Deciphering Neurodegenerative Diseases Using Long-Read Sequencing

Author(s):
Yun Su, MD1,2,3; Liyuan Fan, MD1,2,3; Changhe Shi, MD, PhD4; Tai Wang, MD1,2; Huimin Zheng, MD1,2,3; Haiyang Luo, MD, PhD1,2,3; Shuo Zhang, MD, PhD1,2,3; Zhengwei Hu, MD, PhD1,2,3; Yu Fan, MD, PhD1,2,3; Yali Dong, MD1,2,3; Jing Yang, MD, PhD1,3,5; Chengyuan Mao, MD, PhD4; Yuming Xu, MD, PhD1,3,5

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Equal Author Contributions:
* These authors contributed equally to this work. # Dr. Yuming Xu and Dr. Chengyuan Mao are the corresponding authors.

Corresponding Author:
Yuming Xu
xuyuming@zzu.edu.cn

Affiliation Information for All Authors:
1. Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan, P. R. China; 2. The Academy of Medical Sciences of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan, P. R. China; 3. Henan Key Laboratory of Cerebrovascular Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan, P. R. China; 4. Sino-British Research Centre for Molecular Oncology, National Centre for International Research in Cell and Gene Therapy, School of Basic Medical Sciences, Academy of Medical Sciences, Zhengzhou University, Zhengzhou, Henan, P. R. China; 5. Institute of Neuroscience, Zhengzhou University, Zhengzhou, Henan, P. R. China.

Contributions:
Yun Su: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data
Liyuan Fan: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data
Changhe Shi: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data
Tai Wang: Major role in the acquisition of data; Analysis or interpretation of data
Huimin Zheng: Major role in the acquisition of data; Analysis or interpretation of data
Haiyang Luo: Major role in the acquisition of data; Analysis or interpretation of data
Shuo Zhang: Major role in the acquisition of data; Analysis or interpretation of data
Zhengwei Hu: Major role in the acquisition of data; Analysis or interpretation of data
Yu Fan: Major role in the acquisition of data; Analysis or interpretation of data
Yali Dong: Major role in the acquisition of data; Analysis or interpretation of data
Jing Yang: Major role in the acquisition of data; Analysis or interpretation of data
Chengyuan Mao: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data
Yuming Xu: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Study concept or design; Analysis or interpretation of data

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Abstract

Neurodegenerative diseases exhibit chronic progressive lesions in the central and peripheral nervous systems with unclear causes. The search for pathogenic mutations in human neurodegenerative diseases has benefited from massively parallel short-read sequencers. However, genomic regions, including repetitive elements, especially with high/low GC content, are far beyond the capability of conventional approaches. Recently, long-read single-molecule DNA sequencing technologies have emerged and enabled researchers to study genomes, transcriptomes, and metagenomes at unprecedented resolutions. The identification of novel mutations in unresolved neurodegenerative disorders, the characterization of causative repeat expansions, and the direct detection of epigenetic modifications on naive DNA by virtue of long-read sequencers will further expand our understanding of neurodegenerative diseases. In this paper, we review and compare two prevailing long-read sequencing technologies, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), and discuss their applications in neurodegenerative diseases.

Keywords: neurodegenerative diseases, long-read sequencing, repeat expansions, epigenetic analysis, genetics

Introduction

Neurodegenerative diseases are a class of neurological disorders characterized by a progressive course of cognitive decline, motor disturbance, psychiatric disorders, and eventual disability. The neuropathological features are the selective loss or impairment of specific neurons in the central and peripheral nervous systems. As the second most common
cause of death, neurodegenerative disorders impose a severe burden on individuals, communities, and countries. Therefore, it is crucial to detect and intervene early to slow down the irreversible progression of these diseases.

There are a small number of patients with family histories of neurodegenerative diseases, thus indicating that genetic factors may play an etiologically important role. Since the identification of expanded trinucleotide repeats in Huntington’s diseases\(^1\), cumulative evidence has suggested that repeat expansions may be more involved in the pathogenesis of neurodegenerative diseases than first thought, regardless of whether they reside in coding or non-coding regions including 5’ or 3’ untranslated regions (5’ or 3’ UTR), and introns\(^2,3\). Nevertheless, next-generation sequencing (NGS) technologies are inadequate to resolve the expansions exceeding several kilobases, owing to the limit of short read lengths (150-300bp) and an underrepresentation of GC-rich/poor regions\(^4\), despite innumerable discoveries of mutations facilitated by NGS.

In contrast to NGS, two prevailing long-read sequencers termed LRS, single-molecule real-time (SMRT) sequencing developed by Pacific Biosciences (PacBio)\(^5\) and nanopore sequencing by Oxford Nanopore Technologies (ONT)\(^6\), provide an alternative strategy for single DNA molecule sequencing in real-time. These platforms generate exceptionally long reads of several tens of kilobases, which encompass full-length repetitive regions. Moreover, GC bias can be reduced to a low degree, and more homogeneous genome coverage can be achieved without the requirement of PCR amplification, as opposed to NGS. Recently, CRISPR/Cas9-mediated amplification-free targeted enrichment in conjunction with LRS has emerged as an instrumental tool to achieve targeted sequencing\(^7\). The unique features of LRS have made it well-suited for deciphering neurodegenerative diseases, especially when NGS receives negative results.
Here, we summarize the mechanisms of LRS and their applications in human neurodegenerative disorders.

