BMC Neurology

RESEARCH

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Subclinical peripheral nerve demyelination without overt symptoms in a family with neuronal intranuclear inclusion disease harboring biallelic repeat expansions

Hang Zhang[1,2†], Taiqi Zhao[1,2†], Honglin Zheng[1,3†], Suying Duan[1,2], Chenyang Liu[1,2], Yaochong Zhang[1,2], Qiang Li[1,2], Han Liu[1,2,4*], Haiyang Luo[1,2,4*] and Yuming Xu[1,2,4*]

Abstract

Neuronal intranuclear inclusion disease (NIID) is a rare, progressive neurodegenerative disorder caused by abnormal GGC repeat expansions in the *NOTCH2NLC* gene, leading to multisystem involvement. Electrophysiological abnormalities in NIID have gained increasing attention in recent years. However, subclinical peripheral neuropathy preceding the onset of typical NIID manifestations has not been reported. In this study, we systematically evaluated the clinical characteristics of a family carrying pathogenic *NOTCH2NLC* expansions. Electrophysiological assessments revealed demyelinating changes in some family members, with or without the classic clinical features of NIID. Notably, one individual with biallelic *NOTCH2NLC* GGC repeat expansions exhibited subclinical peripheral neuropathy in the absence of overt NIID symptoms. As NIID is typically inherited in an autosomal dominant manner, cases with biallelic GGC repeat expansions are exceedingly rare. To explore the genotype–phenotype relationship, we employed long-read whole-genome sequencing using Oxford Nanopore and Pacific Biosciences (PacBio) technologies. Our results suggest that biallelic repeat expansions do not necessarily exacerbate the severity or progression of NIID. Although larger studies are warranted to confirm these findings, this investigation broadens the clinical and genetic spectrum of NIID and provides new insights into its underlying pathogenesis.

Keywords NIID, peripheral neuropathy, demyelinating lesions, biallelic GGC repeat expansions, Oxford Nanopore, PacBio

†Hang Zhang, Taiqi Zhao and Honglin Zheng contributed equally to this work and share first authorship.

*Correspondence:
Han Liu
fccliuh2@zzu.edu.cn
Haiyang Luo
fccluohy@zzu.edu.cn
Yuming Xu
xuyuming@zzu.edu.cn

[1]Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Jian-She East Road, Zhengzhou, Henan 450052, China
[2]Henan Key Laboratory of Cerebrovascular Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan, China
[3]The Academy of Medical Sciences of Zhengzhou University, Zhengzhou, Henan, China
[4]Institute of Neuroscience, Zhengzhou University, Zhengzhou, Henan, China

Introduction

NIID is a rare, progressive neurodegenerative disorder characterized by the widespread presence of intranuclear eosinophilic hyaline inclusions across multiple organ systems [1, 2]. Recently, a pivotal discovery identified a GGC repeat expansion in the 5′ untranslated region (UTR) of the *NOTCH2NLC* gene as the genetic basis of both sporadic and familial forms of NIID [3, 4]. Moreover, despite being named "neuronal", these intranuclear inclusions displaying positive staining extend beyond neurons and astrocytes, manifesting in the nuclei of nearly all somatic cells, thus implicating their impact across diverse clinical systems [1].

Increasing attention has been directed toward both clinical and subclinical peripheral neuropathy in the context of NIID. The latter denotes a state devoid of discernible clinical symptoms and signs yet marked by positive findings upon neurophysiological assessment [5]. Tian et al.'s investigation unveiled that while overt manifestations of peripheral neuropathy remained elusive in most NIID patients, neurophysiological evaluations revealed its prevalence in 91.8% of cases, comprising both clinical (42.7%) and subclinical (49.1%) presentations [6]. Nerve biopsies have unveiled mild axonal degeneration coupled with primary demyelinating alterations [5, 7]. In contrast to invasive nerve biopsies, non-invasive electrophysiological assessments are garnering heightened interest. Emerging findings have highlighted a negative association between median nerve conduction velocity and the size of GGC repeats [8].

Given that more than 90% of NIID patients exhibit peripheral neuropathy, the possibility that subclinical neuropathy represents an early or preclinical manifestation is compelling. Yet, the literature currently lacks reports on subclinical peripheral neuropathy preceding the onset of typical NIID symptoms. Moreover, despite the diagnostic and practical value of electrophysiological examinations, comprehensive exploration of pathological mechanisms remains deficient. Moreover, biallelic *NOTCH2NLC* GGC repeat expansions have been documented only in a small subset of patients with NIID and OPDM3 [9–11]. Given its rarity, further investigation into the clinical manifestations and pathological characteristics of biallelic mutations is imperative.

In this study, we performed a comprehensive clinical and electrophysiological evaluation of a family with an NIID genetic background, exhibiting subclinical or clinical peripheral neuropathy. One patient carrying biallelic GGC repeat expansions in the *NOTCH2NLC* gene, presenting without typical NIID symptoms but demonstrating subclinical peripheral neuropathy. Utilizing Nanopore and PacBio long-read whole-genome sequencing technology, we elucidate the genotype-phenotype correlation in this patient. The findings presented herein represent one of the initial investigations into subclinical peripheral neuropathy in the context of NIID.

