REVIEW ARTICLE

Nanopore Sequencing Technology in Oral Oncology: A Comprehensive Insight

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Abstract

Aim: To review the principles and application of Nanopore Sequencing Technology (NPST) in oral cancer.

Background: Oral cancer is a disease caused by aberrations in the genes. Substantial research at the genomic level is still required for in-depth understanding of the molecular mechanism in oral cancer. The advent of the novel nanopore sequencing technique has the potential to detect the alterations at the genomic level. This review highlights nanopore sequencing, its advantages and disadvantages, and how research supports its application in the field of oral oncology.

Materials and methods: Web-based search via PubMed database, internet sources using keywords "nanopore sequencing, third-generation sequencing, next generation sequencing, cancer, oral squamous cell carcinoma, genetic, epigenetic, oncogenic viruses" was performed in this review. Original research, reviews, and short discussions published from 2008 to 2020 were included. The findings are discussed with emphasis on common gene mutations, epigenetic alterations, and oncogenic viruses in oral cancer. A brief mention regarding translational nanopore sequencing research in oral cancer and future perspectives is also discussed.

Results: The results obtained reveal that cost-effectiveness and rapid turnaround time make nanopore sequencing an enticing platform to resolve the ambiguity of genomes, epigenomes, and transcriptomes.

Conclusion: The findings will encourage researchers to further adopt NPST in their studies and give an overview of the latest findings of oral squamous cell carcinoma (OSCC) management. To highlight the importance of NPST application in OSCC studies, this paper not only discusses the use of NPST in identifying the behavior of malignancy but also implies the need for further research using this technique.

Clinical significance: The review suggests that nanopore sequencing can be utilized for diagnosis and achieving personalized treatment in each oral cancer patient.

Keywords: Epigenetics, Genomics, Nanopore sequencing, Oral cancer, Third-generation sequencing.

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Introduction

Oral cancers are characterized by tumoral and intratumoral heterogeneity that drives research at the molecular level.[1] Initiation or progression of carcinogenesis occurs due to the mutations in the genomes of the cancer cells. The function of driver and tumor suppressor genes is primarily affected.[2] The field of genomics is revolutionized because of the advent of next-generation sequencing (NGS) technology. Sequencing of whole genome, whole exome, m-RNA, detection of translocations, copy number alterations, and many more can be achieved through NGS. It also helps to sequence only point mutations referred as focused DNA sequencing.[3] With new advents and modifications in NGS, the third-generation sequencing is coming out with new insight in the field of sequencing. Nanopore-based sequencing has been actively pursued in recent years as the third-generation gene sequencing technique.[4,5] Nanopore is a nanoscaled pore; it facilitates the exchange of ions when embedded on substrates like lipids, graphene, and silicon. The movement of ions through the nanopore disrupts the voltage that can be read out electrochemically thus allowing sequencing of molecules.[5] Nanopore sequencing delivers comprehensive analysis of research samples providing insights into DNA, RNA including human genomics, microbial genomics, plant genomics, cancer research, infectious disease, and personalized medicine.[6,7] Nanopore sequencing possesses advantages over existing commercialized NGS technologies. This new platform has been developed to read longer lengths of DNA/RNA at relatively low cost.[1,8] The present review emphasizes the concept of nanopore technology, highlighting the current and potential applications in the field of oral oncology. Since there are limited studies using this technique in oral cancer, this paper aims at motivating the researchers to pursue this technique in their studies that could further help in oral cancer diagnostics and therapeutics.

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Nanopore Sequencing

Introduction and Evolution

An ease in the identification of mutations in cancer cells is possible because of the sequencing technologies. The first commercialized sequencing method was developed by Frederick Sanger in the 1970s which is referred as the Sanger sequencing or the chain terminator sequencing.[1,5] This technique underwent modifications eventually giving rise to the revolutionary NGS method. NGS technologies can be broadly categorized into two groups, namely short-read sequencing and long-read sequencing techniques. Illumina and Ion Torrent are the cheap short-read sequencers (read data <300 bp) whereas single-molecule real-time sequencing (SMRT) developed by PacBio and Roche are costly long-read sequencers (read data >2.5 kb).[8] Short-read sequencing techniques are mainly used to detect point mutations or genotyping but long-read sequencers are desirable for deciphering highly complicated cancer genomes.[2]

