology, as shown in Fig. 2.

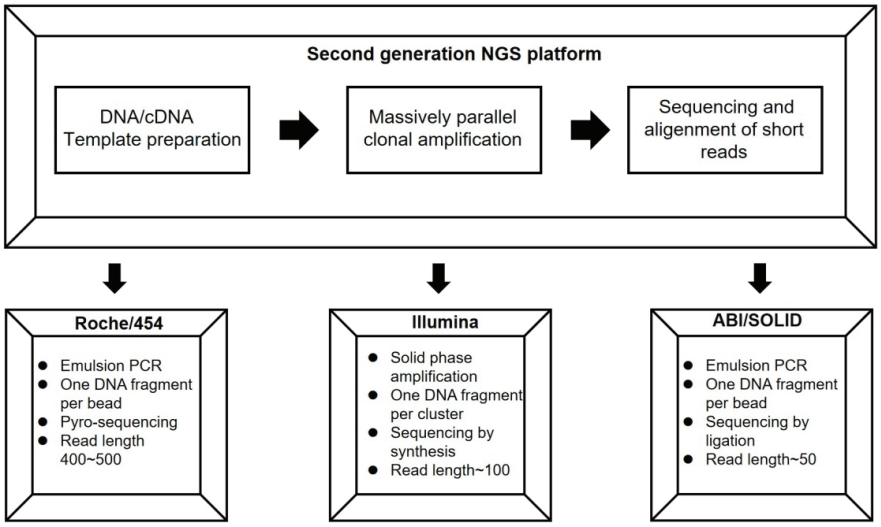

Fig. 1. Characteristics of the three leading second-generation HTS platforms.

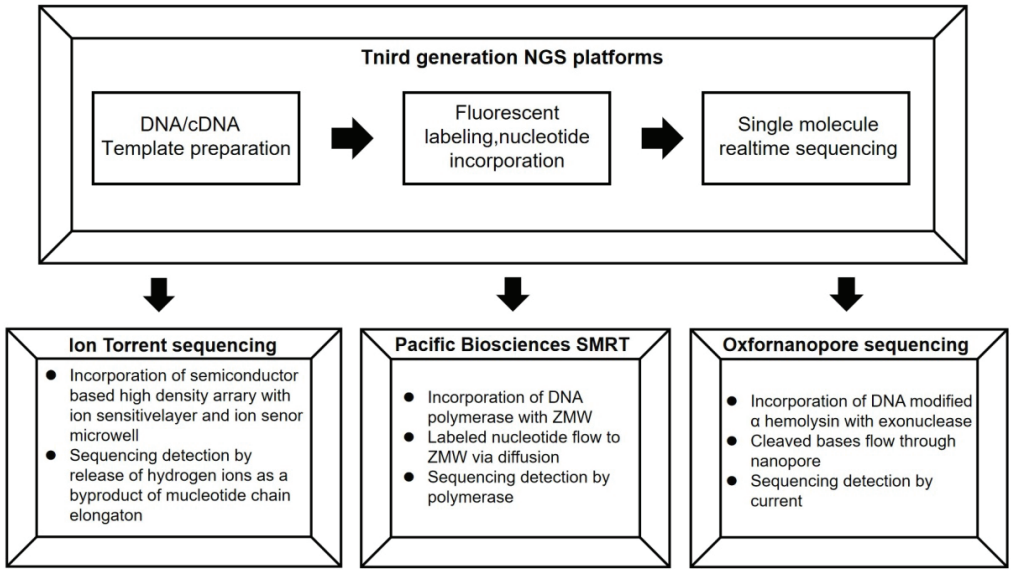

Fig. 2. Characteristics of the three leading third-generation HTS platforms.

Ion Torrent technology

Ion Torrent technology is a nucleotide synthesis-based sequencing (SBS) method, which has many similarities in principle with the 454 pyrophosphate sequencing platform, but the technical means used in detecting the correct insertion of nucleotides differs between the two methods. In Ion Torrent technology, molecules are first fragmented, and then these fragmented molecules are bound to the surface of specific beads. Next, the target molecules on these beads are clonally amplified by emulsion PCR, resulting in a large number of identical target molecules on each beads. This step is a core component of the Ion Torrent technology, ensuring effective capture of target molecules and providing efficient sequencing. After this process, each bead can be regarded as an independent sequencing reaction unit, which lays the foundation for the subsequent sequencing steps [12,17,20,22].

