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REVIEW

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Applications of Nanopore sequencing in precision cancer medicine

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Abstract

Oxford Nanopore Technologies sequencing, also referred to as Nanopore sequencing, stands at the forefront of a revolution in clinical genetics, offering the potential for rapid, long read, and real-time DNA and RNA sequencing. This technology is currently making sequencing more accessible and affordable. In this comprehensive review, we explore its potential regarding precision cancer diagnostics and treatment. We encompass a critical analysis of clinical cases where Nanopore sequencing was successfully applied to identify point mutations, splice variants, gene fusions, epigenetic modifications, non-coding RNAs, and other pivotal biomarkers that defined subsequent treatment strategies. Additionally, we address the challenges of clinical applications of Nanopore sequencing and discuss the current efforts to overcome them.

KEYWORDS

cancer, genomic aberrations, Nanopore sequencing, precision medicine

1 | INTRODUCTION

Genome studies have made a critical contribution to the understanding of the link between genetic alterations and the cause of many human diseases. The Human Genome Project was launched in 1990, having an ambitious goal to sequence and map an entire human DNA sequence for the very first time.[1] Using Sanger sequencing, it took 13 years and 2.85 billion USD to read, assemble, and produce a draft of a single human genome.[2,3] Since then, sequencing techniques have evolved exponentially. With the introduction of next-generation sequencing (NGS), whole genomes can be generated within hours and at a much lower cost.[4] The most frequently used short-read NGS method (second generation sequencing) is clonal amplification, followed by sequencing by synthesis developed by Illumina. Long-read single-molecule real-time sequencing methods (third generation sequencing) have been recently developed by Pacific Biosciences and Oxford Nanopore Technologies.[5,6] Nanopore sequencing (also referred to as ONT) is capable of rapid and ultra-long read sequencing of native DNA or RNA.[7] Although still relatively new, this technique has already been widely employed for various sequencing projects, including the whole human genome assembly and as part of The Human Pangenome Project.[8–10]

2 | ONT AS A SEQUENCING METHOD

The very first research suggesting the usage of a nanoscale pore to characterize a nucleic acid sequence was published in 1996 by Kasianowicz and colleagues.[11,12] Eighteen years later, the first nanopore-based sequencer, the MinION, was released by ONT.[13] In Nanopore sequencing, the nucleic acid passes through a nano-scale protein pore, while alteration in ion current is monitored and decoded into the nucleotide sequence[7] (Figure 1). These nanopores are embedded in an electrically resistant membrane.[7,14,15] A constant voltage applied across the membrane creates a current of ions present in the buffer and promotes DNA and RNA molecules to pass through the nanopore.[15] The latter disrupts the ion current, with a characteristic pattern for each nucleotide passing through the nanopore.[7,14,16] Moreover, signatures for epigenetic DNA/RNA modifications (e.g., 5mC, 5hmC, 6 mA) can be distinguished and thus allow simultaneous acquisition of sequence, epigenome and/or epitranscriptome data.[17] Over the last years, the technique has been continuously refined to improve the base calling precision. Specifically, the motor protein, which is used to unzip double-stranded DNA and decrease DNA translocation speed, and the pore itself are constantly optimized.[18] Currently, the newest R10 flow cells employ a CsgG-derived