Nanopore sequencers are the latest long-read sequencers,[9] first proposed by Church et al. and Deamer and Akeson separately. The concept of nanopore sequencing where DNA translocation through α-hemolysin was described in a publication in 1996 by Kasianowicz et al.[10,11] Nanopore technology is a single-molecule sequencing method since it sequences a single molecule of DNA/RNA without the need for amplifications.[12] Currently it is applied in areas of great interest in many disciplines and has been commercialized by Oxford Nanopore Technologies (ONT; Brown and Clarke, 2016), NabSys, and Sequenom, and widely used in scientific researches.[6] The technology is utilized for sensing of ions, nucleotides, enantiomers, drugs, RNA, DNA, and polypeptides (Flowchart 1).[10,13]

The limitations observed in the older generations were overcome by modifications eventually giving rise to third-generation nanopore sequencing technique.[11,12,14]

Principle of Nanopore Sequencing

The nanopore technique is based on the working principle of the classical Coulter counter or the resistive pulse. The small changes in electric current when ions or nucleotides pass through the nanopore are read out electrochemically. In the nanopore technique, a nanometer-sized aperture embedded in a thin membrane electrophoretically drives the charged polymers. An electrochemical chamber containing conducing buffers separated into cis- and trans-compartments are utilized and the nanopore is located within the chamber. Setting a voltage across this membrane, sensors detect the ionic current changes occupying the pore in real time as the molecules pass through.[10,13]

Classification and Attributes of Nanopores

Nanopores are mainly classified into three groups (Table 1).[10,13]

Advantages of Nanopore Sequencing

Nanopore sequencing possesses a number of advantages over existing commercialized NGS. This technique was relatively developed to read long lengths of DNA/RNA; it potentially reaches long-read length >5 kbp with speed 1 bp/ns offering high-quality reference genomes. They are label-free; detection of bases is fluorescent tag-free. Real-time analysis can be achieved while sequencing is ongoing. The sequencing technique is amplification-free hence simplified, less time-consuming, cost-effective, requires very low sample volume, and less sensitive to changes in temperature throughout the sequencing reaction. It also helps in direct sequencing of DNA/RNA.[5,9,10]

Nanopore Sequencing: Oxford Nanopore Technology

Nanopore sequencing technique is highly commercialized by ONT and this technology has continued to evolve till recently. The technologies have released a device called MinION (2014). It is the

Flowchart 1: Schematic representation of evolution of nanopore sequencing technique (Schadt et al., 2010)