Treated beads are dispensed into tiny holes on the chip during sequencing. These microholes form a microarray, with each hole corresponding to a specific sequencing reaction. When beads are placed in these microholes, a synthetic-based sequencing reaction is performed on each bead [22,29]. During DNA replication, each newly added nucleotide causes a slight change in the pH of the solution. This change is captured by the sensor and converted into a voltage signal, enabling the monitoring of nucleotide addition. Specifically, when a nucleotide pairs successfully with a complementary base on the DNA strand, it releases a hydrogen ion, causing the pH to drop. This pH change is detected by the sensor and converted into a voltage signal. No voltage spike occurs if no nucleotides are added in this round. Two hydrogen ions are released when two neighbouring nucleotide sites are filled with the same nucleotide simultaneously, causing the voltage signal to double. This doubled voltage change provides a direct signal to distinguish between neighbouring nucleotides. By continuously monitoring the addition of nucleotides and the corresponding voltage changes during each sequencing cycle, we can accurately determine the sequence of bases on a DNA strand [17,22].

Ion Torrent technology is favoured in the sequencing field for its highly efficient detection system, which does not rely on expensive cameras, light sources or scanners, resulting in a significant increase in detection speed compared to traditional 454 pyrophosphate sequencing [17]. This advantage has made the Ion Torrent method the preferred choice for various applications. However, the technology is not without its limitations. Studies have shown that while there is a correlation between the number of base integrations and voltage changes, it is not a perfect relationship. As a result, the problem of homopolymer template elongation remains, which is caused by cumulative light intensity changes [30]. Despite this challenge, the Ion Torrent technology remains essential in modern gene sequencing due to its speed and accuracy.

Single-molecule real-time (SMRT) sequencing

In 2011, Single Molecule Real-Time Sequencing (SMRT) technology was introduced by Pacific Biosystems, marking a new advancement in the field of gene sequencing on long-read platforms. This technology is unique because it can sequence up to 30-50 kb or longer DNA fragments, far beyond what conventional sequencing technologies can handle [17,31]. SMRT sequencing centres on the tight binding of a specially formulated DNA polymerase to the target DNA, a process that occurs in SMRT cells [17]. The design of these SMRT cells is unique in that they contain tens of thousands of tiny chambers, each equipped with a DNA polymerase and a zero-mode waveguide (ZMW) well[23]. This innovative design allows the sequencing process to be performed at the level of individual molecules, resulting in efficient and accurate sequencing of DNA sequences. ZMW plays a crucial role, which is a tiny structure that precisely directs light energy to a specific, relatively small-sized region. This property makes the ZMW a critical factor in enabling single-molecule sequencing[17]. In preparation for sequencing, the SMRT technology takes an innovative approach, unlike traditional methods of fixing DNA strands. Specifically, high-fidelity DNA polymerase and single-stranded DNA templates are added to the SMRT cell chamber to serve as templates for DNA replication at the bottom of the ZMW [23,27]. This design allows the DNA replication process to occur at the level of individual molecules, resulting in efficient and accurate sequencing of DNA sequences. An essential step in this process involves the addition of different phosphorylated fluorescein markers to these tiny chambers. These markers are present to enable precise monitoring of DNA polymerase activity. When DNA replication occurs, DNA polymerase must recognize and integrate nucleotides

complementary to the template DNA. Phosphorylated fluorescein markers added to the ZMW minicells play a crucial role in this process. They can interact specifically with DNA polymerase activity, thus enabling researchers to distinguish and detect different events during DNA replication [23].