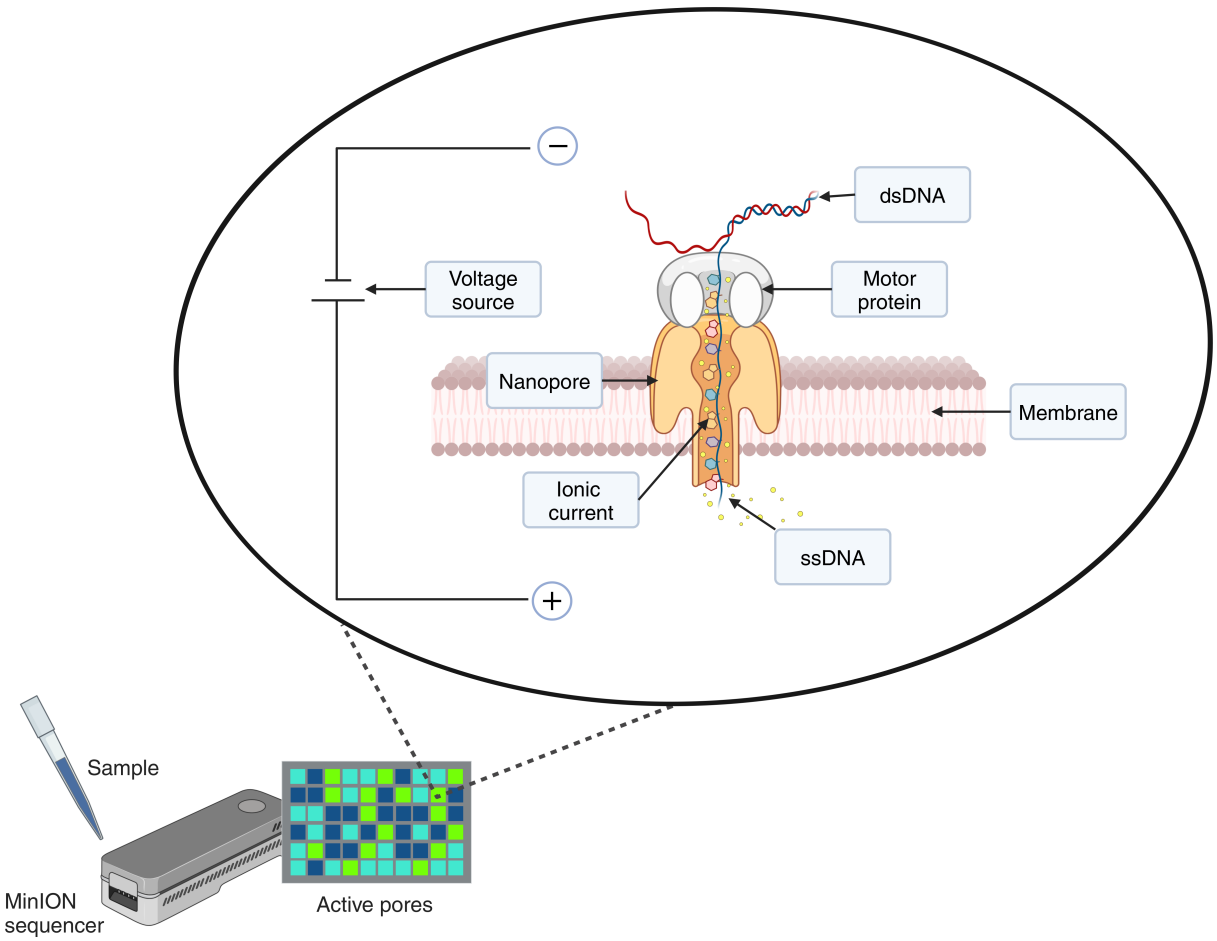

FIGURE 1 Structure of nanopore and principle of Nanopore sequencing.

pore from *Escherichia* coli.[19,20] The sequencing chemistry has also evolved from the initial 1D chemistry that allowed sequencing of a single DNA strand to the newest 1D^2 chemistry, where complementary DNA strand is tethered to the pore for double-strand sequencing.[21–23]

Notably, due to simplicity of the method, an initial investment required for establishing Nanopore sequencing in the laboratory is significantly lower compared to other techniques and is currently around 2000 euro (for MinION device).

Raw electrical current data is provided by the ONT sequencer in POD5 or FAST5 format, and instantly decoded into BAM or FASTQ format; the latter can be further used for direct downstream analysis, e.g., for alignment to reference genomes.[15,24] The initial base calling can be done using various algorithms, most popular of which are Guppy,[25] Megalodon[26] (used for modified bases calling in combination with Guppy), and Dorado[27] (neural networks implemented algorithm for calling of natural and modified bases); these and other tools are reviewed elsewhere.[15,28] Real-time data acquisition provides ample opportunities for various applications, including intraoperative diagnostics.[29] As the native nucleic acid molecules are sequenced, the standard library preparation protocols are quite straightforward and used-friendly. With additional techniques, such as adaptive sampling, amplicon sequencing or CRISPR-based target enrichment, sequencing can be directed to specific targets. Adaptive sampling consists of comparing the first ~180 bases of a nucleic acid fragment migrating through the pore to a target reference in real-time.[30,31] Based on the outcome, the fragments are either selected and further sequenced or expelled from the pore via application of reversed voltage.[32–34] However, the limitation of this method is the usage of rapid and therefore less accurate base calling algorithms that is required for the on-the-fly alignment.[35] In amplicon sequencing the target, a segment of DNA or RNA, is amplified by PCR and then sequenced.[36–40] CRISPR-enrichment of the target is performed by excision of relevant genes followed by sequencing[41–44] (Table 1).

3 | POTENTIAL CLINICAL APPLICATIONS OF NANOPORE SEQUENCING

ONT has a wide spectrum of potential applications in clinical oncology. It is an attractive choice to clinical laboratories due to its affordability in terms of initial investments, straightforward bench workflows, and rapid turnaround times.[45] Notably, ONT has been employed for analysis of both "classical" tumor tissue biopsy and liquid biopsy, that is, circulating tumor DNA (ctDNA), where detection of point mutations as well as larger aberrations have been accessed.[46,47] As a long-read sequencing method, ONT is more suitable for identification of complex structural variants (SV), whereas detection of point mutations, especially with low variant allele frequency (VAF) or ctDNA of low content, is still challenging. This is due to the base detection accuracy, which is currently Q30 (for duplex base calling) and is lower compared to other sequencing techniques.[48] Nevertheless, information on these aberrations can be of extreme use for precision diagnostic as well as for optimization and personalization of the therapy, and further improvements to sequencing protocols are being constantly made.