**Long-Read Technologies**

**SMRT sequencing: PacBio**

PacBio RS, RS II, Sequel, and Sequel II sequencers with increased average read lengths and throughputs have allowed for genomics research at an unprecedented resolution, all of which share a common mechanism, known as synthesis sequencing. First, a template called SMRTbell is created by ligating hairpin adapters to both ends of a target double-stranded DNA (dsDNA) molecule, thereby forming a closed circular DNA (Fig. 1A and B). The library is then loaded onto a flowcell composed of nanoscale wells called zero mode waveguides (ZMWs), where a polymerase is immobilized at the bottom and can combine an SMRTbell adaptor with a complementary primer to initiate the replication process (Fig. 1C). The incorporation of fluorescently labeled nucleotides releases excited fluorescence signals, with the color and duration of the emitted light being recorded in real time (Fig. 1D). A circular consensus sequence (CCS) with higher accuracy (> 99%) is eventually constructed from multiple reads covering the original DNA template. Additionally, the time between adjacent nucleotide incorporations is called the ‘interpulse duration’ (IPD) (Fig. 1D), which reflects variable polymerase kinetics influenced by modification events, and contributes to the epigenetic analysis.

Despite the preponderance of SMRT, raw read errors dominated by deletions or insertions remain problematic. Given the random occurrence of errors, base discrimination can be obtained by constructing a CCS having > 99% accurate consensus with sufficient coverage.
Nanopore sequencing: ONT

To date, ONT has introduced four sequencers: MinION, PromethION, GridION, and SmidgION, which are all competent in electronically detecting individual bases during the disassembling process. To begin with, library preparation occurs in double-stranded DNA fragments, which are end-repaired and adapter-ligated (Fig. 2A and B). The adapters contain 5’ protruding ends, to which a ‘motor’ protein acting as helicase is tightly bound (Fig. 2B). Sequencing then proceeds in nanopores embedded in a synthetic bilayer, through which electric current flows constantly (Fig. 2C). As unwinding DNA or RNA molecules translocate through the nanopore with the assistance of the ‘motor’ protein, individual nucleotides are cut off by an exonuclease (Fig. 2C), and current shifts due to characteristic disruptions are recorded in real time (Fig. 2D).

Nanopore read lengths are confined only by the molecular lengths of the sample as opposed to the limit of the technology itself in SMRT sequencing. It is theoretically possible to sequence templates containing endless nucleotides using nanopore sequencing, supported by recently reported reads of up to 1 Mb\(^9\). Additionally, MinION and SmidgION lead the trend of miniaturization and hold the potential to serve as hand-held and cost-effective devices for epidemiological studies.

A notorious pitfall of ONT is that the raw read error rates can reach up to 15\(^{10}\). Borrowing the mechanism of PacBio CCS, intramolecular-ligated nanopore consensus sequencing has emerged as an effective approach for constructing consensus single-molecule reads with a median accuracy of > 97\(^{11}\).

“[insert Figure 2]”
Applications in neurodegenerative diseases

Fragile X-associated tremor/ataxia syndrome (FXTAS)
The human fragile X mental retardation 1 (FMR1) gene contains a polymorphic CGG trinucleotide repeat in its 5’ UTR on the X chromosome, which causes fragile X syndrome (FXS) and fragile X-associated tremor/ataxia syndrome (FXTAS). The premutation (PM) range of 55–200 repeats underlies FXTAS, while greater than 200 repeats are classified as full mutations, leading to FXS. Although these two neurological diseases are caused by the same repeat motifs, they exhibit distinct clinical presentations and neuropathological features. FXS is considered the most common cause of heritable form of intellectual disability. As a late-onset neurodegenerative disorder, FXTAS is characterized clinically by intention tremor and gait ataxia. Additionally, the premutation range of CGG trinucleotide repeats is prone to expand into a full mutation in the next generation upon maternal transmission, whereas the sandwiched presence of AGG interruptions in every 9 or 10 CGG repeats can reduce this instability. CCS reads of 36 and 95 CGG-repeat-containing plasmids, and a PCR-amplified allele harboring 750 CGG repeats on the PacBio platform clearly showed respective repeat sizes, size distributions, and small AGG interruptions, which overcame the technical limits. More broadly, not only the effect of sequence context on polymerase kinetics but also the strand and position-specific influences of G on the polymerase’s activity were confirmed by the variation in IPD, thus laying the foundation for future investigations of epigenetic modifications and polymerase kinetics. To address the challenging detection and location of AGG interruptions in female PM carriers, SMRT provided a direct demonstration of AGG...
interruptions within the CGG repeat regions and separated a large number of reads containing AGG interruptions based on the X-chromosomes from which they derived. With the valuable information offered by SMRT, it is relatively precise to estimate the expansion risk of CGG repeats in female PM carriers, which can help them to select for preimplantation genetic diagnosis in case of high risk, or invasive prenatal diagnosis within small risk, to check the fragile-X status of the fetus.

