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ORIGINAL ARTICLE

Saliva-Based Triple-Primed PCR as a Non-Invasive Tool for Detecting *NOTCH2NLC* GGC Repeat Expansions in NIID

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ABSTRACT

Background: ***NOTCH2NLC*-related neuronal intranuclear inclusion disease (NIID) is a neurodegenerative disorder caused by abnormal expansions of GGC repeats in the *NOTCH2NLC* gene. Traditional genetic testing relies on blood samples, which can be invasive. This study investigated the feasibility of using non-invasive saliva samples for detecting GGC repeat expansions in NIID patients.**
Methods: **Twenty-four NIID patients and twenty-one controls were enrolled. DNA was extracted from both blood and saliva samples using standard protocols. GGC repeat expansions were detected using triple-primed PCR (TP-PCR), followed by capillary electrophoresis to determine the repeat sizes.**
Results: **All NIID patients exhibited characteristic saw-tooth peaks indicative of GGC expansions in both saliva and blood tests, while control samples showed no such patterns. Saliva and blood genetic testing showed comparable GGC repeat expansions. Notably, one patient showed inter-tissue discrepancies, suggesting somatic mosaicism.**
Conclusions: **Saliva DNA testing offers a reliable, minimally-invasive alternative to blood DNA testing for NIID diagnosis. While tissue-specific mosaicism may cause minor discrepancies, saliva's ease of collection and diagnostic accuracy support its utility in large-scale screening and early intervention strategies.**
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KEYWORDS

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INTRODUCTION

NOTCH2NLC-related neuronal intranuclear inclusion disease (NIID) is a clinically heterogeneous neurodegenerative disorder characterized by a wide spectrum of symptoms, including cognitive impairment, movement disorders, autonomic dysfunction, and episodic symptoms [1]. Pathologically, NIID features widespread p62- and ubiquitin-positive eosinophilic inclusions. Its genetic cause has been identified as pathogenic GGC repeat

expansions (> 60 repeats) in the *NOTCH2NLC* gene. With growing understanding of NIID and the increasing use of DNA testing, the number of reported cases has markedly risen in recent years.

Diagnosis of NIID primarily relies on genetic and pathological testing. GGC repeat expansions in the *NOTCH2NLC* gene are typically detected using blood-derived DNA through long-read sequencing, repeat-primed PCR (RP-PCR), or amplicon length analysis PCR (AL-PCR). Triple-primed PCR (TP-PCR), which combines RP-PCR and AL-PCR, is widely used for diagnosing repeat expansion disorders due to its cost-effectiveness and quick turnaround, making it suitable for large-scale screening [2,3]. However, its dependence on blood samples - requiring trained personnel and clinical settings - limits its utility in large-scale or community-based screening. Therefore, exploring less invasive and more accessible alternative DNA sources for NIID genetic screening is compelling. While saliva-based DNA testing is established for various genetic analyses, its specific application for diagnosing repeat expansion disorders like NIID remains largely unexplored and unreported.

This study aimed to explore the feasibility of using non-invasive saliva DNA samples for detecting GGC repeat expansions in the *NOTCH2NLC* gene. We used a commercially available triple-primed PCR assay for 24 NIID patients and 21 controls. By employing less intrusive sampling techniques, we sought to simplify the diagnostic process for NIID, potentially increasing patient compliance and facilitating earlier diagnosis. This approach could offer a valuable alternative to blood-based testing, aligning with the growing trend towards minimally-invasive diagnostic methods in clinical practice.

MATERIALS AND METHODS

Participants

Twenty-four NIID patients and twenty-one controls were all recruited from Fudan University affiliated Zhongshan Hospital between January 2024 and May 2025. Ethical approval was obtained from the Ethics Committee of Fudan University affiliated Zhongshan Hospital, and written informed consent was obtained from all the patients.

Sample collection and DNA extraction

Whole blood samples were collected in EDTA-containing vacutainer tubes, and saliva samples were obtained in sterile tubes following oral rinsing. Genomic DNA was extracted using the Whole Blood/Amniotic Fluid/Cell Genomic DNA (Centrifugal Column Method) Nucleic Acid Extraction Kit (BIOFAST BIOTECH, Xiamen, China), according to the instruction.

PCR assays and GGC repeat size determination

The TP-PCR was performed using the Human *NOTCH2NLC* Gene Detection Kit (BIOFAST BIOTECH, Xiamen, China) according to the instruction. The amplified products were then analyzed using capillary electrophoresis on ABI3500 SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the data were analyzed using GeneMapper software (Thermo Fisher Scientific) to confirm the presence of abnormal repeat patterns and the number of GGC repeats.