The very first ONT-based assay was approved by US Food and Drug Administration (FDA) in 2020, when the permission was granted to a Clear Dx™ SARS-CoV-2 test.[49] However, as of February 2024, no ONT test is approved for diagnostics of cancer or other genetic-related disorders. Therefore, Nanopore sequencing is not included in standard clinical protocols, though the laboratories can still develop and validate their own ONT-based laboratory-developed tests (LDTs).[50] Hence, the clinical application of ONT is currently limited to

TABLE 1 The main target enrichment techniques used for Nanopore sequencing.

Technique	Analyte	Analysis	Entity	Reference
Adaptive sampling	Genomic DNA	SNV, CNV, methylation	CNS tumors	30
	Genomic DNA	SNV, structural variants	Breast carcinoma	31
Amplicon sequencing	PCR product of vector integration sites	Sequence	T cell therapy	36
	PCR product from FFPE tissue	SNV	Glioma	37
	PCR product from FFPE tissue	SNV	AML, melanoma, NSCLC, hepatocellular carcinoma	38
	PCR product of reverse transcriptase amplified mRNA	Splicing events	Breast carcinoma	39
	PCR product of IGH locus from cfDNA	SNV	ALL	40
CRISPR-enrichment	Extrachromosomal DNA	5-mC CpG methylation	Metastatic melanoma	41
	Genomic DNA	Fusions	ALL	42
	Genomic DNA	SNV, structural variants, fusions	N/A	43
	Genomic DNA	Fusions	Various	44

Abbreviations: AML, acute myeloid leukemia; CNS, central nervous system; CNV, copy number variations; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; SNV, single-nucleotide variants.

translational research performed using patients' samples and to individual case reports encompassing various cancer entities. In the following sections, we discuss selected reports, categorizing them according to the relevant genetic markers/aberrations identified using ONT.

3.1 | Point mutations

Nanopore sequencing can be used to detect point mutations, such as nucleotide substitutions and indels. For instance, Nanopore sequencing has been used to identify driver SNV mutations and their phasing in lung cancer samples.[51] Specifically, c.2369C > T and c.2573 T > G mutations in the *EGFR* gene were identified, which are associated with resistance to gefitinib or erlotinib.[51,52]

ONT sequencing was applied for targeted testing of prognostically relevant mutations in blood malignancies.[53,54] With FDA recently approving *FLT3*, *IDH1*, and *IDH2* inhibitors midostaurin, ivosidenib, and enasidenib for treatment of acute myeloid leukemia (AML), short turn-around time (TAT) testing of point mutations in these genes for therapy stratification is now essential.[55] Specifically, PCR-amplified gDNA from bone marrow mononuclear cells of 22 patients with AML was tested for the presence of hotspot mutations using a customized gene panel including *NPM1*, *FLT3*, *CEBPA*, *TP53*, *IDH1* and *IDH2* genes.[45] The results were generated in less than 24 h and were comparable to those generated by Ion Torrent™ with a longer TAT. Similar workflows were applied for testing *TP53* mutations in CLL patients to identify potential candidates for treatment with Bruton's Tyrosine Kinase (BTK) inhibitors and for identification of *BCR-ABL1* fusions break points in CML patients.[56,57]

DNA fragmentation and cross-linking induced by formalin fixation and a lack of standardized robust protocols represent significant challenges for DNA analysis from formalin-fixed paraffin embedded (FFPE) material. Mimosa et al. analyzed *IDH1* using ONT sequencing of FFPE material.[37] Utilizing an amplicon sequencing method, the authors could circumvent the difficulties associated with formalin fixation and detected all druggable and clinically relevant mutations, for example, R132H, in this gene in 27 glioma patients with TAT and quality outperforming other NGS techniques. Similarly, PCR amplification was used to detect *BRAF* mutations in FFPE tissue of archived cases of Hairy cell leukemia.[58]

To address the challenge of detecting low variant allele frequency (VAF) mutations, an Oncogene Concatenated Enriched Amplicon Nanopore Sequencing (OCEANS) technique was developed. In this method the variants are subsequently amplified and concentrated prior to Nanopore sequencing. OCEANS facilitates the precise identification of somatic mutations with VAF as low as 0.05%. Four different multi-gene OCEANS panels targeting recurrent point mutations in NSCLC, hepatocellular carcinoma, AML, and melanoma have been developed and successfully validated in the clinic. These results indicate potential use of OCEANS for same-day clinical sequencing panels.[38]

Hence, Nanopore sequencing can detect point mutations, which can be employed for stratification and therapy personalization with performance comparable to second generation short-read NGS methods. FFPE material can be used for analysis, however, analysis of fresh frozen tissue is preferred. Due to a rather high error rate, ONT should be used with caution for point mutations detection.[59,60] The accuracy problem can be partially overcome by using specific techniques like amplicon sequencing (Table 1) and computational error corrections.[61] Additionally, ONT is constantly employing novel basecallers which have higher accuracy.