Since the successful sequencing of highly repetitive elements exceeding 2.25kb of 100% GC content by utilizing SMRT sequencing technology, LRS has been the focus of extensive research and broadly applicable to a complete genetic and epigenetic analysis of expanded-repeat elements underlying many other neurodegenerative diseases. More importantly, LRS allows the improvement of genetic counseling in terms of assessing an accurate expansion risk of each allele individually for female PM carriers to achieve optimal fertility.

**Neuronal intranuclear inclusion disease (NIID)**

Neuronal intranuclear inclusion disease (NIID) is a progressive and fatal neurodegenerative disease that presents with great clinical heterogeneity, including dementia, cerebellar ataxia, parkinsonism, peripheral neuropathy, and autonomic dysfunction. Patients with NIID pathologically manifests in the presence of eosinophilic hyaline intranuclear inclusions in the central, peripheral and autonomic nervous systems, as well as somatic cells; therefore, skin biopsy efficiently determines the premortem diagnosis.

Intensive efforts have been made to explore the genetic mechanisms of NIID since the first reported NIID-affected case. Recently, a novel GGC repeat expansion in the 5’ UTR of the human specific gene *NOTCH2NLC* (formerly known as *NBPF19*) has been identified as the
genetic cause in Japanese NIID cases. While no pathogenic copy number variants (CNVs) were found in previous short-read whole-genome sequencing (WGS) data, long-read WGS with either PacBio RS II or the nanopore sequencer PromethION, was subsequently performed. The result of these showed that all the 13 familial and 40 sporadic NIID cases carried GGC repeat expansions ranging from 71 to 183, and was confirmed by repeat-primed PCR (RP-PCR). Neither of unaffected family members nor control individuals carried expanded GGC repeats, indicating family co-segregation. Moreover, nanopore sequencing with the Cas9-mediated enrichment system determined a possible association between a complex repeat structure composed of \((GGA)_n(GGC)_n\) and the muscle-weakness-dominant phenotype of NIID (NIID-M), which requires further validation in the clinical practice. Using ONT, the same causative mutations were also found to be responsible for juvenile-onset, adult-onset, familial, and sporadic NIID in Chinese population, thus further indicating a causative role for GGC repeat expansions in NIID. Intriguingly, GGC repeat expansion was not only observed in 2 cases of Alzheimer disease (AD) but also in three Parkinsonism-affected families displaying typical AD and PD symptoms, respectively. All of them were pathologically presented with eosinophilic intranuclear inclusions in dermal cells, which suggested that NIID has a higher prevalence than first thought, and may account for a portion of dementia and Parkinsonism cases, termed NIID-related disorders (NIIDRD).

Additionally, a direct search strategy named TRhist was initially employed to identify CGG repeats in NIID patients, followed by SMRT to conclusively support the position of CGG repeats located in \(NBPF19\) (recently annotated as \(NOTCH2NLC\)). Note that there are four additional paralogs besides \(NOTCH2NLC\), all of which have sequences with extremely high identities (> 99%). By re-analyzing the data from LRS, no significant methylation difference was detected in
GGC-expanded sequences and adjacent CpG islands between the NIID and control groups, nor was the expression of \textit{NOTCH2NLC}^{20-22}. In contrast, another study demonstrated that the expanded CGG repeats were likely hypermethylated\textsuperscript{23}. The discovery of abnormal anti-sense transcripts\textsuperscript{20}, together with unaltered expression levels of \textit{NBPF19} transcripts irrespective of the hypermethylated status of \textit{NBPF19}\textsuperscript{23}, indicated a potential role for RNA in the molecular pathogenesis of NIID.

Taken together, the success of identifying causative variations for NIID strongly suggests that LRS can be a powerful tool for the discovery of disease-causing mutations, determination of complex repeat structures, analysis of methylated status, and performance of genetic diagnosis, despite the high identities among homologous genes or 100% GC-rich in GGC or CGG repeat expansions.

\textit{Oculopharyngodistal myopathy (OPDM)}

Oculopharyngodistal myopathy (OPDM) is an adult-onset degenerative neuromuscular disorder with unclear inheritance. Patients affected with OPDM suffer from a wide range of muscle involvement, including progressive ptosis, external ophthalmoplegia, and weakness of facial, pharyngeal and distal limb muscles.

