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Nanopore Sequencing of Cell-free DNA: An Emerging Liquid Biopsy Approach for Brain Tumor Molecular Profiling

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Abstract

Background: Brain tumors exhibit significant molecular heterogeneity, complicating diagnosis, prognosis, and treatment. Traditional tissue biopsies are invasive and often fail to capture the full tumor landscape due to intratumoral heterogeneity. Liquid biopsy, which analyzes cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA), offers a minimally invasive alternative for tumor profiling. Nanopore sequencing, a novel long-read sequencing technology, is emerging as a valuable tool in this context.

Purpose: This review explores the application of nanopore sequencing in molecular profiling of brain tumors using liquid biopsy, focusing on its ability to detect both genetic and epigenetic alterations with clinical relevance.

Main body: Brain tumors, such as gliomas and medulloblastomas, are characterized by diverse molecular profiles, which influence patient outcomes and treatment strategies. Nanopore sequencing offers unique advantages for profiling cfDNA from biofluids like cerebrospinal fluid (CSF) and plasma, including the ability to generate long reads and detect structural variants, copy number alterations, and methylation patterns. Several studies have demonstrated its potential to identify key mutations (e.g., IDH1/2, H3K27 M) and track tumor evolution through serial cfDNA monitoring. However, challenges remain, including low ctDNA fractions in biofluids and bioinformatic complexities.

Conclusion: Nanopore sequencing holds significant promise for advancing non-invasive molecular profiling of brain tumors, offering real-time insights into tumor genomics and evolution. This technology could revolutionize personalized brain tumor management, but further validation and optimization are needed before it can be routinely applied in clinical practice.

Keywords: Nanopore Sequencing, Liquid Biopsy, Cell-Free DNA (cfDNA), Brain Tumor Profiling, Molecular Heterogeneity

List of Abbreviations: CFDNA: Cell-Free DNA; CTDNA: Circulating Tumor DNA; CSF: Cerebrospinal Fluid; DIPG: Diffuse Intrinsic Pontine Glioma; IDH: Isocitrate Dehydrogenase; TP53: Tumor Protein p53; EGFR: Epidermal Growth Factor Receptor; MRD: Minimal Residual Disease

Article Highlights

Nanopore sequencing enables non-invasive genetic profiling of brain tumors via liquid biopsy.

It offers real-time, long-read sequencing for detecting mutations and epigenetic changes in cfDNA.

Challenges remain in bioinformatic processing and sensitivity due to low ctDNA levels in biofluids.