In ZMW technology, integrating nucleotides is a crucial step that triggers the release of fluorescein. Once the fluorescein is released from the bottom of the ZMW, it is no longer in the detection state and emits a specific fluorescent signal. The uniqueness of this fluorescent signal means that each nucleotide produces a different fluorescence pattern. In practice, the fluorescence signals from the tiny chamber of the ZMW are captured by a high-definition video system and converted into digital signals. These digital signals are then analyzed to determine the nucleotide sequence on the DNA template. Since each nucleotide has unique fluorescence properties, the sequence of the DNA can be determined by identifying and interpreting these fluorescence signals [15,17,23]. In SMRT cells, up to one million ZMWs are integrated on a single chip. These tiny chambers are the core units in the sequencing process, performing both nucleotide integration and imaging. Each ZMW independently captures and records real-time images of nucleotide integration. This ability to process in parallel dramatically increases the speed and throughput of sequencing [15,17].

Nanopore sequencing technology

As an innovative single-molecule sequencing method emerging in recent years, Nanopore sequencing technology has made significant progress in genomics research. Unlike traditional sequencing techniques based on nucleotide integration, nanopore sequencing technology offers an entirely new strategy. This technology utilizes nanopores as sensors to directly detect biological macromolecules, such as DNA and RNA, at the single-molecule level, thus enabling real-time monitoring at the single-molecule level [15,32].

Nanopore sequencing technology eliminates the cumbersome PCR amplification and chemical labelling steps during operation. This means that researchers do not need to perform complex pre-processing of samples when performing sequencing, greatly simplifying the experimental process. More importantly, this technology allows direct use of cell lysates for sequencing without the need for additional sample preparation, which not only saves experimental time but also reduces experimental costs. These advantages make nanopore sequencing technology more efficient and more widely applicable in single-molecule sequencing [27].

Nanopore sequencing technology is an innovative DNA sequencing method that utilizes protein nanopores embedded in a polymer membrane. In this process, when a DNA molecule passes through a nanopore known as a molecular motor protein, it causes a disorder in the nanopore protein, which generates electrical signals. The conversion of these electrical signals is the core principle of nanopore sequencing technology, which enables researchers to determine the DNA sequence by analyzing these signals[14,27,33,34]. Based on nanopore sequencing technology, researchers can determine the sequence of a DNA molecule by detecting changes in the electrical currents it generates as it passes through the nanopore. The key to this technique is that each nucleotide causes unique current changes that can be detected and recorded precisely. By analyzing these current changes, we can tell the sequence of nucleotides in a DNA molecule [23].

The nanopore technology could offer a wide range of applications in fields such as personalized medicine, agriculture and scientific research. In addition, the portability of nanopore sequencing technology allows sequencers to be taken into the field for direct sequencing, greatly expanding the range of sequencing applications and increasing the efficiency of field studies [14,32-37]. This technology provides a direct and efficient means to study the properties of DNA and is essential

for research in genomics, molecular biology and other related fields.

Application of HTS technology in tumor diagnosis and treatment

Tumors have claimed numerous lives as malignant cells continuously mutate and evolve as they divide. Most patients died from fatal metastasis and cancer recurrence. However, with mature HTS technology, researchers have obtained significant genetic information on tumor pathogenesis, drug resistance and metastasis, which will help in the early screening and identification of tumors, selection of therapies and monitoring of prognosis.

Diagnosis of tumors

Early diagnosis and prompt treatment are critical to controlling the growth of tumors. Genetic testing is characterized by the ease of sampling, which allows researchers to minimize the harm to their subjects. In order to alleviate the pain associated with traditional invasive pathological biopsies, researchers have turned to body-fluid-based sequencing to assist in the diagnosis of cancer. Circulating tumor DNA (ctDNA) is derived from single- or double-stranded DNA and DNA-protein complexes shed by tumor tissue. The presence of mutated DNA fragments is observed at relatively high concentrations in the circulation of most patients with metastatic cancer and low but detectable concentrations in a significant proportion of patients with localized cancer. This characteristic ensures that ctDNA exhibits particular specificity and can serve as a biomarker for clinical purposes [38].