The identification of CGG repeat expansions in the 5' UTR of \textit{LRP12} in 22 out of 88 Japanese individuals with OPDM, termed OPDM1, deciphered a quarter of the genetic etiology in Japanese OPDM cases\textsuperscript{23}. The finding that a large portion of OPDM patients did not necessarily carry repeat expansions in \textit{LRP12} requires further investigation. Although short-read WGS failed to find any likely causal mutations, long-read WGS using the ONT PromethION sequencer successfully identified abnormal expansion of GGC repeats in the 5' UTR of \textit{GIPC1} within both familiar and sporadic Chinese individuals with OPDM\textsuperscript{24}. 
Noncoding GGC repeat expansions in \textit{GIPC1} were also observed in a small fraction of Japanese OPDM cases\textsuperscript{24}, further supporting GGC repeat expansions as the disease-causing variations of OPDM. Intriguingly, an independent strategy but also involving LRS in Chinese OPDM cases identified similar results with a slight difference in the repeat motif, that is, CGG\textsuperscript{25}. There is the possibility that the same repeat expansions are described in different manners, such as based on various kinds of DNA reference sequences of \textit{GIPC1}. \textit{GIPC1} with repeat expansions accounted for 50\% and 51.9\% of Chinese OPDM patients in these two studies, respectively, while only 3.6\% of the Japanese population\textsuperscript{24, 25}. This demonstrates that noncoding trinucleotide repeat expansions in \textit{GIPC1} may be the most frequent cause of Chinese OPDM cohort.

Detection of 5-methylcytosine (5mC) modification in the GGC repeat regions revealed no statistical differences between expanded and non-expanded alleles or between affected individuals and healthy controls\textsuperscript{24}. Despite the low level of methylation around the repeats and the abundance of unchanged protein, the increased level of \textit{GIPC1} mRNA suggests that expanded repeats may have an impact on the process of transcription\textsuperscript{24}.

To date, similar repeat motifs located in two distinct genes (\textit{LRP12} and \textit{GIPC1}) have been found in OPDM by virtue of LRS. This indicates the possibility of genetic heterogeneity and an essential role of repeat expansions themselves in the pathogenic mechanisms of OPDM, irrespective of the genes in which the expanded repeats are located. Furthermore, LRS can be utilized to promote further studies on the molecular mechanisms of OPDM, in addition to establishing the molecular diagnosis.

\textbf{Spinocerebellar ataxias (SCAs)}

Spinocerebellar ataxias (SCAs) are a group of genetically and phenotypically heterogeneous
neurodegenerative diseases with a core symptom of progressive cerebellar ataxia, including gait ataxia, dysarthria and oculomotor abnormalities. Different subtypes of SCAs exhibit various manifestations, such as epilepsy in SCA10\textsuperscript{26}, which are conducive to differential diagnosis.

To date, approximately 50 genetically distinct types of SCAs (SCA1-SAC48) have been registered. Some are caused by CAG repeat expansions in coding regions, resulting in abnormally long polyglutamine (PolyQ) chains, termed PolyQ disorders. Additionally, non-coding repeat expansions and conventional mutations have been attributed to other types of SCAs. This highlights the significance of genetic testing with high sensitivity and specificity to confirm the clinical diagnosis in the presence or absence of family history.

Furthermore, there is a strong inverse correlation between repeat length and age of onset, as well as the severity of symptoms, as illustrated by SCA3\textsuperscript{27}. A second feature is the presentation of clinical anticipation due to the tendency for repeat expansions to change size, mainly enlarging further when transmitted to the next generation\textsuperscript{27}. Therefore, later generations carrying larger expansions would manifest with an earlier age of onset and more severe symptoms.

SCA10, a subtype of the SCA family, is characterized by prominent cerebellar symptoms and seizures, such as focal seizures and generalized motor seizures. A large, non-coding ATTCT pentanucleotide repeat expansion in intron 9 of \textit{ATXN10} on chromosome 22 causes SCA10\textsuperscript{2}. Normal length of ATTCT is 10–32, and intermediate alleles containing 280–850 repeats present with reduced penetrance, while the pathogenic alleles ranging from 850 to 4500 cause full penetrance\textsuperscript{2}. It is well established that interruptions in the expanded ATTCT repeats are strongly associated with an increased prevalence of epileptic attack\textsuperscript{28}. Despite the strikingly long read lengths of the SCA10 expansion, SMRT successfully sequenced across the entire
span of the expansion, ranging from 2.5 to 4.4 kb in length, from three SCA10 patients with different clinical manifestations\textsuperscript{29}. There were remarkably varied structures of interruption motifs among these three patients, such as ATTCC only in one patient, all of which were verified by Sanger sequencing to rule out sequencing or assembly errors\textsuperscript{29}. Although only an extremely low percentage of these rare interruption motifs resided in the typical ATTCT repeat expansion regions, it is possible that repeat motif composition may act as a phenotypic modifier in SCA10, given symptomatic heterogeneity. Intriguingly, the tendency for interrupted SCA10 repeat expansions to contract upon transmission correlated with earlier onset of the disease in successive generations of a family\textsuperscript{29}, which is contrary to the usual anticipation, thus so-called paradoxical anticipation\textsuperscript{30}. However, PCR amplification of repeat expansions to achieve sufficient quantities of DNA for input likely resulted in unpredictable errors\textsuperscript{29}. To this end, the Cas9 capture approach paired with SMRT sequencing without prior amplification of genomic DNA was employed in a Mexican family with multiple affected members, four of whom reported clinical manifestations of SCA10\textsuperscript{31}. The proband uniquely presented with early onset levodopa-responsive Parkinsonism\textsuperscript{31}. Interestingly, a 1400 repeat pattern that consisted of the typical ATTCT pentanucleotide repeats followed by an extra ATTCC interruption motif was observed in the four siblings with representative cerebellar ataxia and seizures, while the sibling clinically diagnosed with PD carried 1304 pure ATTCT repeats with virtually no repeat interruptions\textsuperscript{31}. However, the phenomenon of paradoxical anticipation was not observed due to unavailable information of their father, which deserves further investigations to confirm the uncharacteristic theory and discuss the underlying mechanism.