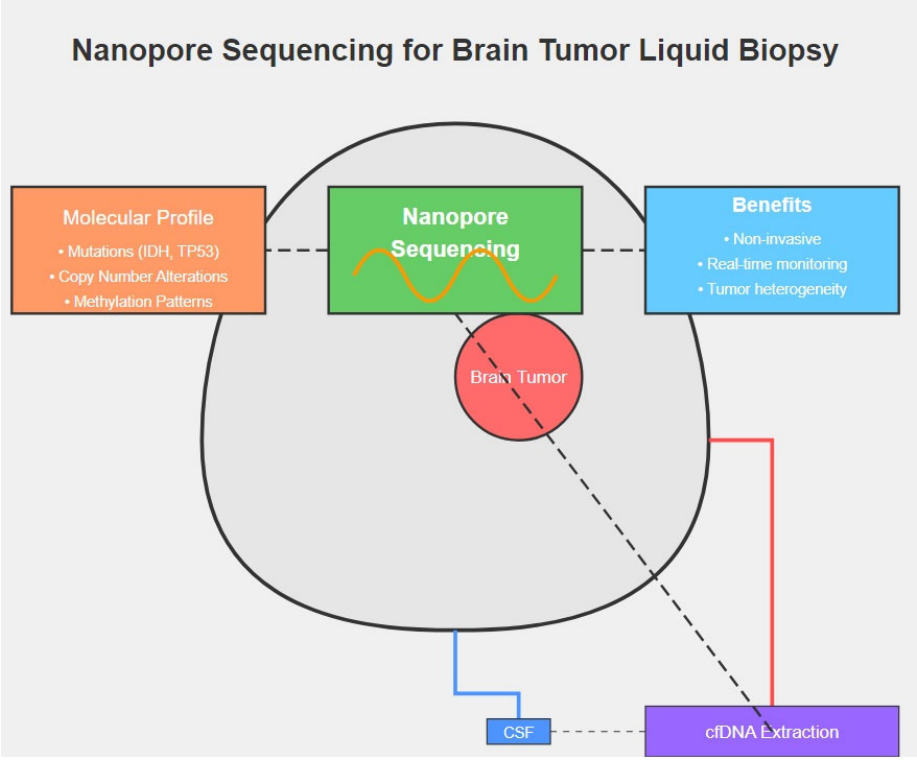

Figure 1. Flowchart of the research collection stages.

Background

Overview of brain tumors and molecular profiling challenges

Brain tumors represent a highly heterogeneous group of neoplasms, composed of both primary and metastatic lesions. Primary brain tumors, including gliomas, medulloblastomas, and meningiomas, exhibit diverse molecular and genetic characteristics, posing challenges for accurate diagnosis, prognosis, and treatment [1]. Traditional classification, based on histological features, often fails to capture the full spectrum of molecular alterations, leading to suboptimal therapeutic strategies. Recent advances in molecular profiling have improved our understanding of tumor biology, but tissue-based biopsies remain invasive, difficult to repeat, and may not fully represent the tumor's genetic landscape due to intratumoral heterogeneity [2-3].

Importance of non-invasive liquid biopsies

Liquid biopsy, a minimally invasive technique, has emerged as a promising alternative to traditional tissue biopsy. By analyzing circulating biomarkers, such as cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) in biofluids, liquid biopsies offer a dynamic and comprehensive view of tumor genetics. This approach allows for serial sampling and real-time monitoring of tumor evolution, making it particularly advantageous for brain tumor patients, where access to tumor tissue can be limited due to the sensitive nature of the brain [4].

Emergence of nanopore sequencing in liquid biopsy

Nanopore sequencing has recently gained attention as a powerful tool for liquid biopsy-based molecular profiling. Unlike traditional short-read sequencing technologies, nanopore sequencing generates long-read data, enabling the detection of complex genomic alterations such as structural variants and methylation patterns. Additionally, nanopore sequencing offers real-time data generation, facilitating rapid clinical decision-making. Its ability to sequence native DNA without amplification makes it particularly suitable for analyzing the fragmented and low-concentration cfDNA from brain tumor patients [5-8].

Brain Tumors: Molecular Heterogeneity and Diagnostic Challenges

Classification of brain tumors – Adult vs pediatric tumors

Brain tumors can be broadly categorized into adult and pediatric types, each with distinct molecular and clinical features. In adults, gliomas are the most common, with

glioblastoma being the most aggressive form. Pediatric brain tumors, including medulloblastomas and ependymomas, often have unique molecular drivers and therapeutic responses [9-11]. The diversity of genetic alterations across different age groups complicates diagnosis and treatment, underscoring the need for precise molecular characterization as presented in **(Table 1)**.

Gliomas, medulloblastomas, and ependymomas: Molecular complexity

Gliomas, medulloblastomas, and ependymomas exhibit substantial molecular complexity. Gliomas, for example, frequently harbor mutations in the IDH1/2 or TP53 genes, as well as alterations in EGFR and PTEN. Medulloblastomas are divided into four molecular subgroups (WNT, SHH, Group 3, and Group 4), each with distinct genetic and epigenetic profiles. Ependymomas also display heterogeneity, with different molecular subtypes correlating with patient prognosis. This level of molecular diversity complicates treatment decisions, as therapies must be tailored to specific genomic alterations [11-14].