Lung cancer is one of the most common cancers, and its early diagnosis plays a crucial role in improving patients' quality of life. However, low-dose computed tomography (LDCT), a widely used method for early detection of lung cancer, cannot accurately distinguish between malignant and benign lung nodules. Many studies have shown that HTS technology can detect specific mutations strongly associated with lung cancer in ctDNA extracted from body fluid samples[39-41]. Sumitra et al. identified tumor-related alterations in 94% of patients with limited-stage small cell lung cancer (LS-SCLC) and 100% of patients with extensive-stage small cell lung cancer (ES-SCLC) by performing targeted ctDNA sequencing and whole genome sequencing on their body fluid samples. This study demonstrates that targeted ctDNA sequencing can identify potential treatment targets in more than half of the patients and shows advantages over traditional invasive biopsy [42].

Additionally, Peng *et al.* have developed a method to determine whether a lung tumor can be surgically removed by performing ultra-deep sequencing on the targeted mutations in ctDNA. Moreover, the accuracy of ctDNA testing is 63%, 83%, 94%, and 100% for stages I, II, III, and IV lung cancer, respectively. The overall sensitivity comes to 80%, considering age and serum biomarker tests, and the specificity rises to 99% [43]. This body-fluid-based screening can reduce the risk of unwanted damage and metastasis and assess the tumor progression by analyzing tumor mutation load.

Based on previously mentioned evidence, it can be concluded that HTS technology is also widely used to diagnose other types of tumors. Himisha et al. have discovered that the genome characterization of castration-resistant neuroendocrine prostate cancer (CRPC-NE) can be identified by analyzing ctDNA in patients' plasma samples. This reduces the trauma associated with traditional invasive pathological biopsies and provides timely guidance for clinical adjustment of medication [44]. Cai et al. developed a genetic diagnosis model based on whole-genome analysis of 5-hydroxymethylcytosine (5hmC) obtained from cfDNA samples of 2,554 individuals. The model demonstrated superior diagnostic performance to AFP and could distinguish between early-stage hepatocellular carcinoma (HCC) and non-HCC with an AUC of 0.884 in the

validation set [45]. In addition, Yurika et al. found that the methylated SEPT9 gene in serum ctDNA was highly significant in the early diagnosis of HCC with both high sensitivity and specificity[46]. However, it should be noted that although HTS-based body fluid biopsy technology provides a new direction for tumor diagnosis, most of the test results still need to be combined with other imaging or serological indicators and cannot be used independently as a diagnostic gold standard.

Furthermore, it is necessary to validate the test in a larger population. In addition, the lack of research on related gene test chips has severely limited its application scope. However, this problem can likely be solved with the development of technology and the related industrial chain.

Treatment for tumors

It is important to note that low-frequency mutations can occur during the division of tumor cells. Therefore, tumor cells can carry different genetic mutations even if they appear histologically similar, while different tumor cells can carry the same mutations. By comparing the gene sequences of primary and metastatic neoplasms, physicians can assess the effectiveness of their treatment and develop personalized therapies. They also gain insight into the potential mechanisms behind tumor drug resistance, laying a solid foundation for accurate diagnosis and treatment of tumors.

Targeted therapy

Today, several signalling pathways and associated mutations associated with tumor formation have become effective drug targets. As a result, targeted therapies based on tumor genes have become mainstream. As the most common BRAF mutation, the V600E mutation is strongly associated with the particular invasiveness of rectal cancer cells in metastatic colorectal cancer cases. Resistance to chemotherapy has been observed in cases with V600E mutations, and resistance to EGFR inhibitors is gradually increasing. Scott et al. demonstrated that using NGS technology, the concomitant use of EGFR and BRAF inhibitors in combination with irinotecan was effective in patients with the V600E mutation. The discovery informed the development of new targeted therapeutic regimens and laid a solid foundation for new targeted therapies [47].