Materials and methods

Clinical evaluation

A systematic clinical history and physical examination were conducted for the proband and his family members. Each participant underwent individualized assessments, including Cranial magnetic resonance imaging (MRI), skin biopsy, nerve conduction studies, the Montreal Cognitive Assessment (MoCA) [12]. For skin biopsy, a 10 cm section of skin above the lateral malleolus was obtained, processed into paraffin sections, and subjected to standard p62 immunostaining following established protocols [13]. In accordance with previous literature, both motor and sensory nerve conduction studies were used to evaluate peripheral neuropathy [5].

Genetic analysis

Genetic analysis workflow

Genomic DNA was extracted from peripheral blood samples of all participants using a whole-blood genomic DNA extraction kit (CWE 9600; Beijing Kangwei Century Biotechnology Co., Ltd.) according to the manufacturer's instructions. Dynamic short tandem repeat amplification detection of NIID-related genes was subsequently performed using repeat-primed PCR (RP-PCR) and fluorescence amplicon length PCR (AL-PCR). As no wild-type *NOTCH2NLC* allele was detected in Patient IV1, long-read whole-genome sequencing was conducted on this sample.

Repeat-primed PCR

RP-PCR was performed as previously described [14]. The DNA fragment of the allelic trinucleotide repeat sequence region was amplified using triple primers. The composition of the triple primer mixture was as follows: 5′-FAM-GGCATTTGCGCCTGTGCTTCGGACCG T-3′, 5′CAGGAAACAGCTATGACCTCCTCCGCCGC CGCCGCC-3′, and 5′-CAGGAAACAGCTATGACC-3′. Electrophoresis was performed on the LifeECO-PCR gene amplification instrument TC-96/G/H(b)C, with LIZ500 used as the standard length. GeneMarker V2.2.0 software was used for analysis.

Fluorescence amplicon length analysis

AL-PCR was performed as previously described [14]. To amplify a dynamically mutated DNA fragment containing a trinucleotide repeat sequence region, specific primers were used. The primer sequences used were as follows: 5′-VIC-CATTTGCGCCTGTGCTTCGGAC-3′ and 5′-AGAGCGGCGCAGGGCGGGCATCTT-3′. Electrophoresis was performed on the LifeECO-PCR gene cycler TC-96/G/H(b)C, with LIZ500 used as the

standard length. The analysis was conducted using GeneMarker V2.2.0 software.

Long-read whole-genome sequencing of samples from patient without wild-type allele of NOTCH2NLC

Long-read whole-genome sequencing was performed on Patient IV1, who lacked a wild-type *NOTCH2NLC* allele. Initially, sequencing was conducted using Oxford Nanopore technology with super base-calling. DNA libraries were prepared with the Ligation Sequencing Kit (SQK-LSK110; Oxford Nanopore Technologies) following the manufacturer's protocol and sequenced on a PromethION platform. Base calling and bioinformatics analyses were then performed. To validate the consensus sequence, additional long-read sequencing was performed using PacBio technology. DNA libraries were prepared with the SMRTbell Express Template Kit 2.0 (PacBio), followed by sequencing on a PacBio Sequel II/IIe platform. The resulting reads were processed and assessed using SMRT Link software, and bioinformatics analyses were subsequently performed.

DNA methylation analysis using PacBio sequencing data

Methylation information was obtained using pb-CpG-tools (v1.0.0) software with default parameters. The CpG/5mC data from PacBio HiFi reads, which were mapped to the reference genome, were analyzed using the custom program pb-CpG-tools (https://github.com/PacificBiosciences/pb-CpG-tools). Visual analysis of the generated file was performed in IGV. If haplotype information was available, the corresponding haplotype result file would be generated.

Results

Clinical manifestations of NIID family

We assessed the proband from a NIID family alongside patients diagnosed with NIID genetically and pathologically. The family pedigree is depicted in (Fig. 1A). The proband (III3), a 53-year-old retired worker, was admitted to the hospital at the age of 47 (in 2017) due to headaches and disorganized speech. Cranial MRI unveiled bilateral frontoparietal and paraventricular white matter demyelinating lesions. At that time, the patient showed no response to empirical treatment for encephalitis, and his symptoms continued to worsen. Muscle biopsy was therefore performed but did not support mitochondrial encephalomyopathy.

Between March 2018 and September 2022, the proband's personal and professional life remained unaffected. However, at the age of 52 (towards the end of 2022), the patient was hospitalized due to episodes of altered consciousness after fever. Skin biopsy demonstrated the presence of ubiquitin-positive inclusion bodies (Fig. 1B). Genetic analysis revealed a heterozygous GGC repeat expansion of 135 units within the *NOTCH2NLC* gene (Fig. 1F). Cranial MRI unveiled demyelination in the bilateral frontoparietal and paraventricular white matter (Fig. 1J). Subsequently, the proband received a diagnosis of neuronal intranuclear inclusion disease based on these findings.