Limitations of traditional tissue biopsies

Intratumoral heterogeneity, the presence of genetically distinct subclones within a single tumor, is a major challenge for tissue biopsies. A single biopsy may not capture the full extent of molecular alterations, leading to incomplete or inaccurate profiles [15,16]. Tissue biopsies carry inherent risks, especially for brain tumors. Surgical procedures can lead to complications such as infection, bleeding, and neurological damage, making them unsuitable for frequent monitoring of tumor progression or treatment response [17-20].

The role of molecular profiling in brain tumor management

Molecular profiling has identified several prognostic markers in brain tumors. For instance, IDH1 and IDH2 mutations in gliomas are associated with better outcomes, while alterations in the H3K27M gene in diffuse midline gliomas predict poor prognosis. Accurate identification of these markers is crucial for guiding patient care [21,22]. Molecular profiling also plays a central role in therapeutic decision-making. By identifying actionable mutations, clinicians can tailor treatments, such as targeted therapies or immunotherapies, to the individual genetic makeup of the tumor. This precision medicine approach holds promise for improving outcomes in patients with brain tumors [23-25].

Liquid Biopsy as a Minimally Invasive Approach

Concept of liquid biopsy

Liquid biopsy refers to the analysis of tumor-derived material, such as cfDNA or ctDNA, present in biofluids like blood, cerebrospinal fluid (CSF), urine, and saliva. It offers a minimally invasive alternative to traditional tissue biopsy, allowing for the detection of genetic mutations, copy number alterations, and epigenetic changes in tumors [26-30].

Cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) in brain tumors

Cell-free DNA (cfDNA) consists of fragmented DNA derived from both normal and tumor cells, while circulating tumor DNA (ctDNA) refers specifically to the fraction of cfDNA that originates from tumor cells. In brain tumors, cfDNA can be

Table 1. Key Genetic and Epigenetic Alterations in Brain Tumors.

Brain Tumor Type	Common Genetic Mutations	Epigenetic Alterations	Clinical Implications	Prognostic Significance
Glioblastoma (GBM)	EGFR, PTEN, TP53, IDH1/2	Promoter methylation of MGMT	EGFR mutations associated with poor response to EGFR inhibitors; MGMT methylation predicts response to TMZ treatment	MGMT promoter methylation is a positive prognostic marker, IDH1 mutations are linked to better outcomes
Diffuse Midline Glioma	H3K27M	Global reduction in H3K27me3	H3K27M mutation defines a distinct tumor subtype with poor prognosis	H3K27M mutation indicates poor prognosis and poor response to treatment
Medulloblastoma	WNT, SHH, Group 3, Group 4	CpG island methylation patterns	WNT subgroup has a better prognosis, while Group 3 is associated with aggressive disease	WNT subgroup has the most favorable prognosis, Group 4 has intermediate prognosis
Ependymoma	RELA, YAP1	DNA hypermethylation	RELA fusion-positive ependymomas are more aggressive	RELA fusion is associated with poor prognosis
Oligodendroglioma	1p/19q co-deletion, IDH1/2	MGMT promoter methylation	1p/19q co-deletion predicts better response to chemotherapy	IDH1/2 mutations and 1p/19q co-deletion are associated with better outcomes

detected in various biofluids, with CSF being particularly enriched for tumor-derived DNA due to its proximity to the central nervous system. ctDNA analysis provides insights into tumor genetics, enabling the detection of mutations and other alterations [31,32].

Advantages of liquid biopsy over tissue biopsy

Liquid biopsy enables serial sampling, allowing clinicians to monitor tumor evolution over time. This is particularly useful for assessing treatment response and detecting emerging resistance mutations [33,34]. By analyzing cfDNA at multiple time points, liquid biopsy provides a dynamic view of tumor evolution. This capability is critical for understanding how tumors adapt to therapies and for identifying new therapeutic targets [35]. Liquid biopsy captures the genetic diversity of tumors more comprehensively than tissue biopsies, as cfDNA reflects contributions from multiple tumor subclones. This enhances the ability to detect rare mutations and improves the overall accuracy of molecular profiling [36].