3.2 | Splice variants

Alternative transcripts of certain genes may drive cancer and therefore represent promising therapeutic targets. Some transcripts can otherwise be considered as biomarkers of specific cancer subtypes, have diagnostic value, and therefore affect a treatment strategy.[62,63] ONT can identify different transcript variants by sequencing either native full-length cDNA or mRNA. For instance, Nanopore sequencing was used to identify new pathogenic splice variants of *BARD1*, an interaction partner of the *BRCA1* gene, in several cancer types using cDNA sequencing.[64,65]

ONT sequencing was used to explore alternative splicing events in colorectal carcinoma (CRC) associated with deficient mismatch repair (dMMR) and microsatellite instability (MSI).[66] dMMR/MSI is observed in CRC and results in an increased mutational burden and production of hyper neoantigens, potentially making tumors more immunogenic. Previously, dMMR/MSI status was used to select patients for immune checkpoint therapy, for example, pembrolizumab (anti-PD1) and to predict response to chemotherapy.[67] In CRC, cancer-associated mutations frequently occur in genes that encode transcripts which are prone to alternative splicing. Using ONT long-read sequencing, Qu et al. constructed CRC mRNA profiles to detect alternative splicing and identified alternatively spliced *INHBA* as a potential biomarker for MSI-H/dMMR solid tumors.[66] More recently, a study of alternative splicing in CRC using ONT has also identified other potential biomarkers of alternative splicing.[68] Moreover, Nanopore sequencing was applied for rapid, shallow transcriptome profiling of various human tumors, which indicated an overactivation of targetable signaling pathways and kinases.[69]

Sequencing of full-length cDNAs facilitates the identification of aberrant cancer-specific splicing variants. These variants, when translated into proteins, are presented by HLA molecules on the cell surface and are recognized as neoantigens. Using Nanopore sequencing, Oka et al. identified novel aberrantly spliced transcripts and potential neoantigens in NSCLC. Integrating aberrant transcript expression profiles with genomic mutational landscape analysis has the potential to enhance the precision of identifying patients who would positively respond to immunotherapy.[70] Yu et al. reported efficacy of the clinically approved pan-RTK inhibitor ponatinib as a new treatment for aggressive meningiomas. In this study, the presence of RTK expression in meningiomas and, therefore, the feasibility of ponatinib

application was evaluated using Nanopore sequencing of full-length cDNA.[71] In the last decade, several druggable genetic alterations were reported in meningiomas, which ultimately resulted in clinical studies (NCT02523014; NCT03071874).[72–75] ONT as a technique, capable of detecting of these alterations, currently paves the way into upcoming clinical trials.

In contrast to short-read methods, long-read sequencing is able to determine the phasing of genetic variants relatively easily. This is achieved by constructing the allelic context of mutations impacting the same gene, which cannot be performed by short read methods, particularly when the genetic alterations are located far apart.[45] Seki et al. compared RNA sequencing and ONT-based cDNA sequencing.[76] Long-read sequencing excels in identifying splice patterns, their combinations, and transcripts of fusion genes. Thus, the authors used full-length cDNA to establish phasing of SNPs previously detected by WES, enabling the identification of allele-specific transcription events. Despite the sequencing accuracy of only 92.3%, the authors postulated the clinical applicability of their developed method when increased numbers of reads is used (which, however, will also increase sequencing coasts).[76]

Direct ONT sequencing of RNA alongside qPCR was utilized to detect nonsense-mediated mRNA decay (NMD) activity in prostate cancer samples.[77] Nanopore sequencing is known to excel in detecting a wider range of isoforms compared to short-read NGS methods, facilitating effective identification of novel isoforms and those with premature termination codons. Therapeutic inhibition of NMD increases truncated proteins acting as neoantigens for T cell recognition, enhancing the efficacy of immunotherapy. The authors showed consistent changes in the transcriptional isoforms of *PRS*, *RPL12*, *SRSF2*, *PPUA*, and *TMEM208* genes, suggesting NMD activity and highlighting potential for its inhibition. Detection of NMD activity may help to stratify patients for NMD inhibition therapy suitability.[77]

Nanopore sequencing has also been used for the detection of minimal residual disease (MRD) in bone marrow and cerebro-spinal fluid (CSF) from patients with ALL using leukemia-specific rearrangements of the *IGH* locus.[40]

3.3 | Copy number alterations

Copy number alterations (CNAs) are deletions and amplifications of segments of DNA, which are either of germline or somatic nature. Somatic CNAs are a subgroup of the SV family and represent a fundamental characteristic in various cancer types, leading to the remodeling of cancer genomes. The analysis of CNAs is used in the identification of tumor entity and therapy susceptibility, making it a valuable diagnostic tool in clinical oncology.[78,79] For instance, Nanopore sequencing has been used to identify CNAs breakpoints in breast cancer susceptibility genes in individuals with a familiar predisposition. Long-read sequencing and chromosome-wide haplotyping enabled accurate breakpoints resolution, which may uncover silent carriers to improve prevention.[80]