Patients II2, II4, and II5 exhibited analogous symptoms, including cognitive impairment and ataxia. Additionally, they manifested other symptoms associated with NIID. For instance, II2 and II5 suffered from apathy, irritability, and urinary retention, while II4 presented symptoms indicative of dementia. Furthermore, Patient III6 reported experiencing headaches and intermittent memory loss.

Given the prevalence of NIID within the patient's family, we conducted clinical and genetic screenings on other family members. The Patient III5 experienced episodic alterations of consciousness eight years prior, at the age of 40. These episodes were characterized by a loss of temporal and spatial awareness, which resolved after several hours of rest. Similarly, the Patient III10 developed episodic dizziness three years ago, at the age of 42, presenting as lightheadedness. Skin biopsy samples from Patient III5 and Patient III10 exhibited positive p62 staining for intranuclear inclusions (Fig. 1. C-D), while Cranial MRI revealed white matter lesions.

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The electropherogram of RP-PCR displayed a zigzag pattern, indicative of GGC repeat amplification in *NOTCH2NLC* (Fig. S1). To ascertain the size of the *NOTCH2NLC* GGC repeat amplification, we employed AL-PCR. The AL-PCR findings for Patient III5 and Patient III10 demonstrated repeat numbers of 17/132 and 13/120, respectively (Fig. 1G-H), implying the presence of heterozygous mutations.

In the AL-PCR analysis of Patient IV1, we observed 118 repeats along with a subtle bulge, indicative of repeat instability (Fig. 1I).

Notably, no signal peak corresponding to the wild-type allele was discernible in Patient IV1, signifying the occurrence of biallelic GGC repeat expansions. It's noteworthy that Patient IV1 did not manifest typical clinical symptoms or white matter lesions associated with NIID (Fig. 1M). However, both skin biopsy and AL-PCR results suggested the presence of subclinical-related lesions