Potential biofluids for brain tumor liquid biopsy

Cerebrospinal fluid (CSF): CSF is the most relevant biofluid for brain tumor liquid biopsy, given its direct contact with the central nervous system as presented in **Table 2**. cfDNA concentration in CSF is typically higher than in plasma, making it a valuable source for detecting tumor-derived alterations [37,38].

Plasma: Plasma is a convenient and widely used biofluid for liquid biopsy. However, cfDNA from brain tumors is often present at lower concentrations in plasma compared to CSF, which may limit its sensitivity for detecting certain mutations [39-42].

Urine: While less commonly used for brain tumors, urine can serve as a non-invasive source of cfDNA for detecting systemic tumor-derived DNA. Its utility for brain tumor profiling is still under investigation [43-45].

Saliva: Saliva offers another non-invasive biofluid for cfDNA analysis, though its use in brain tumor liquid biopsy is limited. Further research is needed to determine its potential for detecting tumor-specific alterations [46].

Nanopore Sequencing Technology

Overview of nanopore sequencing

Mechanism through Electrical Current Disruption as DNA Passes through Nanopores. Nanopore sequencing works by measuring changes in electrical current as DNA strands pass through protein nanopores. Each nucleotide disrupts the current in a unique way, allowing the sequence of the DNA to be determined in real time [47]. Several nanopore sequencing platforms are available, including the portable MinION, the mid-throughput GridION, and the high-throughput PromethION. These devices offer flexibility in terms of scale and application, making them suitable for both small and large sequencing projects [48].

Advantages of nanopore sequencing for liquid biopsy

Nanopore sequencing generates long reads, which are particularly useful for detecting structural variants, copy number alterations, and complex rearrangements in cfDNA as depicted in **Figure 1**. These features are often challenging to detect using short-read sequencing technologies [49]. Nanopore sequencing provides real-time data, enabling rapid analysis and clinical decision-making. This is a significant

Table 2. Liquid Biopsy Biofluids for Brain Tumor Detection.

Biofluid	Advantages	Limitations	Applications	Sensitivity for Brain Tumors
Cerebrospinal Fluid (CSF)	High concentration of ctDNA due to proximity to the CNS	Invasive collection procedure (lumbar puncture); limited sample availability	Ideal for detecting tumor-specific mutations, methylation profiling, and monitoring tumor evolution	High (especially for CNS tumors like gliomas)
Plasma	Widely accessible, non-invasive	Lower ctDNA concentration for brain tumors; potential contamination from non-tumor sources	Useful for serial monitoring of tumor evolution and treatment response, but less sensitive for brain tumors	Moderate to low (depends on tumor shedding)
Urine	Non-invasive, easy to collect	Very low ctDNA concentration for brain tumors; limited research on brain tumor detection efficacy	Primarily used for systemic cancers; limited application in brain tumor profiling	Low (limited utility for brain tumor analysis)
Saliva	Non-invasive, easy to collect	Limited ctDNA from brain tumors due to distance from the CNS; contamination from oral microbiota	Under investigation for other cancers (e.g., head and neck); not yet established for brain tumor detection	Low (not yet validated for brain tumors)