Statistical analysis

Statistical analysis was performed using SPSS version 20 and Graphpad Prism version 9. To assess the agreement between two gene testing methods in detecting GGC repeat counts, Bland-Altman analysis was performed.

RESULTS

The main clinical manifestations in our NIID patients included cognitive impairment, parkinsonism, paroxysmal symptoms, muscle weakness, and autonomic symptoms. Skin biopsy was performed in 12 NIID patients, and the results showed P62-positive eosinophilic intranuclear inclusions in fibroblasts (Table 1). DNA was successfully extracted from all blood and saliva samples. As shown in Table 2, saliva samples yielded higher DNA concentrations and slightly better purity metrics, suggesting their suitability for downstream PCR analysis (Table 2).

NIID diagnosis using saliva and blood DNA relies on identifying both saw-tooth patterns and expansion-associated amplification peaks, which confirm the presence and estimate the size of *NOTCH2NLC* GGC repeat expansions. In our cohort, all NIID patients exhibited characteristic saw-tooth peaks in both blood and saliva samples. The median GGC repeat number was 135.5 (IQR 111.2 - 206.8) in blood and 124.5 (IQR 109.0 - 207.5) in saliva (Figure 1A). In contrast, control samples from either source showed only normal-range GGC repeats, without expansion-associated sawtooth peaks (Figure 1B). Notably, one patient showed a marked difference in repeat size between saliva (114 repeats) and blood (144 repeats) (Figure 1C). Another case displayed two distinct repeat sizes in blood but only a single repeat size in saliva, suggesting somatic mosaicism or tissue-specific repeat instability of the *NOTCH2NLC* GGC expansion (Figure 1D). Saliva and blood tests demonstrated high concordance, with comparable repeat counts (Figure 1E).

DISCUSSION

NIID exhibits a broad spectrum of clinical manifestations, requiring careful differentiation from other disorders. In 2011, Sone et al. [4] first observed eosinophilic intranuclear inclusions in fibroblasts and sweat gland cells from skin biopsies of NIID patients, establishing

Table 1. Summary of clinical manifestations of participants.

	Cases (n = 24)	Controls (n = 21)
Age (years)	68.0 (51.0 - 70.0)	54.0 (40.0 - 67.0)
Gender ratio (male/female)	9/15	7/14
Clinical manifestations		
Cognitive impairment	16/24 (66.7%)	0/21 (0%)
Parkinsonism	12/24 (50.0%)	0/21 (0%)
Paroxysmal symptoms	5/24 (20.8%)	0/21 (0%)
Muscle weakness	15/24 (62.5%)	0/21 (0%)
Autonomic symptoms	13/24 (62.5%)	0/21 (0%)
DWI high signal	14/24 (58.3%)	0/21 (0%)
Skin biopsy	12/12 (100%)	0/21 (0%)
Repeat expansion size (blood DNA test)	135.5 (111.2 - 206.8)	21.0 (19.0 - 23.5)
Repeat expansion size (saliva DNA test)	124.5 (109.0 - 207.5)	21.0 (19.0 - 23.5)

DWI: diffusion-weighted imaging.

Table 2. Comparison of DNA yield and quality according to DNA collection method.

Methods of DNA collection	Blood	Saliva
Total amount of samples	45	45
Amount of sample used (mL)	0.4 (0.2 - 0.5)	0.6 (0.5 - 1.0)
DNA concentration (ng/μL)	20.3 (13.0 - 36.4)	49.6 (22.7 - 137.8)
260/280 nm ratio	1.75 (1.67 - 1.79)	1.72 (1.68 - 1.73)
260/230 nm ratio	1.86 (1.18 - 2.17)	2.30 (2.18 - 2.38)

skin biopsy as an effective and less invasive diagnostic tool for NIID. In 2019, GGC repeat expansion in the *NOTCH2NLC* gene was identified as a major genetic cause in East Asian populations with NIID [5-7]. Diagnosis now relies on genetic and pathological testing. Although skin pathology is considered a diagnostic gold standard, false negatives can occur, underscoring the importance of genetic testing. Previous studies have identified *NOTCH2NLC* GGC repeat expansions in patients with ALS [8], PD [9], OPDM [10], and essential tremor [11], especially in cases with unclear etiology. These findings highlight the value of genetic screening for NIID in patients with unexplained neurodegenerative or systemic degenerative disorders.