Dasatinib is a small-molecule tyrosine kinase inhibitor that can be used as a first- and second-line treatment for gastrointestinal mesenchymal tumors (GIST). Zhou et al. found that dasatinib can be employed as a preferred treatment option for patients with wild-type GIST or GIST with the D842V mutation who are unable to take regorafenib, as they identified genetic variations related to the tumor signalling pathway by NGS technology [48]. Christian et al. investigated the clinical efficacy of the combination of ibrutinib, rituximab and high-dose methotrexate (HDMTX) in central nervous system lymphoma (CNSL). The results proved that the combination is safe and effective. In addition, the analysis of ctDNA in cerebrospinal fluid samples enables researchers to monitor disease progression in patients with CNSL and to adjust targeted therapy [49] promptly. In recent years, site-specific therapies (including HTS-guided targeted therapies) emerged as a promising option for patients with metastatic cancer of unknown primary site (CUP). A phase 2 clinical trial conducted at 19 institutions in Japan by Hidetoshi et al. demonstrated that CUP site-specific therapy based on NGS technology had favorable survival outcomes.

Moreover, targeted therapy based on the tumor-associated mutations showed excellent therapeutic responses, even in patients with CUP [50]. In conclusion, NGS technology can provide a reference for clinical drug selection and efficacy evaluation by detecting target genes and other tumor-related genes, which has great potential in pursuing individualized treatment today. Nevertheless, it is essential to acknowledge the current limitations of the evidence base, primarily de-

rived from small, single-ethnic clinical trials. When the mutation type is considered rare, the subjective decision to administer chemotherapy over targeted therapies in the clinic may also affect the study results.

Chemotherapy

Chemotherapy has consistently been the focus of research. as one of the leading traditional means of treating tumors. In addition, gene sequencing technologies, represented by HTS technology, have undoubtedly facilitated the chemotherapy of tumors. Most patients diagnosed with triple-negative breast cancer (TNBC) receive neoadjuvant chemotherapy. Approximately one-third of them can achieve complete pathological remission with neoadjuvant chemotherapy, but two-thirds will have residual disease and a high risk of recurrence. Milan et al. conducted ctDNA sequencing using HTS technology in 196 early-stage TNBC patients with residual disease after neoadjuvant chemotherapy and circulated tumor cell (CTC) analysis in 123 patients. The results showed that the presence of ctDNA and CTC in early-stage TNBC patients after neoadjuvant chemotherapy was associated with distant tumor metastasis. The detection of ctDNA and CTC in early-stage TNBC patients after neoadjuvant chemotherapy was found to be independently associated with disease recurrence and serve as an important indicator for assessing patient status after future neoadjuvant chemotherapy [51]. Colorectal cancer is one of the most common tumors worldwide, with surgical resection, chemotherapy, radiotherapy, and targeted therapy representing the principal treatment modalities. Resistance to chemotherapy drugs in some tumor patients has also posed a significant challenge for clinicians. Li et al. from Peking University combined the i-CR platform with HTS technology to construct a new in vitro tumor model, enabling personalized drug testing and personalized treatment for patients within 2-3 weeks and providing an entirely new treatment option for colorectal cancer [52].

Osteosarcoma is one of the malignant tumors for which early and effective preoperative chemotherapy is crucial to the survival of patients. However, the previous combination of methotrexate (MTX), doxorubicin (DOX), and cisplatin (DDP) has been found to have significant differences in therapeutic efficacy among patients, with a higher incidence of adverse effects. Thus, there is an urgent need to develop new drug combinations. Zhang et al. from Central South University revealed the heterogeneity of potential therapeutic target genes. They elucidated the synergistic mechanism of DOX and HDACs inhibitors for treating osteosarcoma through RNA sequencing and second-generation sequencing (HTS) of osteosarcoma samples. This provides a foundation for developing entirely new chemotherapeutic drug combinations and will undoubtedly inspire the subsequent researchers [53]. Researchers can also develop effective chemotherapy sensitizers by studying drug-resistance genes and resistance mechanisms. A team of researchers has already demonstrated that SMOi inhibitors can release the drug resistance of breast cancer tumor cells and improve their chemosensitivity to doxorubicin by studying the resistance mechanism in breast cancer dependent on the Hh signalling pathway [54]. Based on sequencing relevant tissue or body fluid samples from patients using HTS technology, physicians can comprehensively assess the expected therapeutic effects before chemotherapy to appropriately risk-stratify patients in a clinical setting. However, it is important to consider whether the relevant experimental results exclude potential interactions with other types of treatment given after chemotherapy, and another vital influence is the duration of follow-up, as a short follow-up period may lead to biased experimental data. In conclusion, further clinical studies of NGS results are required before they can be relied upon as a risk assessment tool.