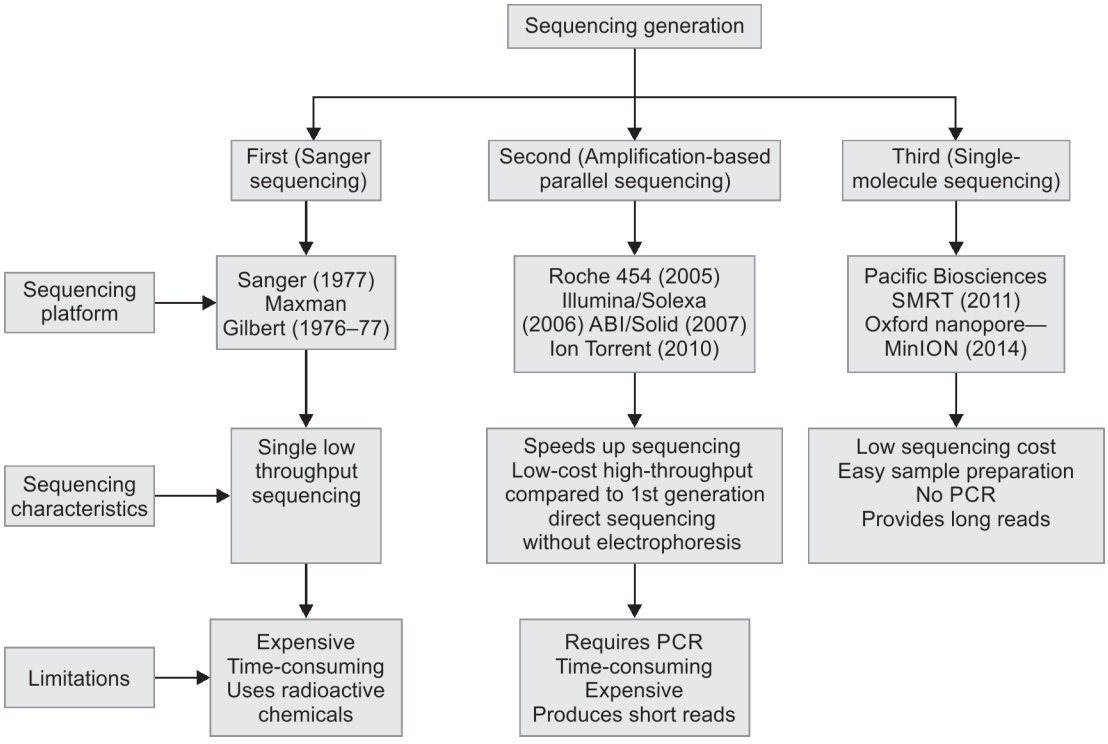

Table 1: Summarizes various nanopores with advantages and disadvantages, respectively

Types of nanopores	*Substrates used*	*Advantages*	*Disadvantages*
Biological nanopores	Planar lipid membranes	Extremely reproducible	Mechanically fragile
Common biological pores studied	Liposomes	Harvested in cells in large quantities at low cost	Susceptible to extreme solution conditions, such as pH, temperatures, and salt environments
a. α-Hemolysin			
b. MSPA (*Mycobacterium smegmatis* porin A)	Polymer membranes	Industrial large-scale production is possible	
c. Bacterial virus Phi29 connector			
Synthetic nanopores/ solid-state nanopores	Silicon based—silicon nitride and silicon dioxide Highly stable chemically, thermally, mechanically	Substrates are durable	Fabrication process is labor intensive
	Aluminum oxide	Reusable	
	High dielectric constant materials—titanium oxide, hafnium oxide	Integrated into nanodevices	
	Graphene sheets		
Hybrid nanopores	The concept was demonstrated by inserting α-hemolysin channel in solid-state nanopores	Fragile lipid bilayer of α-hemolysin is circumvented by placing it inside a mechanically robust solid-state nanopore creating advantages of both	Leakage currents are significant due to deformation of biological pore when it contacts with solid-state pore

size of USB flash drive and can be easily connected to laptop. Being economical, portable and easy operational on MinION instrument may prove to be a valuable tool for clinical testing. ONT also launched scaled-up GridION commercially in 2017 and PromethION bigger version of MinION which can hold 48 flow cells with 3,000 channels each. ONT launched real-time RNA sequencing and cDNA analysis in 2017. Flongle the adapter for MinION, GridION for rapid small tests was launched in 2019, mobile phone compatible SmidgION is also launched by ONT.[7,15]

In early 2020, ONT was put to use in the surveillance of coronavirus outbreak.[16,17] Researchers have also showcased nanopore sequencing has potential for adaptive sampling, can real time detect cancer genes or exomes in a whole-genome sample on the device.[18–20] In India, genotypic technology is appointed as the first certified service provider for ONT and is employed in research studies.[21]

Oral Cancer

Although the diagnostic approaches for oral cancer have improved over decades yet it ranks as the sixth most common cancer in the world.[22,23]

Distant and cervical lymph node metastasis causing poor prognosis and survival rate in oral cancer is a matter of major concern too.[24] Most oral cancers are classified as squamous cell carcinoma (OSCC) based on the cell that proliferates predominantly.[1,23] Tobacco, alcohol, and betel quid use are primarily associated with OSCC.[23] But a change in the trend where young patients with no associated habits are also observed in OSCC.[25–27] Therefore, other factors like genetic, epigenetic alterations, oncogenic viruses, oro dental factors, dietary deficiency, and chronic candidiasis should also be considered in the etiopathogenesis.[28] Unveiling the aspects of genetic, epigenetic, and oncogenic viruses in OSCC are essential in this review that helps us understand the better implementation of nanopore sequencing for patient benefit.

Genetic and Epigenetic Alterations

Evidence suggests that solid tumors are genetically unstable and several extensive studies have been performed to determine the genetic origin of solid tumor cells. Defects in the segregation of chromosomes, copy number alterations, loss of heterozygosity, telomere stabilities, regulation of cell-cycle checkpoints, and DNA damage repairs lead to genetic instability in tumor cells.[23] Aberrant expression on *TP53, NOTCH1* (*Notch homolog 1* genes), EGFR (epidermal growth factor receptor), CDKN2A (cyclin-dependent kinase inhibitor 2a), STAT3 (signal transducer and activator of transcription 3), Cyclin D1, and retinoblastoma has been reported in OSCC development. Inheritable alterations are transferred to the cell clones by the genetically unstable precancerous keratinocytes. Various signaling pathways transform these normal oral keratinocytes into precancerous lesions, which further deteriorate into malignant tumors (Table 2).[29,30]