As mentioned above, the genetic architecture of the SCA10 repeat expansion may serve as a phenotypic modifier, with the feat of SMRT revealing novel and various structures of
interruption motifs\textsuperscript{29, 31}, and demonstrating a phenotype-genotype correlation between Parkinsonism and \textit{ATXN10}\textsuperscript{31}. These discoveries may shed light on the research of many other types of SCA. Further studies are required to better understand how different compositions of interruption motifs serve as phenotypic modifiers, which relies heavily on advanced sequencing technology, particularly LRS.

\textit{Huntington’s disease (HD)}

Huntington’s disease is an adult-onset neurodegenerative disorder with an autosomal dominant inheritance that is clinically characterized by progressive dystonia, chorea, cognitive dysfunction, and personality disorders.

As a notable PolyQ disorder, HD is caused by CAG repeat expansions in the Huntingtin (\textit{HTT}) gene on chromosome 4\textsuperscript{32}. The repeat is up to 26 CAGs long in the normal population, while the intermediate alleles containing 27–35 repeats have a tendency to expand into the disease-causing range in the next generation. In patients with HD, the CAG repeats tend to expand above 36 units, such as 36–39 repeats presented with reduced penetrance and \geq40 with full penetrance\textsuperscript{33}. Given the correlation between distinct sizes and disease phenotypes, fragment analysis is indicated in the clinical setting to determine repeat sizes. However, with the concern of PCR stutter and extreme GC content, there is a need for more advanced approaches. To this end, the CRISPR/Cas9 system paired with SMRT sequencing has been employed, followed by a mapping-independent algorithm to analyze and visualize the repeat sequence in clinically relevant HD samples\textsuperscript{34}. In addition to \textit{HTT}, researchers have evaluated the multiplexing efficiency of additional targets involving \textit{FMR1}, \textit{ATXN10}, and \textit{C9ORF72}, causing FXS, SCA10, amyotrophic lateral sclerosis, and frontotemporal dementia (ALS/FTD), respectively\textsuperscript{34}. These support the feasibility of No-Amp targeted sequencing in
multiplexing of different targets in the same run. More importantly, the variability in the number of CAG repeats within expanded alleles of the same patient indicated somatic mosaicism, especially for larger repeat expansions\textsuperscript{34}. The sizes of CCG repeats flanking the CAG repeats have also been resolved, with 7 of the most frequent and the subsequent 10, consistent with previous data\textsuperscript{35}. Nevertheless, the off-target effect on chromosome 9 could be explained by the presence of a single nucleotide polymorphism (SNP) increasing homology to the ATXN10 gRNA\textsuperscript{34}.

In summary, No-Amp targeted sequencing generates an exact estimation of the repeat count to confidently investigate somatic variations without PCR amplification, and provides a more direct insight into the exact location of interruption motifs, such as in \textit{FMRI}. Furthermore, it is practically possible to target and capture a large number of targets by well-designed gRNA and high-fidelity Cas9 enzymes, with the potentially wrong cleaved sites induced by SNPs taken into consideration.

\textbf{Benign adult familial myoclonic epilepsy (BAFME)}

Benign adult familial myoclonic epilepsy (BAFME) is a slowly progressive disease mainly clinically characterized by cortical myoclonus tremors and infrequent epileptic seizures, and electrophysiologically by giant somatosensory evoked potentials and long-latency cortical reflex\textsuperscript{36}. It is generally known as familial cortical myoclonic tremor with epilepsy (FCMTE) in China and autosomal dominant cortical myoclonus and epilepsy (ADCME) or familial adult myoclonic epilepsy (FAME) in Europe.