Typically, analyzing tumors requires an invasive tissue biopsy. Sequencing of cfDNA isolated from plasma or other liquids is therefore an attractive low-invasive alternative. However, to date cfDNA analysis using short-read NGS methods is not cost effective, mostly due to initial instrumentation costs.[81] Recently, Martignano et al. reported the very first successful ONT-based CNA profiling of cfDNA, making it applicable for further liquid biopsy-based studies.[81] The authors developed a low-coverage approach to detect CNAs using only 2 million reads, which allowed to reconstruct whole genome karyotypes of lung cancer patients. Importantly, Nanopore long-read sequencing identifies genetic variations overlooked by short-read methods. However, because cfDNA is presented by short fragments of around 166 bp,[82] cfDNA sequencing with ONT presents certain challenges. These limitations include, among others, a lower number of cfDNA reads compared to Illumina sequencing. The authors achieved appropriate sequence quality by optimizing the library preparation protocol by increasing the magnetic bead-to-sample ratio at the purification steps.[81] Another attempt to optimize ONT for short fragment sequencing was done by Baslan et al.[83] Analysis of size-selected short tumor DNA fragments (~500 bp vs. standard 10 kb) derived from patients with AML resulted in a 4–6-fold increase in read numbers, while the generated results were in accordance with Illumina sequencing data. The enhanced sequencing yield facilitates the multiplexing of clinical samples via barcoding, thereby reducing the costs associated with CNA analysis in cfDNA.[83]

3.4 | Other structural variations

Structural variations other than CNAs are common aberrations detected in all cancers and often of clinical relevance. Long-read ONT sequencing can be applied to resolve large or complex genomic aberrations, that are otherwise difficult to detect using short-read methods.[68,84,85] For example, Nanopore sequencing was used to identify an array of structural aberrations in lung cancer, some of which have both therapeutic and prognostic value.[86] Moreover, 30% of AML[87] and ALL patients[88] carry hugely clinically relevant gene fusions. These are often missed by short-read NGS methods, require rapid TAT and multiple probes for fluorescent in situ hybridization (FISH). Nanopore sequencing was shown to detect these gene fusions including those with complex breakpoints or infrequent fusion partners that cannot be detected by FISH.[89–93]

Nanopore sequencing was further optimized for gene fusion analysis by application of CRISPR/Cas9 enrichment of a panel of targeted gene fusions (Table 1). In this method CRISPR/Cas9/sgRNA complexes bind to the 5′ and 3′ ends of sequence-specific loci of genomic DNA, preventing them from being degraded by exonucleases and therefore enriching the target. This enrichment method was applied to detect gene fusions to evaluate prognosis and guide the treatment in patients with acute promyelocytic leukemia (APL), CML, and AML.[43] Notably, Yeung et al. used CRISPR/Cas9 enrichment for detection of gene fusions in leukemia patients within a very short turnaround time of 6–7 h, which resulted in a delay-free clinical

decision. In this study, the peripheral blood or bone marrow specimen from patients with CML, AML, and APL were analyzed and *PML-RARA*, *BCR-ABL1*, and *KMT2A-AF4* fusions were detected, ultimately resulting in modification of the treatment strategy.[42] Notably, ONT was also combined with Hi-C technique to characterize novel genomic rearrangements in AML.[94]

Another enrichment method, Fusion Detection from Gene Enrichment (FUDGE), combines target-selective and strand-specific CRISPR/Cas9 activity for fusion gene enrichment with the bioinformatics pipeline NanoFG. The assay allowed rapid detection of gene fusions, which enabled prompt therapy adjustment and suggested specific genetic markers for tracking of MRD.[44]

Finally, nanopore-based WGS can, in certain cases, rapidly characterize gene fusions without prior target enrichment or PCR amplification in certain cases. For example, Au et al. could identify translocation breakpoints resulting in *DUSP13-GRIN2B* and *NUP98-NSD1* gene fusions using bone marrow-derived DNA of an AML patient. This method can further facilitate personalized disease monitoring.[95]

3.5 | Non-coding RNAs

Unlike other sequencing techniques, ONT can sequence native RNA, which significantly simplifies and accelerates the analysis of non-coding RNAs. The family of non-coding RNAs (ncRNAs) includes lncRNAs, miRNAs, and circRNAs. These regulatory molecules control the development, promotion, and metastasis of cancers. Along with mRNA, various non-coding RNAs have recently been identified as promising prognostic and predictive biomarkers as well as potential therapeutic targets in many cancers.[96,97] The Nanopore-Induced Phase-Shift Sequencing (NIPSS) method was developed to directly sequence miRNAs. NIPPS can distinguish different identities, isoforms and epigenetic variants of miRNAs.[98] Future development of this technique is believed to assist the development of miRNA-based early diagnostic, prognostic and therapy-selective purposes and novel cancer therapeutics.[99]