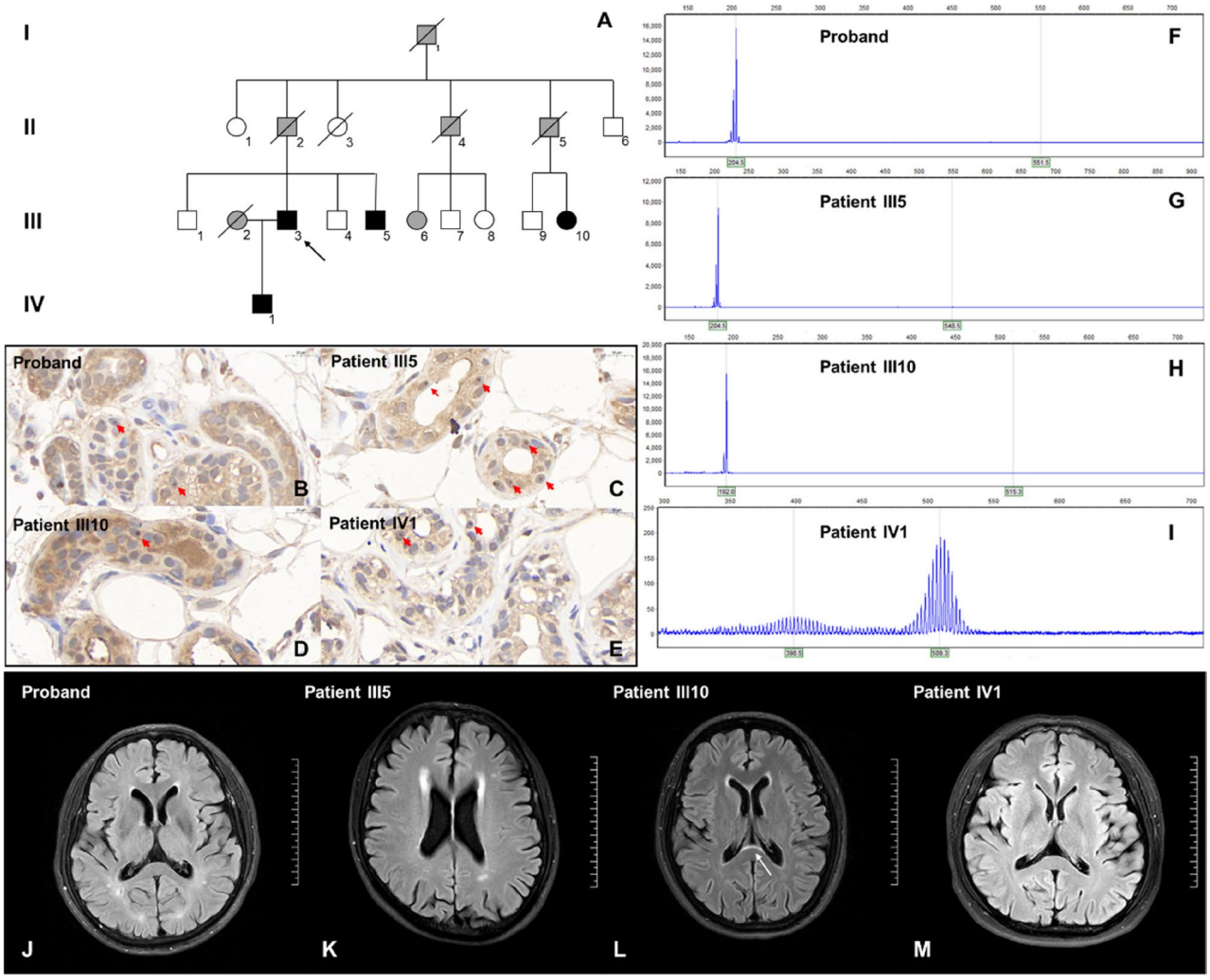

Fig. 1 Pathological, genetic, and imaging findings in a family with biallelic GGC repeat expansion in *NOTCH2NLC*. **A** A familial pedigree comprising gray symbols indicating the suspected patients without genetic tests; **(B-E)** Immunohistochemical staining of skin biopsies of the family, showing p62-positive intranuclear inclusions (arrows); **(F-I)** AL-PCR results of the pedigree, with DNA length (bp) shown in the figure. The number of allele repeats is calculated as (DNA length − 154)/3; **(J-M)** Fluid-attenuated inversion recovery (FLAIR) results. The proband, patient III5, and patient III10 exhibited white matter lesions, while patient IV1 showed normal signal

(Fig. 1E). A summary of the clinical characteristics of these patients is presented in Table S1-2 of the supplementary material.

Subclinical peripheral neuropathy in patients with or without typical NIID symptoms

Subclinical peripheral neuropathy is a common finding in patients with NIID, highlighting the discordance between subjective symptoms and objective examination. Neurological assessments can reveal severe peripheral neuropathy even when clinical manifestations are mild [15]. Furthermore, there is currently a dearth of reports documenting cases solely presenting with subclinical peripheral neuropathy in the absence of typical NIID symptoms. Consequently, we undertook a meticulous analysis of electrophysiological alterations in these patients.

Despite the absence of overt symptoms such as muscle atrophy, weakness, or sensory impairment, Patient III5 (heterozygous) and Patient IV1 (homozygous) exhibited diminished conduction velocities across various peripheral nerves, including the median, ulnar, sural, and deep peroneal nerves. Moreover, Patient III5 demonstrated deceleration in peripheral motor conduction velocity specifically in the left tibial nerve. Nerve conduction study results indicated reduced MCV and SCV in Patient III5 and Patient IV1, while CMAP and SNAP remained normal, suggesting demyelinating lesions. Patient IV1 carries subclinical peripheral nerve demyelination without typical NIID symptoms.

Consensus sequence of biallelic GGC repeat expansions

The disease spectrum of NIID demonstrates significant heterogeneity. Previous studies have suggested that this

variability may be associated with factors such as the GGC repeat copy number [16], interruptions in the GGC repeat sequence [17], and DNA methylation status [18]. Analysis of these factors can aid in predicting the phenotype of NIID patients. The consensus sequence of the biallelic GGC repeat expansion within the *NOTCH2NLC* gene was delineated. Nanopore super basecalling technology was employed to extract the base sequence from the original image and translate the changing current value into the corresponding base sequence. This approach achieved a single-base accuracy exceeding Q30 (99.9%). The repeat copy number histogram and waterfall plot depicted in Fig. 2A-B demonstrate that the repeat numbers of a pair of alleles in Patient IV1 are too closely aligned to distinguish. The GGC repeat genotypes derived from Nanopore whole-genome sequencing were determined based on the number of inserted bases within the tandem repeat region. Among the 23 reads analyzed, 15 reads exhibited nearly identical repeat lengths (Fig. 2C).