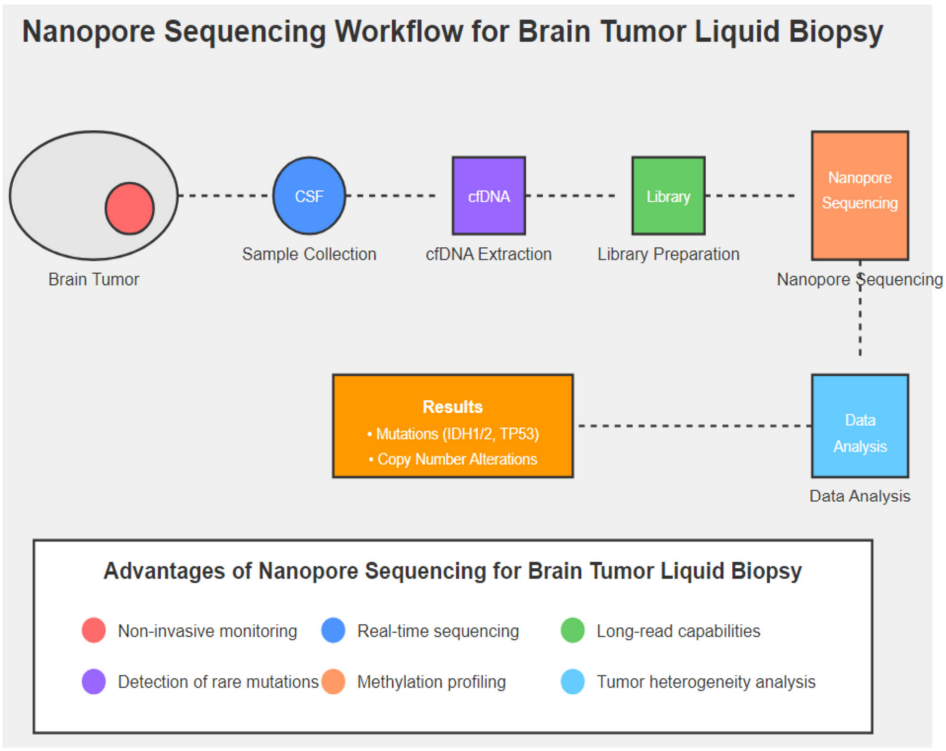

Figure 1. Advantages of Nanopore Sequencing for Liquid Biopsy.

advantage for applications where timely results are critical, such as monitoring treatment response [50]. Nanopore sequencing can directly sequence native DNA without the need for amplification, preserving important epigenetic information, such as DNA methylation. This feature is particularly valuable for brain tumor profiling, where epigenetic changes play a key role in tumor biology [51-53].

Comparison with other sequencing technologies (Illumina and PacBio)

Nanopore sequencing differs from other technologies like Illumina and PacBio in several ways as presented in **Table 3**. While Illumina provides high accuracy and short-read sequencing, it lacks the ability to detect large structural variants

Table 3. Comparison between Nanopore Sequencing and Illumina Sequencing.

Parameter	Nanopore Sequencing	Illumina Sequencing
Sequencing Technology	Single-molecule, long-read sequencing via protein nanopores	Short-read sequencing via reversible dye terminators
Sample Preparation	Requires less complex library preparation; can sequence native DNA	Requires more complex library preparation; amplification often needed
Turnaround Time	Real-time sequencing; data available as sequencing progresses	Batch-based sequencing; data available after the entire sequencing run
Length of Reads	Long reads (up to 2 Mb, typical reads >10 kb)	Short reads (150–600 bp, depending on the run type)
Data Output	Lower throughput compared to Illumina in terms of Gb/run	High throughput with enormous data output (up to several Tb per run)
Error Rate	Higher error rate (~5-15%) but improving with bioinformatic tools	Lower error rate (~0.1%) due to short-read accuracy and error correction
Data Analysis Pipeline	Requires specialized tools (e.g., Guppy, Medaka, Minimap2) to handle long reads and correct errors	Mature and widely used pipelines with extensive support (e.g., BWA, GATK)

Epigenetic Profiling	Direct detection of DNA methylation and other modifications	Requires bisulfite treatment or additional steps for methylation analysis
Structural Variant Detection	Excellent for detecting large structural variants, copy number alterations, and complex rearrangements	Limited due to short reads; requires paired-end reads or assembly for larger variants
Cost per Sample	Lower initial cost for small-scale projects; cost-effective for smaller labs	Higher initial equipment cost, but cost per sample lower for high-throughput projects
Portability	Highly portable (MinION fits in a pocket)	Requires large, specialized equipment (e.g., HiSeq, NovaSeq)
Applications	Best for real-time sequencing, long-read applications, structural variants, epigenetics	Best for high-throughput genomics, population-scale studies, and diagnostics
Suitability for Liquid Biopsy	Well-suited for cfDNA and ctDNA analysis due to long-read capabilities and real-time sequencing	Commonly used for cfDNA analysis, but limited in detecting large structural variants without additional tools

and methylation patterns. PacBio offers long-read sequencing but requires more expensive and complex equipment compared to nanopore devices. Nanopore sequencing combines the advantages of long-read sequencing with lower cost and portability, making it an attractive option for liquid biopsy applications [54,55].