While short-read techniques struggle to determine the sequence of full-length non-coding RNA, ONT has been successfully applied for the detection of cancer-associated circRNA and prognostic miRNA that can further be used for early diagnostics of cancer.[100,101] Moreover, Nanopore sequencing has already been utilized for direct analysis of epigenetic changes in lncRNA in glioblastoma. For instance, Krusnauskas et al. compared Nanopore-based m6A analysis in lncRNA glioblastoma samples versus standard immunoprecipitation followed by sequencing (MeRIP). The authors found that the direct ONT lncRNA sequencing allows higher-resolution analysis of m6A-RNA modifications compared to more labor-intensive MeRIP sequencing.[102] They also reported an association of m6A-lncRNA profiles with glioblastoma grade and patient survival. Therefore, Nanopore sequencing provides a very promising platform for cancer-associated non-coding RNA analysis, however, to date most studies are still preclinical and further in-patient investigations are warranted.

3.6 | Epigenetic markers

Epigenetic modifications may impact the expression of various genes and contribute to cancer initiation, progression, and drug resistance.[103,104] As a tool for epigenetics analysis, Nanopore sequencing has a significant advantage over other methods, as it offers a direct analysis of DNA and RNA base modifications, eliminating the need for prior PCR amplification, reverse RNA-to-DNA transcription, or bisulfite conversion.[105,106] Nanopore Cas9 Targeted Sequencing (nCATS) was applied for accessing methylation of *MGMT* promoter in glioblastoma biopsies, which has a direct clinical impact and, if found, suggests a sensitivity to temozolomide. Unlike previous methods that exclusively focused on the *MGMT* promoter region, ONT allowed an extended analysis encompassing CpG sites proximal to the promoter, including the entire exon 1, and sections of intron 1.[107] Moreover, ONT-based epigenetic analysis can be used to subclassify a tumor and therefore assign an appropriate therapeutic strategy, as it has been shown for gliomas, where classification based on their methylation signatures is well established.[108,109] Kuschel et al. developed an ad hoc random forest algorithm capable of accurately classifying Nanopore-sequenced brain tumor samples with as little as 1000 CpG sites (80.4% sensitivity, 100% specificity).[110]

Epigenetic profiling of cfDNA can reveal tissue-specific signatures. Therefore, interrogation of cfDNA for cancer-associated biomarkers can reveal the presence and origin of ctDNA.[111] Lau et al. developed an ONT-based epigenetic classifier which determines whether an individual read originates from a tumor cell, and is thus ctDNA, or from healthy blood cell. Moreover, they could define the tissue of origin of ctDNA as well as tumor burden.[112] Hence, Nanopore sequencing is particularly interesting for epigenetic profiling due to remarkable its simplicity and low cost compared to other methods. However, robust standardized protocols utilizing Nanopore sequencing for this purpose are still to be developed.

3.7 | Multimodal analysis

Integrative analysis of various types of molecular alterations often results in a better overview of available personalized treatment options available. In multimodal analyses, information on specific point mutations, CNAs, simple and complex structural variants as well as epigenetics markers and ctDNA fragmentation signatures can be effectively analyzed simultaneously to render a more comprehensive picture of a specific cancer case.[113] In this regard, Nanopore sequencing is an attractive technique as it can produce all these data in question within a single sequencing experiment.

CNS tumors are highly heterogeneous and require accurate classification for therapy selection. Several groups applied Nanopore sequencing for this purpose. Euskirchen et al. developed a one-day ONT-based workflow for brain tumors classification based on the combined CNA, methylation and mutation analysis, which is critical for further treatment strategy selection. In this study, fresh frozen tumor tissues were analyzed using ultra-low pass WGS to detect

CNA, focal alterations and methylation of CpG sites. Using this approach, the authors could classify gliomas into *IDH*-mutated and wild-type and confirm the tissue of origin. Additionally, deep amplicon sequencing facilitated the detection of SNVs in the promoter regions.[29] To further accelerate CNS tumors classification, adaptive sampling was applied.[30] This approach enabled selective sequencing of clinically relevant alterations, as well as DNA regions applicable for tumor classification, and has advantages over standard NGS-based and EPIC arrays.[30] Djirackor et al. used shallow Nanopore sequencing-based analysis of CNA and DNA methylation profiling combined with machine learning to develop a rapid brain tumor classification algorithm. This approach was tested in 105 patients. The developed method could support intraoperative decisions as results were reported within 90–160 min, and overcomes limitations of current intraoperative histomorphological techniques.[114]