Although the candidate genes have been mapped to several loci including 8q23.3-q24.1 (BAFME1)\textsuperscript{37}, 2p11.1-q12.2 (BAFME2)\textsuperscript{38}, 5p15.31-p15.1 (BAFME3)\textsuperscript{39}, 3q26.32-3q28 (BAFME4)\textsuperscript{40}, 16p21.1 (BAFME6)\textsuperscript{41}, and 4q32.1 (BAFME7)\textsuperscript{41}, none of the
causative mutations had been reported until 2018\textsuperscript{41}. A study identified TTTTA pentanucleotide repeats and ‘extra’ TTTCA repeat sequences in intron 4 of Samd12 in Japanese families affected with BAFME\textsuperscript{1}. SMRT sequencing of bacterial artificial chromosome (BAC) clones from two patients exactly determined two repeat configurations: (TTTTA)\textsubscript{exp}(TTTCA)\textsubscript{exp} and (TTTTA)\textsubscript{exp}(TTTCA)\textsubscript{exp}(TTTTA)\textsubscript{exp}\textsuperscript{41}. By means of ONT, the former pattern was clearly confirmed in Chinese FCMTE1 pedigrees\textsuperscript{42}. In addition, the PacBio sequel system not only showed an approximately 4.6kb Samd12 intronic repeat insertion but also estimated the size of insertion\textsuperscript{43}. This is broadly consistent with the results of Southern Blot analysis, proving the versatility and effectiveness of LRS. However, the inserted sequence was considered to be TTTCT instead of TTTCA as mentioned above\textsuperscript{43}, probably owing to the high error rates of LRS. Of note, TTTTA-repeat expansion also contained impure repeat sequences of TTTTTA or TTTA\textsuperscript{41}, possibly due to the susceptibility of SMRT sequencing to insertion and/or deletion. Given that contractions of expanded repeats may occur during BAC cloning, nanopore sequencing of genomic DNA was further performed to recount TTTTA and TTTCA motifs, the lengths of which were comparable and variable\textsuperscript{41}, indicative of somatic instability and/or artifacts introduced by nanopore sequencing. With regard to neuropathological findings, loss of Purkinje cells as the degenerative feature was observed in the cerebellar cortex of BAFME1 patient with homozygous mutations, and RNA foci was noted in the cortical neurons\textsuperscript{41}, suggesting that transcription was a key step and RNA foci may be involved in the potential pathogenesis. Note that there were two families whose abnormal expansions of TTTCA and TTTTA pentanucleotide repeats did not reside in Samd12, but in TNRC6 (BAFME6) and RAPGEF2 (BAFME7), respectively\textsuperscript{41}.

Recently, an intronic ATTTC expansion in STARD7 causing FAME2 and unstable
TTTTA/TTTCA expansions in \textit{MARCH6} responsible for FAME3 were reported by short-read WGS and RP-PCR\textsuperscript{44,45}. These were also suggested to be somatically variable by both SMRT and ONT\textsuperscript{44,45}. Nevertheless, the presence of ATTTTC or TTTTCA pentamer did not affect the transcript or protein abundance of \textit{STARD7} or \textit{MARCH6} among expansion carriers and control individuals\textsuperscript{44,45}, indicating that the repeat sequence alone may be pathogenic. More recently, SMRT sequencing detected intronic expansions of TTTTA and insertions of TTTCA repeats in \textit{YEATS2} as the genetic etiology of BAFME4 in a Thai family\textsuperscript{46}.

In conclusion, the expansion of noncoding TTTCA or ATTTTC repeats in multiple independent genes shared by all six types of BAFMEs (BAFME1, 2, 3, 4, 6, and 7) raised the possibility that repeat motifs themselves rather than genomic loci may play pivotal roles in the pathogenesis of BAFMEs, and the same expanded repeat motifs could result in overlapping clinical spectra of diseases. LRS is of diagnostic value with a sensitivity of 100% despite the low GC content, and may assist to the development of therapeutic strategies targeting similar repeat motifs.

\textit{Amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD)}

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are fatal neurological diseases with an autosomal dominant inheritance. In ALS, progressive muscle wasting, weakness, spasticity, and eventually general paralysis result from the degeneration of upper and lower motor neurons in the motor cortex, brainstem, and spinal lord\textsuperscript{47}. As the second most common presenile dementia, FTD is characterized by personality changes and inappropriate behavior with relative preservation of memory owing to the degeneration of the superficial frontal cortex and anterior temporal lobes of the brain\textsuperscript{48}. There is mounting evidence that ALS and FTD represent a clinicopathological continuum of disease starting
from the presence of frontotemporal dysfunction and motoneuron impairment in both ALS and FTD, to the pathological discovery of the transactive response DNA binding protein with $M_r$ 43 kDa (TDP-43) throughout the central nervous system$^{49}$.

To date, a large number of genes harboring mutations have been identified, such as $TARDBP^{50}$, $FUS^{51}$, and $OPTN^{52}$ in ALS, and $GRN$ in FTD$^{53}$. Nevertheless, all mutations rarely coexist in either ALS or FTD. Linkage analysis of familial ALS and FTD implicated a major locus for both diseases on chromosome 9p21$^{54}$, and confirmed in sporadic cases$^{55}$, further supporting the related association between these two diseases termed 9p-linked ALS/FTD. Furthermore, the identification of an expanded GGGGCC hexanucleotide repeat in the noncoding region of chromosome 9’s open reading frame 72 ($C9orf72$) in 9p-linked ALS/FTD$^3$, genetically connected ALS with FTD. As the most common cause, this $G_4C_2$ repeat expands hundreds to thousands of times in affected cases, while 2–30 in control individuals$^{56}$. Given the extreme length, high GC content, and tendency to form G-quadruplexes in both DNA and RNA$^{57,58}$, the $G_4C_2$ repeat expansion may be the least advantageous for sequencing. Nevertheless, both the PacBio and ONT sequencing platforms can traverse these challenging repeat expansions$^{59}$. ONT MinION may more closely resemble expected read lengths because of its tighter distributions of read lengths than PacBio, indicating great promise for future ONT MinION applications. With regard to base calling accuracy, the PacBio RS II and the ONT MinION sequencers using consensus sequence attained approximately 99.8% and 26.6% accuracy, respectively$^{59}$, showing the superiority of PacBio RS II. As for affected carriers with $G_4C_2$ repeat expansions, PacBio Sequel can accurately distinguish the expanded allele with 1324 repeats from the unexpanded allele with 8 repeats, even with a low coverage (8×)$^{59}$. Additionally, the PacBio No-Amp targeted sequencing method with a higher coverage (800×) was capable of closely estimating
expansion size and measuring nucleotide content with the result of > 99% GC content in the repeat regions. However, there were G₃C₂, G₄C₁ and non-GC interruptions in addition to G₄C₂ motifs, possibly due to insertions and deletions as the most common error in PacBio sequencing, which requires cautious interpretation and larger studies.