Since the implementation of DNA testing in the diagnosis of NIID, the identification of NIID cases has surged. For diagnostic confirmation, DNA is extracted from blood to detect and quantify GGC repeats in the *NOTCH2NLC* gene via long-read sequencing, or RP/AL-PCR. The gold standard for diagnosing *NOTCH2NLC*-related NIID is third-generation sequencing, which enables accurate quantification of GGC repeat expansions. However, due to its high cost, it is not suitable for large-scale screening. Triple-primed PCR (TP-PCR) is a method that combines RP-PCR and AL-PCR. Briefly, primers are designed to target sequences outside and within the GGC repeat region for PCR amplification, allowing for the generation of *NOTCH2NLC* gene amplification product and multiple GGC amplification products that vary by 3 bp in size. These amplified fragments are subsequently analyzed using capillary electrophoresis to assess the characteristic sawtooth pattern and the length of repeats. However, due to limitations in PCR efficiency, amplification becomes challenging for GGC repeats exceeding 300 repeats.

Saliva, a body fluid obtained non-invasively, contains a high concentration of oral exfoliated epithelial cells, making it possible to extract substantial amounts of DNA. Compared to blood sampling, saliva collection demonstrates distinct advantages in resource-limited areas due to its simplified storage requirements without stringent cold-chain logistics. Saliva has been employed in diagnosing and researching various diseases. However, reports on its use for screening and diagnosing re-