3.8 | Liquid biopsies

The term "liquid biopsy" indicates a low-invasive sampling and analyses of the body liquids, usually blood, urine, CSF. Interrogation of liquid biopsy-derived ctDNA can give an invaluable information of the genetic background of the primary tumor and metastases.[115] Afflerbach et al. performed methylation and CNA profiling of cfDNA isolated from 129 CSF samples, therefore utilizing a minimally-invasive liquid biopsy diagnostic approach. The authors were able to detect tumor DNA in 45% of the samples and ultimately 17.1% of cases could be precisely classified into the right brain tumor entity according to the methylation profile. Further protocol and classifier optimization should improve the outcome of this approach.[116] Another technique used for the selective and rapid analysis of diagnostic targets is nCATS. This method uses panels of guide RNAs which enable the simultaneous assessment of SNVs, structural variants and CpG methylation.[117]

The multifactorial analysis proved to be efficient not only in plasma-derived, but also in urine-derived cfDNA studies. Profiling of urine cfDNA of bladder cancer patients informed about tumor burden within 24 h therefore being an attractive approach for non-invasive disease monitoring.[118] Thus, analysis of liquid biopsies using ONT has the potential to provide a comprehensive fingerprint of the tumor, which can be used for both tumor classification and therapy planning. The generalized analysis workflow as well as various application of ONT are represented on Figure 2.

4 | LIMITATIONS OF ONT IN ONCOLOGY

Nanopore sequencing still holds certain limitations which restrict its broad implication in the clinic. A major limitation of ONT to date is the accuracy of base identification. Though recent updates in chemistry have elevated sequencing precision up to 99% (Q20) for simplex reads (or 99.9%, Q30, for duplex reads when Super accuracy model is used), it is still at the lower limit of the acceptable NGS reads.[48] Therefore, correct base calling and distinguishing a true mutation from sequencing artifacts requires greater sequencing depth, and better sample quality and purity. The latter is challenging to achieve in the case of tumor samples, especially when isolated from FFPE-material or cfDNA from liquid biopsy. Similar to second generation NGS, Nanopore sequencing exhibits lower accuracy when sequencing GC-rich regions; additionally, substitutional errors within purine (A↔G) and pyrimidines (C↔T) are rather common.[119] Moreover, the precision of ONT struggles when low complexity regions like homopolymers and STR are sequenced, as false indels are often called here.[8] These regions are responsible for roughly half of all sequencing

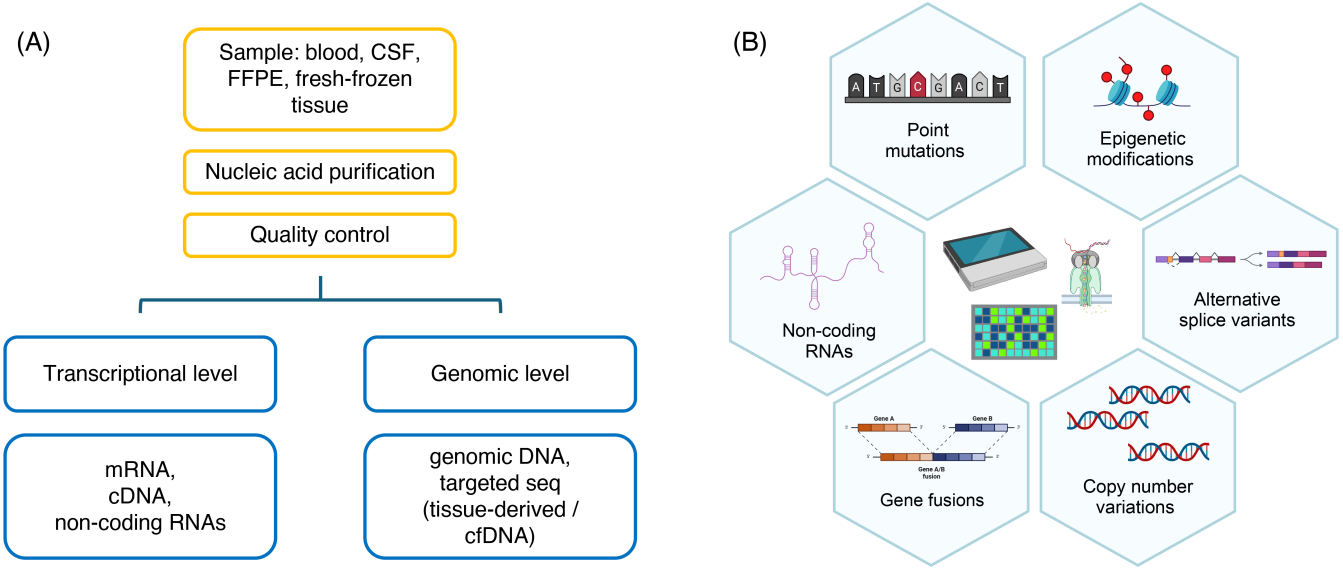

FIGURE 2 (A) General workflow of sample analysis using ONT. (B) Main potential applications of Nanopore sequencing in precision cancer medicine. CSF, cerebro-spinal fluid; FFPE, formalin-fixed paraffin embedded; ONT, Oxford Nanopore Technologies.

errors done by ONT.[119] Of note, several errors come from the base calling complexity as the electrical signal needs to be converted to the nucleotide sequence.