In general, LRS is ideal for characterizing repeat expansions, such as their locations, sizes, and content, despite the extraordinary stretches of repeats with pure GC content. Moreover, No-Amp targeted sequencing allows investigators to achieve a considerably in-depth degree of coverage and accurately elucidate the nucleotide-level features of known and undiscovered mutations. LRS shows great promise in both research and clinical settings. However, the errors, such as insertions and deletions, should be taken seriously.

Conclusions and prospects
To date, LRS has already proven their outstanding ability to identify causative repeat expansions, recognize repeat configurations, estimate repeat sizes, and detect base modifications in neurodegenerative diseases. We summarize the extensive applications of LRS in neurodegenerative diseases as mentioned above (Table 1). The modification of repeat configurations to clinical phenotypes, the definition of threshold between pathogenic and normal ranges based on repeat sizes, the age at onset and severity of symptoms inversely associated with repeat sizes, and the silencing of transcription and translation due to epigenetic modifications all contribute to our expanding knowledge of neurodegenerative diseases. We may safely draw the conclusion that repeat expansions underlying neurodegenerative diseases are good targets for LRS, thanks to its extremely long read lengths, while NGS may result in negative results.

Although noncoding repeat expansions have been detected by LRS to cause a number of
neurodegenerative diseases over the last three years, the pathogenic mechanism remains largely unclear. The presence of anti-sense transcripts and RNA foci containing the repeat transcripts implicates the pathogenicity of RNA, known as toxic gain-of-function. For example, anti-sense transcripts have the capacity to regulate gene expression by binding to and sealing off complementary RNAs. In addition, repeat-associated non-AUG translation may lead to aggregation of aberrant protein products. Therefore, further research is needed to investigate the potential pathway at the transcriptional and translational level, in addition to reaching to gene level.

In this review, LRS has been shown to mainly characterize repeat expansions, its ability is not confined to only one type of mutation. The successful identification of a pathogenic structural variant in a patient suffering from the Carney complex using LRS indicate that LRS is capable of discovering causal structural variants (SVs)\(^6\), and this is also applicable to neurodegenerative diseases. In terms of clinical utility, LRS has great potential to secure genetic diagnosis of neurodegenerative diseases, contribute to differential diagnoses and provide vital information for genetic counseling. Additionally, the promising prospects of LRS in medical research lie in detecting novel disease-causing and disease-modifying variations, exploring the underlying molecular mechanisms, and developing targeted therapeutic strategies.

Nevertheless, there still remains much room for improvement with regard to variant-calling accuracy, bioinformatic analysis, cost of deciphering the human genome, and throughput. First, the raw read errors were fundamentally derived from interrogating a single DNA molecule by the polymerase or exonuclease. Deletions, insertions, and mismatches were primarily attributed to unlabeled nucleotide contamination, double count mediated by the failure of a base to translocate through the nanopore, and spectral misassignments of the dyes,
respectively. Additionally, single nucleotide discrimination by ONT remains difficult, given that the polynucleotides within the nanopore rather than one single nucleotide result in the current disruption, suggesting the superiority of PacBio over ONT in terms of variant-calling accuracy. Purer fluorescently-labeled nucleotides, spectrally compatible dyes, and CCS construction are expected to improve read accuracy. Nevertheless, given the intrinsic restriction of a single molecule, the maximal accuracy of LRS may barely match that of NGS. Beyond the read accuracy challenges for LRS, sequencing data analysis will be conducted. For example, unique polymerase kinetics generated by epigenetic modifications and various forms of DNA damage allow discrimination between modified nucleotides in the DNA template; however, there is a demand for unmodified reference data. As we work on refining bioinformatics algorithms, de novo modification profiling may be achievable. The last major issue relates to the relatively low throughput and high cost, which was around several hundred dollars per gigabyte base pair. With the active development of LRS, it is anticipated that by virtue of sophisticated mathematical algorithms, LRS-based genome-wide sequencing could accurately discover all genetic variants in an individual’s genome at a reasonable cost in the near future.