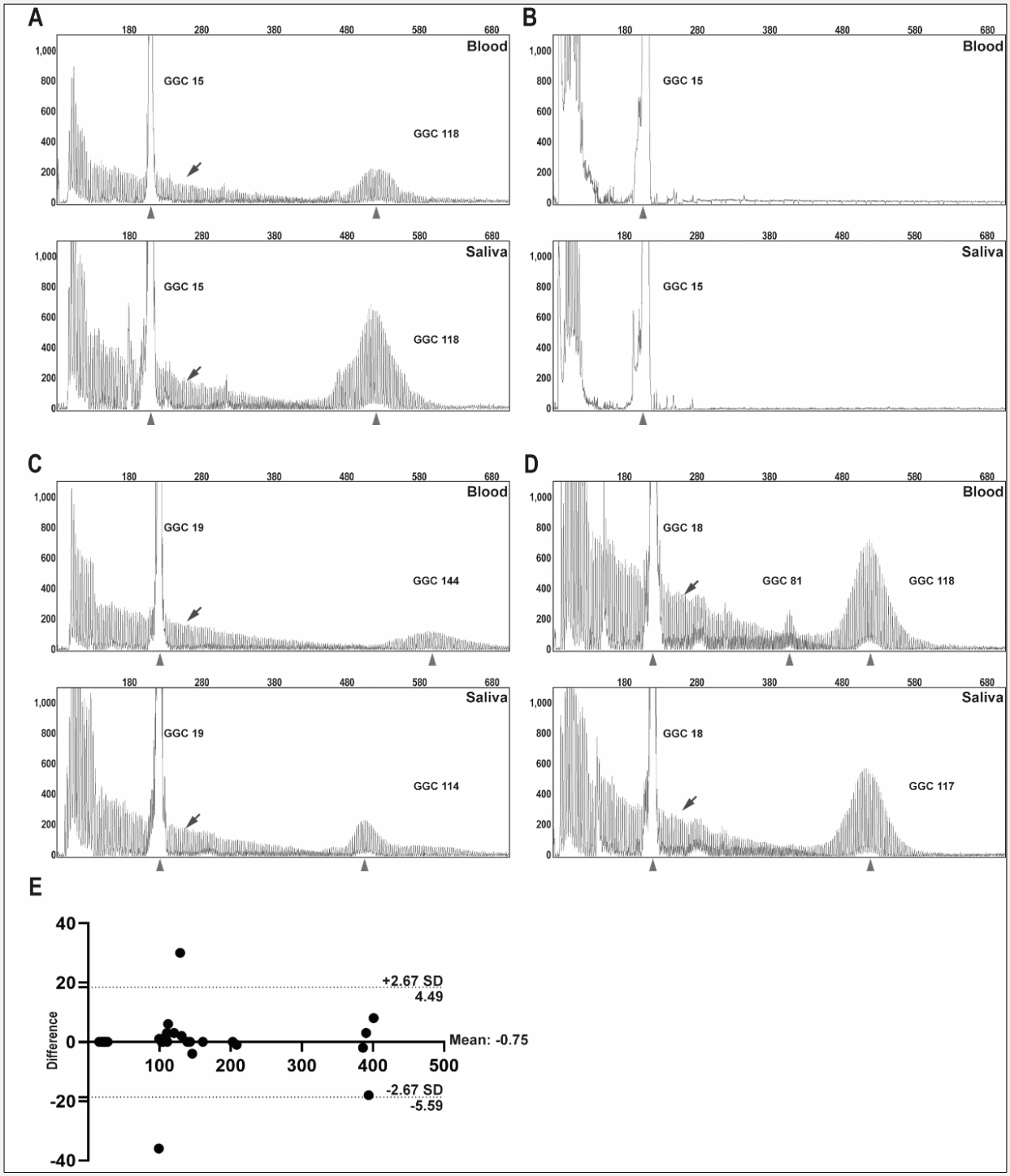

Figure 1. Detection of the GGC repeat expansions in blood and saliva DNA.

A - D: Representative electropherograms of triple-primed PCR (TP-PCR). Saw-tooth patterns and amplification signal peaks were detected in both blood and saliva DNA of one NIID patient (A), but not in a control individual (B). A discrepancy in GGC repeat expansion size between saliva and blood DNA was detected in one NIID patient (C). Biallelic expansions in blood but monoallelic expression in saliva was detected in one NIID patient (D).
E: Bland-Altman analysis showed a high concordance between blood and saliva DNA tests. Arrows indicate saw-tooth patterns. Arrowheads indicate normal and mutation amplification signal peaks.

peat expansion disorders remain scarce. In our study, we extracted DNA from both the blood and saliva of individuals diagnosed with NIID and controls. We employed TP-PCR to identify GGC repeats in the *NOTCH2NLC* gene. Our findings indicate that the accuracy and sensitivity of salivary genetic testing for NIID are comparable to those of traditional blood-based genetic tests. Saliva sampling eliminates phlebotomy needs and cold-chain logistics, enabling cost-effective NIID screening in resource-limited settings. Given the reliability of saliva DNA testing results and the ease of sample collection, salivary genetic testing can be used for large-scale screening of NIID.

Interestingly, our study identified notable discordance in GGC repeat counts between matched saliva and blood specimens in some NIID cases, with one striking case demonstrating 144 repeats in blood versus 114 in saliva. This 30-repeat inter-tissue discrepancy strongly suggests postzygotic somatic mosaicism arising during cellular differentiation. Moreover, one NIID patient carried two different pathogenic sizes of GGC repeats, also suggesting repeat instability or somatic mosaicism in the GGC repeats of the *NOTCH2NLC* gene. This aligns with the findings of Fukuda et al. [12] and Deng et al. [13], who observed somatic instability of short tandem repeats (STRs) in the *NOTCH2NLC* gene in NIID patients. Huntington's disease (HD), a neurodegenerative disorder driven by CAG trinucleotide repeat expansions, exhibits clinically significant somatic instability where expansion size-dependent phenotypic variability correlates with disease progression [14]. This established paradigm of dynamic somatic mosaicism underscores the biological relevance of detecting analogous somatic mutations in NIID pathogenesis. Given the shared ectodermal origin of salivary glands and neural tissue, contrasting with the mesodermal derivation of hematopoietic lineages, saliva DNA may better reflect central nervous system molecular profiles, advocating combined biofluid analysis for comprehensive somatic instability assessment.

This study has limitations. The sample size is relatively small and limited to a single center. Furthermore, TP-PCR may fail to detect extremely large repeat expansions, and third-generation sequencing remains necessary in such cases.

In summary, this study explored the feasibility of using non-invasive saliva DNA samples to detect GGC repeat expansions in the *NOTCH2NLC* gene. Employing less intrusive sampling techniques, such as saliva testing, aimed to simplify the diagnostic process, potentially increasing patient compliance and facilitating earlier diagnosis. Our findings validate saliva as a practical alternative for NIID diagnosis and large-scale NIID screening, particularly in resource-limited settings.

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Data Availability Statement:
The data presented in this study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to Participate:
This study was approved by the Ethics Committee of Fudan University affiliated Zhongshan Hospital (no. B2024-478). The patients or their immediate family members were fully informed about the purpose of the study and signed the informed consent forms.

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Declaration of Interest:
The authors declare that they have no competing interests.

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