Radiotherapy

Radiotherapy is an effective and cost-efficient treatment. In recent years, with the continuous improvement of radiotherapy equipment and technology, the cure rate of radiotherapy has increased, with the side effects gradually decreasing. While genetic therapies are gradually replacing chemotherapy and targeted therapy, radiotherapy remains an important treatment option. However, based on the patient's sensitivity to radiotherapy, HTS technology can still guide the use of radiotherapy. In a recent study, Raffaello et al. statistically employed HTS technology to investigate the relationship between ctDNA and tumor regression grade (TRG) of surgical specimens in 25 consecutive patients with locally advanced colorectal cancer (LARC) who had undergone long-term neoadjuvant chemo-radiotherapy (Na-ChRT). The results showed that the side effects of Na-ChRT were significantly associated with positive liquid biopsies on the day of surgery. This suggests that ctDNA assessment using HTS technology may identify LARC patients with a poor response to NA-ChRT to avoid potentially ineffective treatment [55].

Radiotherapy is one of the critical tools in treating lymphoma, which can improve the therapeutic effect and alleviate the symptoms. However, some patients still experience relapse or a poor prognosis due to resistance to radiotherapy. Luo *et al.* employed CRISPR to construct an activated cell line library, screened for radiotherapy-resistant cells and performed HTS and bioinformatics analyses to identify genes associated with radiotherapy resistance. Sixteen of the screened genes were identified as potential genes associated with lymphoma radiotherapy resistance. These genes are not only expected to be used as potential biomarkers or new targets for therapy but have also demonstrated the advantages of HTS technology in potential target screening [56]. The assessment of the overall condition and prognosis of patients after radiotherapy is another crucial part of the treatment, and it has been a hot topic of research in recent years to indirectly assess the prognosis of patients by monitoring the relevant genes in their peripheral blood-free DNA (cfDNA) using NGS technology. Jae et al. found the value of HPV cfDNA in evaluating treatment response by dynamically monitoring the cfDNA of cervical cancer patients using targeted HTS. They found that HPV cfDNA is valuable for monitoring and predicting treatment response, providing new insights for management and evaluation after radiotherapy [57].

Radiotherapy is an effective cancer treatment, but it still inevitably has some side effects. Most patients tolerate and respond well to radiotherapy after surgery for early-stage breast cancer. However, a proportion of long-term survivors still develop radiotherapy-related complications, with subcutaneous fibrosis and capillary dilatation being the most common cutaneous complications of radiotherapy for breast cancer. In order to identify their susceptibility genes and genotoxicity, Sarah et al. performed targeted HTS on germline DNA samples from 48 breast cancer patients with extreme late-stage cutaneous toxicity phenotypes and identified a total of five single-nucleotide variants in three genes (TP53, ERCC2, and LIG1) with possible effects. This discovery can provide a possible way for radiotherapy to avoid patients susceptible to the side effects and serious consequences of inappropriate radiotherapy58 and promote the development of individualized medication. HTS technology accelerates the discovery of radiotherapy-resistant genes, effectively avoiding ineffective radiotherapy and significantly improving the effectiveness of treatments. However, there are fewer studies on applying HTS to radiotherapy complications, and radiotherapy is mainly used as an adjuvant treatment. Therefore, the influence of chemotherapy or targeted therapy cannot be excluded from the research, and its application as a risk assessment tool still needs to be further explored.

Tumor prognosis

Currently, following surgical intervention and related treatments, tumor patients need to be assessed for treatment and monitored for tumor recurrence through regular serum tumor marker tests and conventional imaging tests. If tumor recurrence can be anticipated at an earlier stage than the traditional examinations, early interventions can be carried out, thus prolonging the survival time of patients. Remaining tiny tumor foci after surgery or radiotherapy treatment are important factors affecting patients' prognosis, but it is more difficult to detect their presence by traditional imaging and serology screening. Fortunately, ctDNA detection based on HTS technology can provide a new idea to solve this problem. Based on this, Jeanne et al. performed ctDNA analysis on stage III colon cancer patients who had undergone chemotherapy and surgery and found that ctDNA analysis in patients undergoing surgery can serve as a marker of prognosis.