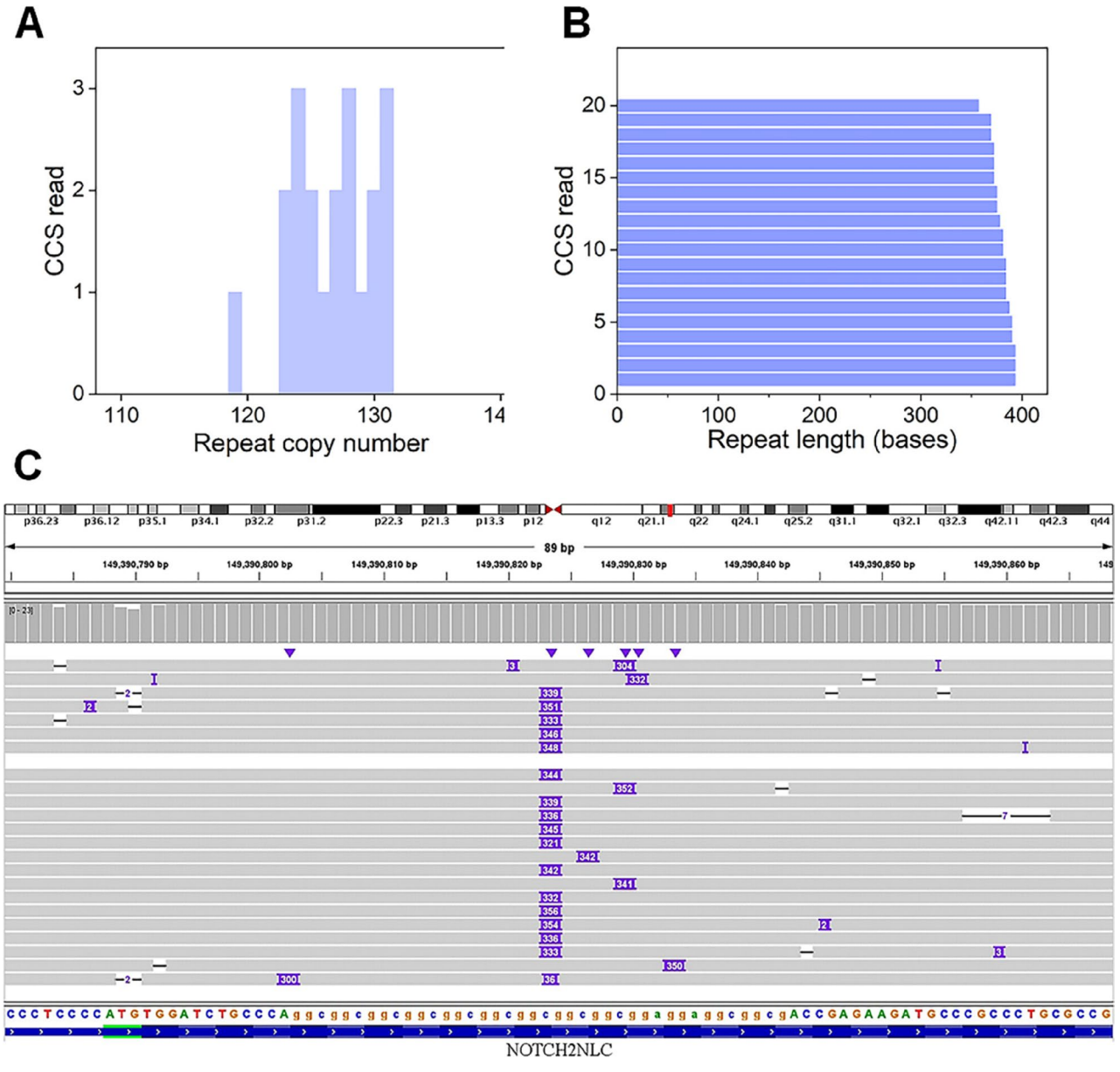

Fig. 2 Repeat expansion analysis of NOTCH2NLC using Nanopore whole-genome sequencing. **A** Histograms display the distribution of circular consensus sequence (CCS) reads based on the repeat copy number in NOTCH2NLC as observed by the AL-PCR method. **B** Waterfall-style plots illustrate the precise repeat structure of expanded alleles in Patient IV1. The horizontal axes represent the length of repeat expansions in bases, while the vertical axes indicate the number of CCS reads. The repeat lengths of CCS reads in Patient IV1 were similar, suggesting a homozygous NOTCH2NLC repeat expansion. **C** The results of Nanopore whole-genome sequencing revealed the number of inserted bases (bp) within the GGC tandem repeat region compared to the reference genome

The reads from Patient IV1 could not be definitively classified, further supporting the presence of homozygous repeat expansions. Among the 8 reads obtained from PacBio sequencing, 7 displayed nearly identical repeat lengths (Fig. 3A). To elucidate the specific base sequences of the repeated amplifications, Integrative Genomics Viewer (IGV) was employed to visualize the genomic data (Fig. 3B). The analysis revealed that Patient IV1 harbored a sequence with interruptions of (GGA)n. To corroborate the findings of biallelic NOTCH2NLC gene repeat amplification obtained through PacBio sequencing, Nanopore super basecalling was conducted, and the genomic data were visualized using IGV (Fig. S2). By eliminating mismatches and comparing identical sequences, we derived the accurate sequence of the biallelic GGC repeat expansion: (GGC)68 (GGA) {(GGC)3(GGA)2}2 (GGC)3 (GGA) (GGC)19 {(GGA)2 (GGC)2}2 (GGC)9 (GGA)2 (GGA)2 (GGC)2.