Limitations of nanopore sequencing

Nanopore sequencing has a higher error rate compared to other sequencing technologies, particularly in homopolymeric regions. However, ongoing improvements in base-calling algorithms and error-correction methods are helping to reduce these errors [56].

The analysis of nanopore sequencing data presents several bioinformatic challenges, including the need for specialized tools to handle long-read data and correct sequencing errors. Tools such as Guppy, Medaka, and Minimap2 are commonly used for base calling and alignment, but further optimization is required to improve data accuracy [57-59].

Applications of Nanopore Sequencing in Brain Tumor Molecular Profiling

Mutation detection in cfDNA from brain tumors

IDH1/2: Mutations in IDH1/2 are common in gliomas and have prognostic significance. Nanopore sequencing can detect these mutations in cfDNA, enabling non-invasive molecular profiling of gliomas [60].

H3K27M: The H3K27M mutation is a hallmark of diffuse midline gliomas and is associated with poor prognosis. Nanopore sequencing offers a rapid and sensitive method for detecting this mutation in cfDNA, particularly in pediatric patients [61].

EGFR, TP53 mutations: Nanopore sequencing can also identify mutations in EGFR and TP53, which are common in glioblastoma and other aggressive brain tumors. These mutations have therapeutic implications, as they may inform the use of targeted therapies or immunotherapies [62].

Copy number alteration and structural variant detection

Nanopore sequencing excels at detecting copy number alterations and structural variants, which are often missed by short-read sequencing technologies. These alterations can have significant clinical implications, as they can influence tumor behavior and treatment response [63].

Epigenetic profiling using nanopore sequencing

Nanopore sequencing can directly detect DNA methylation, a key epigenetic modification involved in brain tumor development. Methylation profiling can aid in the classification of brain tumors and provide prognostic information [64]. Nanopore sequencing can also provide insights into chromatin accessibility, which reflects the regulatory landscape of the tumor genome. This information can help identify potential therapeutic targets and improve our understanding of tumor biology [65].

Real-time monitoring of treatment response

Nanopore sequencing enables real-time monitoring of tumor evolution through serial cfDNA analysis. This approach allows clinicians to detect emerging resistance mutations and adapt treatment strategies accordingly. By analyzing cfDNA at multiple time points, nanopore sequencing can identify resistance mutations that arise during treatment, allowing for timely modifications to therapy [66,67].

Pediatric vs adult brain tumor profiling using nanopore sequencing

Pediatric brain tumors often have unique molecular features and lower cfDNA concentrations, posing challenges for liquid biopsy. Nanopore sequencing's sensitivity and ability to detect complex genetic alterations make it well-suited for profiling pediatric tumors. Nanopore sequencing can aid in

the molecular subtyping of pediatric brain tumors, which is critical for guiding treatment decisions and predicting patient outcomes [68-70].

Key Proof-of-Concept Studies in Nanopore Sequencing for Brain Tumors

CSF cfDNA in glioma patients

Wadden *et al.* study demonstrated the feasibility of using nanopore sequencing to analyze cfDNA from cerebrospinal fluid (CSF) in glioma patients. The authors successfully identified key mutations, including IDH1 and TP53, highlighting the potential of nanopore sequencing for non-invasive brain tumor profiling [71].

Plasma and CSF cfDNA in pediatric DIPG

Chicard *et al.* used nanopore sequencing to analyze cfDNA from both plasma and CSF in pediatric patients with diffuse intrinsic pontine glioma (DIPG). The study demonstrated the ability of nanopore sequencing to detect the H3K27M mutation, providing a non-invasive method for diagnosing and monitoring DIPG [72].