In contrast, ctDNA analysis in chemotherapeutic patients could screen out patients who have completed the standard therapy but are still at high risk of recurrence [59]. HTS technology, as an emerging test method, has advantages over traditional serological and imaging tests. Ma et al. assessed ctDNA in breast cancer patients using NGS technology while using the molecular tumor burden index (mTBI) in the samples to monitor tumor burden and found that it may have a higher sensitivity in indicating disease progression and distant metastasis than CT imaging [60].

Although most patients with advanced tumors have lost the opportunity for radical surgical treatment, appropriate radiotherapy or interventional therapy, as well as an assessment of prognosis, are still necessary. By sequencing the RAS gene in ctDNA from 47 plasma samples from 37 patients with RAS-mutated colorectal cancer (CRC) with unresectable metastases, Elena *et al.* found that the RAS-mutated allele score had an independent prognostic value for CRC survival and could be used as a non-invasive decision-making tool in first-line treatment for cancer [61]. Zhao et al. performed targeted capture sequencing on 1,021 genes frequently mutated in unresectable primary HCC cases. The results showed that ctDNA abundance correlated more closely with tumor size than AFP levels. It was also associated with the Barcelona Clinic Liver Cancer (BCLC) staging system. Dynamic changes of ctDNA showed consistent or higher sensitivity compared with imaging in assessing the response to interventions and a high degree of consistency with tumor mutational load of tissue and blood samples [62].

As a focus in the realm, immunotherapies represented by immune-checkpoint inhibitor therapies have revolutionized the treatment of late-stage solid tumors. Immune checkpoint inhibitors and the immunotherapy they represent have revolutionized the treatment of advanced solid tumors as a hotspot in cancer therapy. Valsamo et al. analyzed the clonal dynamics of ctDNA and tumor exogenous TCR parameters during immune checkpoint blockade in non-small cell lung cancer (NSCLC) employing the NGS technology. They assessed the value of liquid biopsy monitoring as a surrogate indicator of treatment response. The research results indicate that ctDNA testing after treatment can enable patients with immune checkpoint blockages and primary drug resistance to be quickly identified for alternative treatment [63].

Because of the side effects and drug resistance that immunotherapy can cause, only a small proportion of patients can benefit from it in the long term, and it is particularly important to provide the necessary monitoring throughout the process[64]. By measuring ctDNA levels and dynamic changes using HTS technology, the prognosis and outcome of immunotherapy can be predicted both before and during treatment to avoid the trauma and misinterpretation that traditional monitoring can cause. How-

ever, it should be noted that monitoring tumors after treatment is a long-term process, and there is still room for improvement in terms of price and variety compared to traditional means. Before HTS can be promoted and applied as a decision-making tool, it is still necessary to carry out comparative experiments on a large scale with traditional means of detection in order to further prove its reliability.

CONCLUSION

As a high-throughput detection technology, HTS can still be applied to large-scale genetic or genomic testing, thus providing a powerful tool for researching the mechanisms of tumor genesis and clinical diagnosis and treatment. As sequencing technology continues to evolve, it is anticipated that the goal of routine screening, prevention, diagnosis, treatment and prognosis of tumors will be achieved through this technology.

ACKNOWLEDGMENTS

The authors thank all the Department of Clinical Laboratory colleagues for their comments on earlier versions of this manuscript.

Funding

This study received funding from the Major Project of Humanities and Social Sciences Research in Anhui Universities (grant no. SK2021ZD0032) and Key Project of Natural Science Research of Higher Education Institutions in Anhui Province (Grant No. 2024AH050739).

Availability of data and materials

Not applicable.

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Authors' contributions

CX and DZ wrote the original draft and edited and critically revised the manuscript. MZ substantially contributed to the conception and revision of the work.. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have declared that no competing interest exists.

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