The cancer phenotype is driven by the epigenetic changes which are known to cooperate with genetic alterations in cancer cells. The most common epigenetic changes observed in cancer are the DNA methylation which includes 5-methylcytosine (5-mc), 5-hydroxymethylcytosine (5-hmc), and 6-methyladenine (6-mA). The other epigenetic modifications include the histone modifiers and readers, chromatin remodelers, microRNAs (miRNA), and other components of chromatin.[43,44] These epigenetic alterations have also been reported in oral cancer.[45,46] The first epigenetic alterations in cancer are the DNA methylation changes targeting tumor suppressor genes (TSG) and have been related to the early stages of

Table 2: Common gene mutations in OSCC and their roles in tumorigenesis

Genes	*Variant type*	*Location of mutation*	*Regulatory function*	*References*
TP53	Missense (31,33–36,38–40,42) Nonsense (31,33,34,36,38–40,42) Deletion (34,36,38,40) Insertion (36,40) Splice-site mutation (34,36,38–40,42)	DNA-binding domain (33,34,36)	Regulates cell cycle and apoptosis tumor suppressor (27,29)	31,33–42
NOTCH1	Missense (31,32,34,36,39,40) Nonsense (31,40) Deletion (36) Insertion (34,36) Splice-site mutation (34,36)	EGF-like repeats (31–34,36)	Cell proliferation, differentiation, apoptosis (31)	31,32,34,36,39,40
CDKN2A	Missense (34,36,39,40) Nonsense (34,36,38,40,42) Deletion (38,40) Insertion (36,40) Splice-site mutation (34,36,40,42)	Ankyrin repeats (36)	p16 and p14 function as tumor suppressors (29,36,70)	33,34,36,38–42
PIK3CA	Missense (34,38,40,42) Nonsense (34,42) Splice-site mutation (40)		Cell growth and division (37)	33–35,37–42
HRAS	Missense (38,40,42)		Cell growth and division (70)	33,35,38–42
PTEN	Missense (35) Nonsense (40) Deletion (35)		Tumor suppressor (45)	35,40
EGFR	Missense (36) Deletion (38) Splice-site mutation (36)	Furin-like repeats (36)	Regulates cell proliferation (47,70)	36,38
CASP8	Missense (36) Nonsense (36) Deletion (36)	Caspase homologues domain (36)	Regulates cell apoptosis (36)	36
RB1	Missense (38,40) Nonsense (40)		Tumor suppressor (45)	38,40

carcinogenesis. Methylation of several TSGs, more specifically $P14^{ARF}$ and $P16^{INK44a}$ epigenetic silencing has been observed in OSCC tissues and precancerous oral lesions.[47] Elevated levels of oncogenic protein could be related to the overexpression of oncogenic miRNA or the loss of tumor-suppressor miRNA expression.[46,48,49] All these changes contribute to initiation and progression of tumorigenesis in oral cancer.[48] In this context, nanopore sequencing can be well tested to evaluate the mutational status of genes, epigenetic modifications, transcriptomes, etc., which helps us to acquire more biological information about the tumor.

Oncogenic Viruses

Oncogenic capability of certain types of human papillomaviruses (HPV), Epstein-Barr virus (EBV), and human herpes simplex virus (HSV) have been reported in cancers.[50] Syrjanen et al. (1983) first proposed the involvement of HPV in oral cancers.[51,52] E6 and E7 are the two important viral oncoproteins of HPV. The function of p53 is interfered by E6 and E7 inactivates the function of the retinoblastoma protein (pRb), leading to cellular proliferation by promoting inhibition of apoptosis and dysregulation of the cell cycle, respectively.[3,52] Association of subtype of HPV 16 and HPV 18 with oral cancers was declared by the International Agency of Research of Cancer (IARC) in 2012.[51,53] It is also observed that HPV-related oral cancers differ from HPV-negative cancers in their clinical response and overall survival rates.[53] The seropositivity to Herpes simplex virus HSV-1 and HSV-2 may modify the risk associated with exposure to tobacco, alcohol, or HPV in OSCC patients.[50] Other less commonly associated viruses in OSCC are EBV and hepatitis C virus (HCV). Keisuke et al. concluded from their studies that EBV virus infection of oral squamous epithelium may be carcinogenic or the virus may merely exist in epithelial cells of squamous cell carcinoma, carcinoma *in situ,* and leukoplakia. Different grades of OSCC and EBV DNA positivity showed a positive correlation in a study done by González-Moles et al. Nagao et al. suggested that high prevalence of anti-HCV antibodies in patients with oral lichen planus, which is histologically a disease of squamous cells, may further lead to the development of OSCC.[51] Analysis of HPV or other viruses through nanopore sequencing can potentially be utilized as a diagnostic tool or in research areas in OSCC.