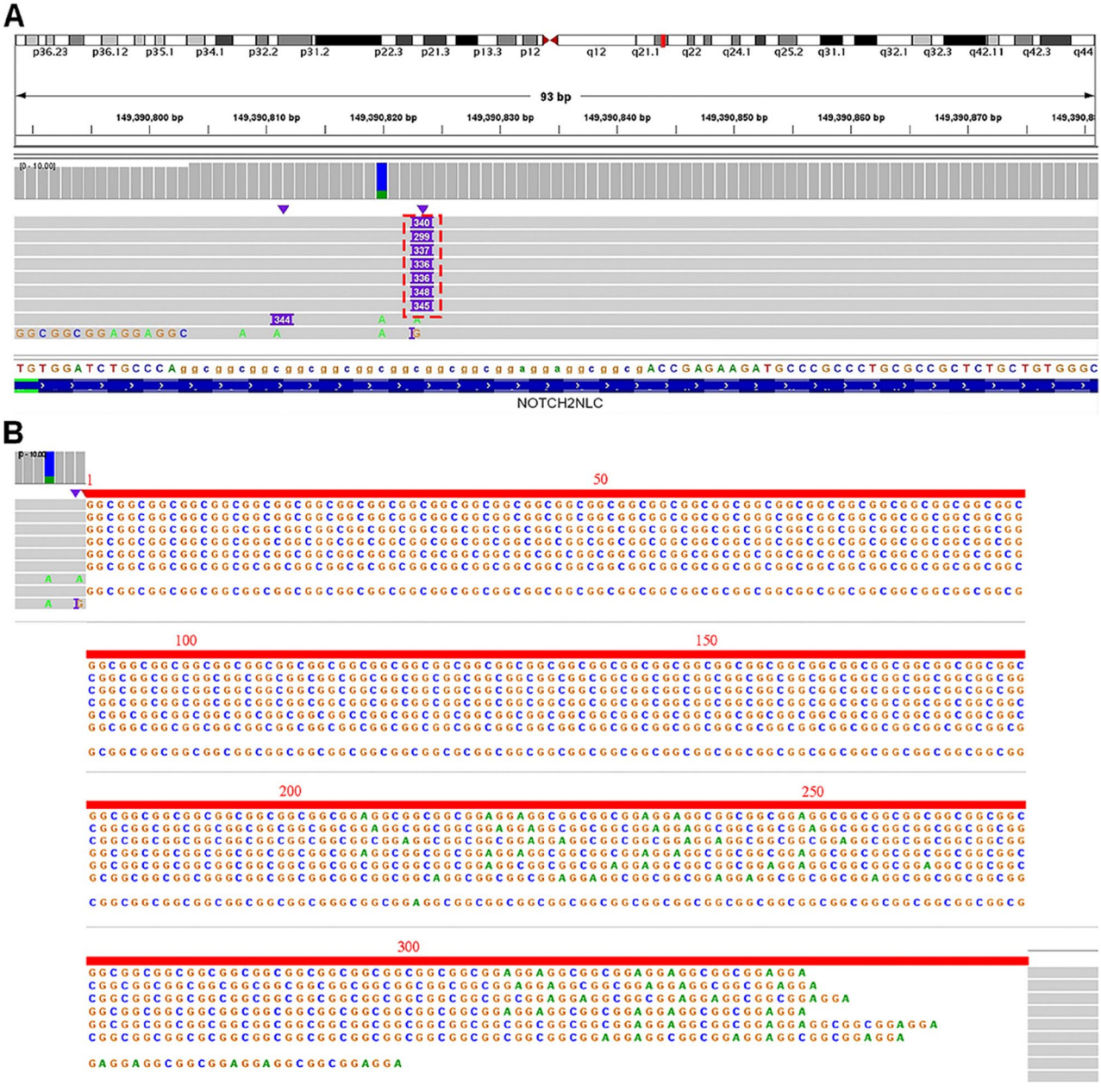

Fig. 3 Repeat analysis of NOTCH2NLC using PacBio sequencing. **A** The PacBio sequencing results showed the number of inserted bases (bp) within the GGC tandem repeat region, respectively, relative to the reference genome. **B** Concrete sequences inserted by all reads at the same insertion site for PacBio sequencing are shown using IGV