Another limitation is the need for a relatively high sample input. Nanopore sequencing DNA input can be as low as 20 ng/flow cell (low data output and no preservation of methylation information), which is comparable to input needed for Illumina sequencing (25–200 ng/flow cell). However, a PCR-free library preparation for Nanopore sequencing requires at least 1000–2000 ng of DNA. At the same time, the cost per Gigabase for ONT is still higher than the newest high-throughput Illumina platforms, which, however, require significant initial capital investments and ongoing maintenance.[120] Otherwise, the throughput of the most advanced ONT sequencing machine PromethION, which simultaneously uses 48 high-capacity flow cells, is currently comparable with the latest Illumina platforms.[121,122]

Finally, ONT lacks standardization, which is essential for in-clinic application starting with the fact that the human genome reference has been generated by a short-read platform and that ONT flow cells are not yet produced using the required standards for diagnostic devices inducing significant batch variation.[123,124] As it is still a rather new technique, no common consensus on data processing has been reached.[125] This may lead to a variability in data interpretation among different diagnostics and research laboratories as various analysis workflows and even base calling tools may be used.[126] Moreover, medical accreditation following International Organization for Standardization (ISO) 15189 or US FDA approval to introduce this technology into routine clinical practice are mostly lacking.

5 | DIRECTIONS FOR THE FURTHER IMPROVEMENT OF THE TECHNIQUE

Nanopore sequencing is a novel and developing technique. Its further improvement should overcome the problems listed above. There are two main directions of ONT development.[127] The first path comprises continuous optimization of nanopore structure along with other aspects of sequencing chemistry; this should result in higher quality and consistency of raw signals, higher throughput, a longer longevity of the pores, and ultimately in better nucleotide identification accuracy. Additionally, the development of sample preparation protocols and quality control methods is likely to reduce the number of sequencing artifacts caused by nucleic acid degradation.[127,128]

The second direction is an evolution of base-calling algorithms that includes both development of new tools and fine-tuning of already existing ones.[129] Here, approaches utilizing machine learning and artificial intelligence might be of great use.[28,130,131]

Resolving the precision problem as well as developing the technique overall will result in a reduction of sample input requirement. Further evolution of the ONT should result in cost reduction and ultimately in increased utilization and standardization of Nanopore sequencing both in research and the clinic. Arguably, the development of hybrid sequencing methods combining long- and short-read NGS techniques might be a single albeit costly solution to many problems listed above.[132,133] However, the future of ONT sequencing is believed to be in the refining of the technique itself and removing of a need for a short-read based polishing of the long-read data.[120,134]

Finally, several recent large-scale clinical studies like NCT01856296 (WINTHER) and NCT01976741 have shown that combining genome and transcriptome sequencing data for patient stratification is more efficient than using either technique alone.[135,136] As ONT can be used for both analyses within the same device without much time delay, it may become a cutting-edge technique in this regard. This approach is also applicable to single-cell analysis,[137] which is increasingly being adopted in clinical settings.[138,139] However, to prove general clinical applicability and eventual superiority of ONT over NGS methods, multiple clinical trials would be needed.

Overall, ONT currently undergoes a rather straightforward way of development and optimization, which other NGS techniques have already gone through. Updates of both hard- and software are to be expected. However, full integration of ONT into routine clinical use, although announced to be the main strategic goal of the company,[50] may yet require a considerable amount of time.

6 | CONCLUSION

Nanopore sequencing is a young and elegant method of single-molecule DNA, RNA and epigenome sequencing with huge potential. Several current drawbacks of this technique are likely to be overcome soon. Due to its simplicity, speed, variety of output data, and low initial cost, ONT may become the leading sequencing method in the future and will certainly make sequencing much more accessible worldwide. Moreover, multifactor analysis approach may open up new avenues for research and clinical applications. ONT is expected to advance precision diagnostics and catalyze the transition of the medical paradigm from a "one-size-fits-all" to an individualized treatment approach.

AUTHOR CONTRIBUTIONS

Sergey A. Dyshlovoy: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Stefanie Paigin:** Data curation; formal analysis; investigation; methodology; software; visualization; writing – original draft; writing – review and editing. **Ann-Kristin Afflerbach:** Data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Annabelle Lobermeyer:** Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Stefan Werner:** Formal analysis; investigation; resources; validation; writing – review and editing. **Ulrich Schüller:** Formal analysis; investigation; resources; validation; writing – review and editing. **Carsten Bokemeyer:** Formal analysis; investigation; resources; validation; writing – review and editing. **Anna H. Schuh:** Formal analysis; investigation; resources; validation; writing – review and editing. **Lina Bergmann:** Data curation; formal analysis; investigation; resources; software; validation;

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CONFLICT OF INTEREST STATEMENT

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