Combined cfDNA and methylation profiling

This study combined cfDNA analysis with methylation profiling using nanopore sequencing to improve the classification of brain tumors. The authors demonstrated that methylation patterns could be used to distinguish between different tumor subtypes, providing valuable prognostic information [73-75].

Other recent studies and their clinical implications

Several other studies have explored the use of nanopore sequencing for brain tumor profiling, demonstrating its potential for detecting mutations, structural variants, and epigenetic changes. These studies underscore the clinical utility of nanopore sequencing for guiding treatment decisions and monitoring disease progression [76].

Challenges and Limitations

Low ctDNA fraction in biofluids

One of the main challenges of liquid biopsy in brain tumors is the low concentration of ctDNA in biofluids, particularly in plasma. This can limit the sensitivity of nanopore sequencing, making it difficult to detect rare mutations [77].

Sequencing error rates and bioinformatic complexity

Nanopore sequencing has a higher error rate than other sequencing technologies, necessitating the use of robust error correction algorithms. Tools such as Guppy and Medaka have been developed to improve base-calling accuracy, but further improvements are needed [78]. Several bioinformatics tools are available for processing nanopore sequencing data. Guppy and Medaka are used for base calling and error correction, while Minimap2 and NGMLR are commonly used for alignment. Optimizing these tools is critical for improving the accuracy and reliability of nanopore sequencing data [79].

Sample collection and processing variability

The quality of cfDNA analysis depends on several pre-analytical factors, including the type of collection tubes, storage conditions, and cfDNA extraction methods. Standardizing these protocols across institutions is essential for ensuring consistent and reliable results [80].

Need for larger validation studies

Larger validation studies are needed to assess the analytical and clinical sensitivity and specificity of nanopore sequencing for brain tumor liquid biopsy. These studies will help determine the accuracy of nanopore sequencing compared to traditional tissue biopsy [81,82]. Concordance between liquid biopsy results and tissue biopsy results is critical for establishing the clinical utility of nanopore sequencing. Further studies are needed to assess how well nanopore sequencing reflects the molecular profile of the tumor [83].

Future Directions and Innovations

Optimization of pre-analytical and analytical protocols

Improving cfDNA extraction and enrichment techniques is essential for maximizing the sensitivity of nanopore sequencing. New methods for isolating ctDNA from biofluids, particularly in low-yield samples like plasma, will enhance the overall performance of liquid biopsy as presented in **Figure 2**. Standardizing pre-analytical and analytical protocols across institutions will be critical for ensuring the reproducibility and reliability of nanopore sequencing data in clinical settings. This includes harmonizing cfDNA extraction, sequencing, and data analysis methods [84,85].

Integration with multi-omics approaches

Integrating nanopore sequencing with other omics approaches, such as proteomics and transcriptomics, will provide a more comprehensive view of tumor biology. Multi-omics profiling will enable the identification of novel biomarkers and therapeutic targets, improving the precision of brain tumor diagnosis and treatment [86,87].

Machine learning and AI in nanopore sequencing data analysis

Machine learning and artificial intelligence (AI) techniques are being developed to enhance the sensitivity of nanopore sequencing data analysis. These models can help identify