Nanopore Sequencing in OSCC

Nanopore sequencing is an era of genomic research and personalized treatment, and it has the potential to determine both. Nanopore sequencing technique detects alterations at genomic level that includes genome sequencing (de novo genome sequencing, resequencing of genomes, metagenome sequencing),[54–57] RNA sequencing (direct RNA sequencing, cDNA, metatranscriptome sequencing),[58–60] epigenetic sequencing (5mc, 5hmc),[61] amplicon sequencing/targeted sequencing (full length 16s microbiome profiling, Ampli-Seq).[62,63] Genomic changes are a fundamental step for understanding exome genome dysfunction in cancer.[64] Hence in the future, mechanisms at the molecular level will play an important role in classifying cancer than merely based on symptoms. Molecular alterations found within the tumor can help in treatment modality hence in-depth characterization of individual tumors is of potential benefit to patients.[65] As discussed earlier, genetic, epigenetic, and oncogenic viruses are involved in OSCC but there are many areas that remain ambiguous and demand advanced research for instance-genetic alterations from distinct ethnic and geographic areas, other genes as potential diagnostic biomarkers, complete coding regions of genes that are more frequently mutated in OSCC. In the context of genetic-based risk assessment to predict prognosis and better guide treatment,[32,33,38] methylations of genes and its role in pathogenesis of OSCC[66,67]mutations in miRNA and the alterations in OSCC,[46] the association of HPV with oral cancer is an incidental finding or a sole etiologic factor, further research related to prognosis, research on the role of HPV vaccine as prophylaxis,[52] and many more. The aim should be to improve the technology not only to the experimental setting but also toward its use in routine clinical practice to diagnose and determine the treatment and prognosis of oral cancer patients. One of the characteristics of MinION is its convenience for use, including its portability, simplicity, cost-effectivity, and easy settings for the library preparation and sequencing.[68] This device can be well utilized for the procedures to identify driver genes in each OSCC patient for personalized medicine.

Translational Nanopore Sequencing Research in OSCC

Targeting and disrupting the cellular deregulations using targeted drugs in cancer can be facilitated by advances in translational research. It can help in therapeutic drug development and improve the safety and efficacy of the drug. Important clues revealing the treatment modalities for normal responders can be improved which prove to be biologically significant.[69] Targeted molecular therapies include small molecule drugs or monoclonal antibodies and gene therapy. In comparison with present modalities like surgery, chemotherapy, and radiotherapy, targeted molecular therapy possesses minimal side effects.[70] Chemotherapeutic agents paclitaxel, cisplastin, doxorubicin, docetaxel, methotrexate, fluoropyrimidine 5-fluorouracil are widely used in the treatment of OSCC.[71] Chemoresistance to cisplastin or cancer cell resistance to conventional therapies is observed in OSCC, which is one of the major causes of tumor recurrence and metastasis.[71,72] In such cases, targeted cancer therapies personalized to the patient like EGFR inhibitors (Lapatinib, cetuximab, panitumumab, zalutumumab, and nimotuzumab), EGFR tyrosine kinase inhibitors (gefitinib, erlotinib, afatinib, and dacomitinib), or gene therapy by replacing mutated p53gene with wild type p53 or p16, immunotherapy, etc. would be beneficient.[70,71]

Today, advancements in nanopore sequencing can prove to be an attractive platform for comprehensive analysis of cancer cells. Nanopore sequencing is used in various cancer studies like lung cancer,[68] brain tumors,[73] cervical cancers,[74] leukemias,[75] and detection of infectious pathogens.[7,60] The results obtained through these studies revealed that nanopore sequencing could detect diverse patterns of major driver genes including point mutations, fusions, epigenetic modifications, single nucleotide variants, HPV infections, and microbiomes effectively.[74,75] Researchers were able to gain unprecedented access to the human cancer genome through nanopore sequencing. Hence, simple methods of MinION sequencing would be beneficiary to small/mid-scale research centers and hospitals to conduct research studies and promote suitable therapeutics.[68] Considering oral cancer is accessible and can be easily detectable; why not this simple technique be implemented in research as well as hospital settings for assessing the risk associated with genetic instability in OSCC patients and drive them to the molecular targeted therapy?