In conclusion, LRS has drastically revolutionized sequencing technology and will advance our understanding of the genetic etiology and molecular pathogenesis of neurodegenerative diseases, thereby further facilitating effective treatment.
## Appendix I: Authors

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yun Su, MD</td>
<td>Zhengzhou University, Zhengzhou, Henan, China</td>
<td>Study concept and design; analysis and interpretation of data; creation of figures and manuscript draft</td>
</tr>
<tr>
<td>Liyuan Fan, MD</td>
<td>Zhengzhou University, Zhengzhou, Henan, China</td>
<td>Study concept and design; analysis and interpretation of data; creation of figures and manuscript draft</td>
</tr>
<tr>
<td>Changhe Shi, MD, PhD</td>
<td>Zhengzhou University, Zhengzhou, Henan, China</td>
<td>Study concept and design; analysis and interpretation of data; creation of figures and manuscript draft</td>
</tr>
<tr>
<td>Tai Wang, MD</td>
<td>Zhengzhou University,</td>
<td>Analysis and interpretation of data</td>
</tr>
</tbody>
</table>
Chengyuan Mao, MD, PhD
Zhengzhou University, Zhengzhou, Henan, China
Study concept, design, analysis and interpretation of data; study supervision and critical review of manuscript for intellectual content

Yuming Xu, MD, PhD
Zhengzhou University, Zhengzhou, Henan, China
Study concept, design, analysis and interpretation of data; study supervision and critical review of manuscript for intellectual content

References


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Figure legends

Figure 1 Overview of single-molecule real-time sequencing (SMRT) technology.

(A) Library preparation starts with cutting double-stranded DNA to the right size. (B) The template termed ‘SMRTbell’ is created by ligating hairpin adapters (light blue) to both ends of double-stranded DNA. (C) The library is thereafter loaded onto a SMRT flowcell containing millions of zero mode waveguides (ZMWs) (grey). In the best case, a SMRTbell diffuses into a ZMW, and the adaptor binds to a DNA polymerase (white) immobilized at the bottom, thereby initiating the incorporation of fluorescently labeled nucleotides. As a nucleotide is held in the detection volume by the polymerase, a fluorescence pulse (orange) on illumination is produced and recorded, which identifies the base. (D) Not only the fluorescence color is registered, but also the time between adjacent nucleotide incorporations, termed the interpulse duration (IPD), indirectly reflective of epigenetic modification.
Figure 2 Schematic representation of Oxford Nanopore Technology.

(A) Double-strand DNA fragments often undergo an optional DNA repair step. (B) End-repaired DNA fragments are tagged with sequencing adapters (light blue) preloaded with a motor protein (red) on the 5’ protruding ends. (C) The DNA template is loaded onto the flow cell containing thousands of nanopores (dark blue) embedded in a synthetic membrane (grey). The membrane divides the sequencing dimension into two compartments (cis and trans). Once the adapter inserts into the opening of the nanopore, the motor protein begins to unwind the double-stranded DNA and drives the single-stranded DNA through the pore under the action of electric current. As the DNA molecule translocates through the pore, individual nucleotides will be cut off, which causes characteristic disruptions to the current. (D) Changes in current correspond to a readout known as a ‘squiggle’.
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<tr>
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Abbreviations: FXTAS = fragile X-associated tremor/ataxia syndrome; _exp_ = expansion; 
_FMR1_ = fragile X mental retardation 1; PacBio = Pacific Biosciences; PCR = polymerase chain reaction; NA = not available; NIID = neuronal intranuclear inclusion disease; NOTCH2NLC = notch 2 amino-terminal-like protein C; ONT = Oxford Nanopore Technologies; NBPF19 = neuroblastoma break point family member 19; OPDM = oulopharyngodistal myopathy; GIPC1 = GIPC PDZ domain containing family member 1; SCA10 = spinocerebellar ataxia type 10; ATXN10 = ataxin 10; No-Amp targeted sequencing = no-amplification targeted sequencing; PD = Parkinson’s disease; HD = Huntington’s disease; HTT = huntingtin; BAFME1 = benign adult familial myoclonic epilepsy type 1; SAMD12 = sterile alpha motif domain containing 12; BAC = bacterial artificial chromosome; FAME2 = familial adult myoclonic epilepsy type 2; STARD7 = StAR related lipid transfer domain containing 7; FAME3 = familial adult myoclonic epilepsy type 3; MARCH6 = membrane-associated ring finger (C3HC4) 6; BAFME4 = benign adult familial myoclonic epilepsy type 4; YEATS2 = YEATS domain containing 2; ALS/FTD = amyotrophic lateral sclerosis/frontotemporal dementia; C9ORF72 = chromosome 9’s open reading frame 72.

* NBPF19 is recently annotated as NOTCH2NLC.

# The inserted sequence was suggested to be (TTTCT), rather than (TTTCA) as reported previously, possibly due to the high sequencing error rate of long-read sequencing technologies.
Deciphering Neurodegenerative Diseases Using Long-Read Sequencing
Yun Su, Liyuan Fan, Changhe Shi, et al.
Neurology published online August 13, 2021
DOI 10.1212/WNL.0000000000012466

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