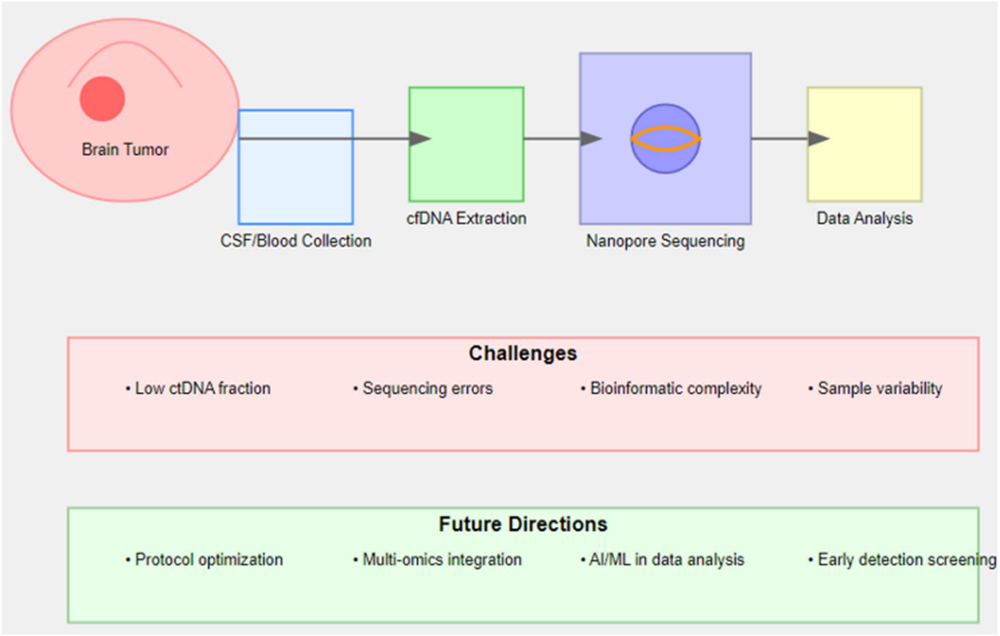

Figure 2. Future directions and challenges.

patterns in large datasets, improving the detection of rare mutations and other alterations in cfDNA. AI-powered models can also integrate serial cfDNA timepoints to track tumor evolution over time. This dynamic monitoring capability will enable clinicians to detect emerging resistance mutations and adjust treatment strategies in real-time [88-90].

Potential of early detection and screening using liquid biopsy

Nanopore sequencing has the potential to be used for early detection and screening of brain tumors in high-risk patients. By analyzing cfDNA from biofluids, clinicians could identify molecular alterations at an early stage, improving the chances of successful treatment. Nanopore sequencing could also be used to detect minimal residual disease (MRD) in brain tumor patients. By analyzing cfDNA after treatment, clinicians could identify residual tumor cells, enabling early intervention and reducing the risk of recurrence [91,92].

Conclusions

The use of nanopore sequencing in liquid biopsy for brain tumor profiling offers a transformative approach to non-invasive molecular diagnostics. Its ability to provide long-read sequencing, detect structural variations, and analyze methylation patterns directly from cfDNA in real-time has the potential to dramatically enhance the accuracy and timeliness of molecular profiling. This is particularly relevant for tracking tumor evolution and tailoring personalized treatment strategies. However, the field faces several limitations, including the low concentration of tumor-derived DNA in biofluids, sequencing error rates, and the need for more robust bioinformatic pipelines. The integration of nanopore sequencing into clinical practice will require larger-scale validation studies, standardization of pre-analytical processes, and improvements in sequencing accuracy. Future research should focus on optimizing cfDNA extraction methods and developing machine learning algorithms to improve data interpretation.

Recommendations

To fully harness the potential of nanopore sequencing for brain tumor liquid biopsy, several key areas require further development. First, standardized protocols for cfDNA collection, storage, and processing must be established across institutions to ensure reproducibility and comparability of results. Second, bioinformatic tools need to be refined to handle the unique challenges posed by long-read sequencing and to correct for sequencing errors. Third, integrating nanopore sequencing with other omics technologies, such as proteomics and transcriptomics, could provide a more comprehensive view of tumor biology. Finally, prospective clinical trials are imperative to validate the clinical utility of this technology, particularly in detecting minimal residual disease and monitoring treatment response. These advancements will be crucial for establishing nanopore sequencing as a routine tool in brain tumor diagnostics and personalized therapeutic decision-making.

Declarations

Ethical approval and consent to participate

Not applicable.

Clinical trial number

not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data are available and sharing is available as well as publication.

Competing interests

The author hereby declares that they have no competing interests.

Funding

The corresponding author supplied all study materials. There was no further funding for this study.

Authors' contributions

The author completed the study protocol and was the primary organizer of data collection and the manuscript's draft and revision process. The corresponding author wrote the article and ensured its accuracy.

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