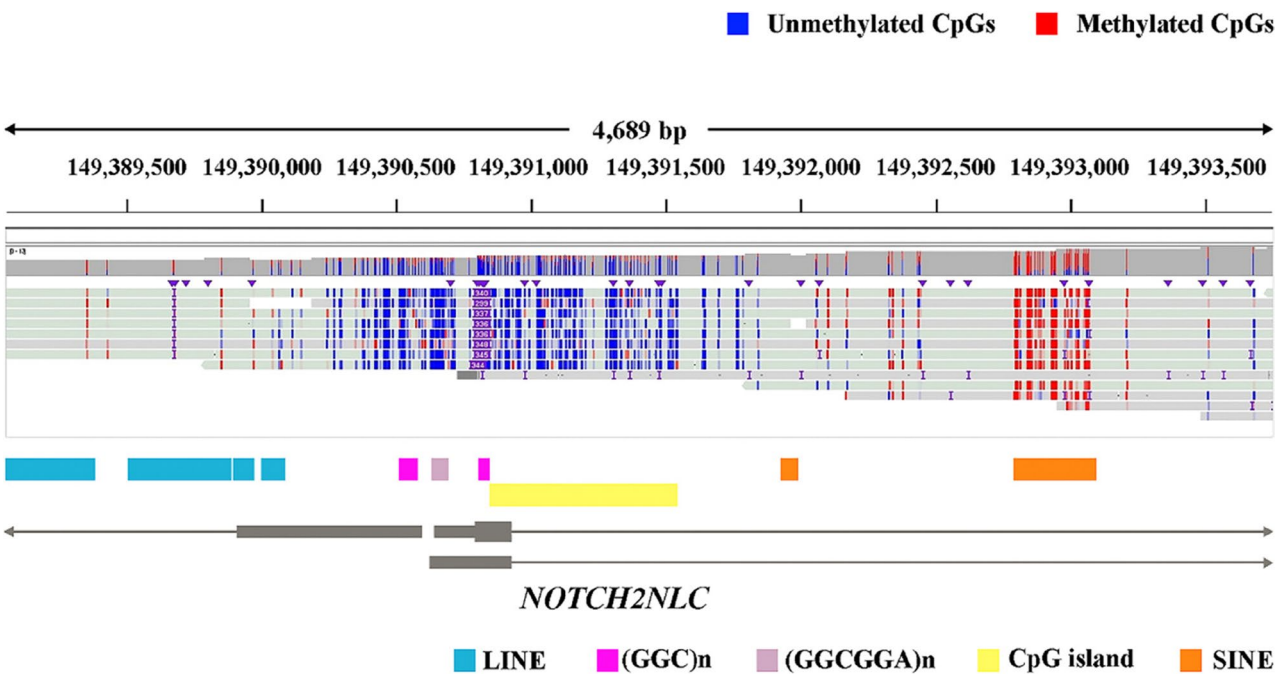

Fig. 4 PacBio sequencing was used to analyze the DNA methylation landscape of the approximately 5-kb NOTCH2NLC region. The upper read-based plots in IGV visually depict the methylation status of CpG sites in the reads, with methylated and unmethylated CpGs shown as red and blue lines, respectively

Biallelic hypomethylation of CpGs in the nearest CpG island and the GGC repeat

When the biallelic GGC trinucleotide tandem repeat region or adjacent CpG island undergoes hypermethylation, the hypermethylated allele experiences transcriptional silencing, potentially resulting in a pathophysiology similar to that observed in heterozygous repeat expansions commonly observed in autosomal dominant mutations in NIID [18, 19].

To mitigate the influence of hypermethylation on alleles, we employed PacBio whole-genome sequencing to ascertain the DNA methylation status of the GGC tandem repeat region and adjacent CpG islands. Analysis of the bisulfite mode in IGV revealed hypomethylation in both alleles at the CpG island nearest to the GGC repeat and within the expanded GGC repeat itself (Fig. 4). This suggests that both expanded alleles may retain transcriptional activity.

Discussion

NIID represents a heterogeneous disorder with widespread involvement across multiple systems, characterized by the presence of intranuclear inclusions detectable in the nuclei of all somatic cells [1]. As a slowly progressive neurodegenerative condition, patients typically present with mild clinical manifestations juxtaposed with pronounced objective examination findings, a pattern consistent with the patients in our study. Considering subsequent clinical symptoms and genetic test results, the proband's phenotype aligns with CNS-dominant NIID. At age 52, the patient experienced a more severe episode of encephalopathy, leading to a diagnosis of NIID. Notably, the proband did not initially exhibit clinical symptoms associated with peripheral neuromuscular disease, such as muscle weakness or sensory impairment. However, muscle biopsy was performed to exclude mitochondrial disorders given the clinical suspicion at that time, and the findings did not support a primary myopathy. Subsequent genetic testing ultimately confirmed the diagnosis of NIID, offering valuable insights into the diagnostic approach for similar cases.

In recent years, a growing body of literature has highlighted that the incidence rate of peripheral neuropathy associated with NIID can surpass 90%, underscoring the vulnerability of peripheral nerves in NIID patients [6, 20]. Notably, a recent study focusing on complex hereditary peripheral neuropathy identified NIID as a prevalent genotype within this specific patient cohort [21]. Electrophysiological examinations have emerged as invaluable tools for detecting, managing, and mitigating peripheral neuropathy [5]. Despite the absence of clinical symptoms or signs indicative of peripheral neuropathy in Patient III5 and Patient IV1, nerve conduction studies revealed the presence of subclinical peripheral neuropathy, characterized by demyelination of peripheral nerves without axonal damage. On the other hand, the proband's nerve conduction results revealed decreased MCV, SCV, CMAP, and SNAP, indicative of peripheral

nerve demyelination coupled with axonal damage. Interestingly, in Patient IV1 with homozygous mutations, subclinical peripheral neuropathy was observed even in the absence of typical NIID clinical symptoms and white matter lesions. It is worth noting that F-wave studies, which have been identified as sensitive markers of proximal demyelination in NIID [22], were not systematically conducted during the original electrophysiological evaluations, as they were not yet integrated into the routine clinical diagnostic protocol at that time. This limitation should be considered when interpreting our findings.

Given the current dearth of preclinical status studies within the realm of NIID research, our findings suggest that subclinical peripheral neuropathy may represent an early pathological change in NIID. However, this conclusion warrants further substantiation through additional cases. Nevertheless, it's important to acknowledge that asymptomatic carriers seldom seek genetic screening or clinical evaluation. Patient IV1, identified in this study, was serendipitously discovered during familial screening of the proband's family and subsequent analysis. Therefore, the widespread adoption of electrophysiological examinations among individuals harboring NIID pathogenic mutations could facilitate early detection of subclinical lesions, enabling prompt intervention and preventive measures.