There are only few published papers using nanopore platform in oral cancer. Research studies using MinION, a nanopore sequencer was done to sequence the mitochondrial genomes of OSCC cell lines namely H103, SAS, and the tumor spheres against the chemotherapeutic agent cisplatin by Aminuddin et al. Comparing the results obtained by MinION with Sanger sequencing, they found that 95.7% of the variants were precise.[76] In a pilot study conducted by Wongsurawat et al. whole shotgun metagenome sequencing of fecal samples obtained from 10 head and neck cancer patients (study included 5 oral cancer patients) was performed using ONT. A comparison of results was done with those obtained using Illumina Technology (IT). Higher sequencing depth was achieved by IT whereas ONT produced longer mean read length (longest >30 kb on average). In conclusion, they found ONT may be used for taxonomic profile filing of gut microbiome clinically which could serve as a useful tool for rapid screening of microbiota.[77] CyclomicsSeq, which is based on Oxford Nanopore sequencing was used by Marcozzi et al. to detect levels of circulating tumor DNA (ctDNA) in liquid biopsies of head and neck cancer patients. They demonstrated that CyclomicsSeq can be applied to any genomic locus. TP53-specific CyclomicsSeq assay helped in achieving accurate diagnosis in their study. Implementation in point-of-care clinical workflows and monitoring tumor burden during treatment for head and neck cancer patients were achieved through this assay.[78]

Limitations and Future Perspectives

Compared to short-read sequencers nanopore sequencer produces lower read accuracy and a high error rate (approximately 10–15%) with their 9.4 and earlier-version flow cells. This is the major drawback of nanopore sequencers. Nanopore reads are not considered optimal for single nucleotide variation (SNV) detection since insertions and deletions are included in the errors and they do not have the possibility of sequencing the same strand multiple times.[7,9,15] Pore chemistry and the base-calling algorithm (Patel et al., 2018) play a crucial role in improving the accuracy of nanopore sequencers. Data analysis can be achieved precisely if users have bioinformatics expertizations that aid in improving base-calling accuracy.[79] Optimization of existing bioinformatics tools and development of many more devices are actively performed by ONT to overcome aforementioned limitation. Some of the recent advances in ONT are LamPORE COVID-19 to detect SARS-CoV2,[80,81] improvements in PromethION enabling 10 terrabase sequencing record with modal single read accuracy

Table 3: List of bioinformatics software used in the analysis of nanopore data[84]

	Base callers	*Assembler*	*Variant calling and phasing*	*Aligners*	*Others*
Softwares	Albacore	ABruijn	Clair	BWA	DeepMod
	Basec	Canu	HapCUT2	GraphMap	EPI2ME
	RAWller	Cobbler	IDP-ASE	Kart	FLAIR
	Chiron	Flye	Medaka	LAMSA	Flappie
	DeepNano	HINGE	NanoPipe	LAST	Mandalorion
	Fast QC	LINKS	Nanopolish	Minimap2	MetaG
	Flappie	MECAT	PBHoney	Minialign	NanoDJ
	Guppy	Miniasm	Sniffles	NGMLR	RUBRIC
	Metrichor	Racon	WhatsHap	MarginAlign	Tandem-genotypes
	Nanocall	RAILS			
	Scrappie	SMART denovo			
		SPAdes			
		wtdbg2			

of 99.1%, Bonito CRF new analysis algorithm, which increases accuracy of single read base calling, expertise to automate library preparation to enable users to run high number of samples,[82] Cas9-based protocol for enrichment of specific genomic regions to perform targeted sequencing.[75,83] Advancements in technology and diagnostic approaches of nanopore sequencing can be adopted as a viable option in the near future for clinical research in the field of oral oncology (Table 3).

Conclusion

Although many platforms for NGS exist, the development of simple and cost-effective procedure to identify driver genes in oral cancer is needed. Nanopore sequencing serves as a promising platform in understanding the omic mechanisms in cancers. However, only a few studies have discovered the research application of novel nanopore sequencing in oral oncology. As improvements continue to be made toward higher accuracy and robust performance, the next goal is to expand this approach not only to the research phase but also in the clinical setting to diagnose and promote personalized medicine in each OSCC patient.

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