To ascertain the phenotypic impact of biallelic GGC repeat expansions, we employed Nanopore super basecalling and PacBio technology to accurately determine GGC repeat expansion sequences. The genomic data were visualized using IGV. In Patient IV1, homozygous GGC repeat expansion sequences were observed, comprising (GGC)68 (GGA) {(GGC)3(GGA)2}2 (GGC)3 (GGA) (GGC)19 {(GGA)2 (GGC)2}2 (GGC)9 (GGA)2 (GGA)2 (GGC)2. This repeat expansion number corresponds to the dementia-dominant subtype, typically presenting with a median age of onset at 60 years (IQR 54.50–63.00) [6]. Considering the clinical manifestations and repeat expansion size, this NIID family appears to exhibit the dementia-dominant phenotype. Visualizing the results in IGV revealed discontinuities in GGA trinucleotide repeat expansion within the patient's GGC repeat expansion. Previous studies have suggested that GGA discontinuities in GGC repeat expansion may serve as genetic modifiers, influencing the age of symptom onset [17], and potentially elucidating the onset age within this family. However, despite harboring biallelic GGC repeat expansions, Patient IV1 did not present with typical clinical symptoms or white matter lesions associated with NIID. It's noteworthy that GGC repeat copy numbers exceeding 200–300 can induce DNA methylation and subsequent transcriptional silencing of GGC, resulting in asymptomatic carriers. In our study, patients exhibited biallelic repeat expansion sizes below 300 copies and demonstrated hypomethylation status, suggesting the involvement of both alleles in the production of toxic RNA and polyglycine proteins.

As a polyglycine disease, NIID arises from GGC repeat expansions in the 5'UTR of the *NOTCH2NLC* gene, resulting in the generation of RNA with hairpin-like structures and the production of misfolded polyglycine proteins [23]. Similarly, Fragile X-associated tremor/ataxia syndrome (FXTAS) stems from CGG repeat expansions in the 5'UTR of the FMR1 gene and shares substantial overlap with NIID in terms of clinical presentation, imaging findings, and pathological features, indicating potential common pathogenic mechanisms [24]. Recent studies have demonstrated that the severity of FXTAS remains unaffected by homozygous mutations in the FMR1 gene [25]. NIID is postulated to be inherited in a fully dominant manner, with the pathogenic mechanism likely involving a non-dose-dependent gain-of-function mechanism triggered by GGC repeat expansion. Our study further corroborates that biallelic repeat expansions do not exacerbate the severity or progression of NIID. Nevertheless, given the rarity of biallelic repeat expansions, additional research is warranted to substantiate this conclusion.

Conclusion

In this study, we performed a comprehensive clinical assessment of a family with NIID genetic background presenting with subclinical peripheral neuropathy. Through electrophysiological examinations, our findings support the concept that subclinical peripheral neuropathy may serve as a preclinical lesion. Furthermore, leveraging Nanopore and PacBio whole-genome sequencing, alongside DNA methylation analysis, we genetically verified a patient harboring homozygous biallelic *NOTCH2NLC* repeat expansions. Our study provides additional evidence that biallelic repeat expansions do not exacerbate the severity or progression of NIID.

Abbreviations

NIID	neuronal intranuclear inclusion disease
CNS	central nervous system
FXTAS	fragile X-associated tremor/ataxia syndrome
OPDM3	oculopharyngodistal myopathy type 3
PacBio	Pacific Biosciences
MoCA	Montreal Cognitive Assessment
RP-PCR	repeat-primed polymerase chain reaction
AL-PCR	fluorescence amplicon length polymerase chain reaction
MCV	motor nerve conduction velocity
SCV	sensory nerve conduction velocity
CMAP	compound muscle action potential
SNAP	sensory nerve action potential

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12883-026-04898-2.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements
We sincerely thank the patients and their families for participating in this study.

Authors' contributions
Conceptualization: Haiyang Luo, Yuming Xu, Han Liu; Methodology: Haiyang Luo; Formal analysis and investigation: Honglin Zheng, Suying Duan, Chenyang Liu, Hang Zhang, Yaochong Zhang and Qiang Li; Writing - original draft preparation: Taiqi Zhao; Writing - review and editing: Hang Zhang, Taiqi Zhao, Suying Duan; Funding acquisition: Yuming Xu; Resources: Yuming Xu; Supervision: Haiyang Luo, Yuming Xu.

Funding
This work was supported by the National Natural Science Foundation of China (Grant No. U1904207 to Dr. Yuming Xu) and the Non-profit Central Research Institute Fund of the Chinese Academy of Medical Sciences (Grant No. 2020-PT310-01 to Dr. Yuming Xu).

Data availability
The raw sequencing data generated in this study are available in the NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA1377206, with SRA run accessions SRR36349560 and SRR36349561. The BioProject page is publicly accessible at: [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1377206]

Declarations

Ethics approval and consent to participate
Ethical approval for patient data collection was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval number: 2022-KY-0386-002). All participants or their legal guardians provided written informed consent, in accordance with the ethical standards of the Declaration of Helsinki and its subsequent amendments.

Consent for publication
The authors affirm that all human participants provided informed consent for the publication of any identifying images or clinical data. For participants with cognitive impairment, written informed consent for such publication was additionally obtained from their legal guardians.

Competing interests
The authors declare no competing interests.

Received: 16 October 2025 / Accepted: 9 April 2026
Published online: 18 April 2026

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