The Role of Presenilin Associated Rhomboid-Like Transcript Variants in Leber's Hereditary Optic Neuropathy Expression

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ABSTRACT

A primary mtDNA mutation, mt.11778G>A, is essential but not sufficient to cause blindness in Leber's Hereditary Optic Neuropathy (LHON). This study aimed to elucidate the role of presenilin associated, rhomboid-like (*PARL*), a candidate nuclear modifier gene of LHON identified previously through linkage and association study. *PARL* transcript variants were screened using reverse transcription and gel electrophoresis to compare the difference in pattern of expression between affected and unaffected mutation carriers and non-carrier healthy normal controls. The splice variants of *PARL* transcripts in affected and unaffected mutation carriers and normal controls were similar. We concluded that the different isoforms of *PARL* transcript variants do not play a role as a modifier in LHON disease expression.

Keywords: Leber's hereditary optic neuropathy, Nuclear modifier gene, *PARL*

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Introduction

Leber's hereditary optic neuropathy (LHON) is one of the most common mitochondrial diseases characterized by defect in subunits of the respiratory chain complex I or NADH dehydrogenase (ND) of the OXPHOS system (Yu-Wai-Man et al., 2009). A meta-analysis in European populations estimated that the prevalence of LHON was 1:45,000 (Mascialino et al., 2012). The major pathogenesis is the degeneration of the retinal ganglion cells (RGCs) and their axons forming the optic nerve (Huoponen, 2001). The disease starts with acute blurring in central vision of one eye followed by the other eye in a few weeks or months later. Blindness in LHON is usually painless and exhibits no inflammation. Interestingly, most patients are young adult men. In Thailand, the age of onset ranges from 6 to 53 years (mean = 21.5 years) (Chuenkongkaew et al., 2001). The nature of the disease progression is rapid with very rare chance of recovery.

Primarily LHON patients harbor one of the three non-synonymous point mutations in the mitochondrial DNA (mtDNA) encoding subunit of the respiratory complex I. Mutations in mt.11778G>A of ND4, mt.14484T>C of ND6 and mt.3460G>A of ND1 (Ruiz-Pesini et al., 2007) account for more than 90% of the patients. The most common mutation worldwide, as well as in Thai population, is mt.11778G>A mutation (Chuenkongkaew et al., 2001). Importantly among these mutation carriers, approximately 50% in men and 10% in women develop the visual failure in their life (Kirkman et al., 2009). The incomplete penetrance nature of the disease implies that the mtDNA mutation alone is not sufficient to cause the disease. The additional genetic and/or environmental factors are suggested to modify the expression of LHON.

Disease modifiers are not the causal agent of the disease. They are neither necessary nor sufficient to develop the disease. However, modifiers can affect the expression of the disease. Recent studies on LHON attempt to identify the disease modifiers by the effects of the genomic, transcriptomic, proteomic profiles as well as by the environmental factors. Considering that 99% of mitochondrial proteins are encoded by nuclear genes, the potential influence of nuclear genes on the mitochondrial disease should be highly focused on.

The male predominance pattern of disease expression suggested that nuclear modifier genes might be on the X chromosome. Although some evidences for X-Linked modifier loci including the first STR (DXS7) in Finnish pedigrees have been identified (Shankar et al., 2008; Vilkki et al., 1991), the linkage signals from various studies on chromosome X were inconsistent (Carvalho et al., 1992; Ji et al., 2010; Man et al., 2002). The association study has been done in OPA1 gene because its mutations can cause the disease of another type of optic atrophy. However, no association between that gene and LHON expression was found (Hudson et al., 2010).

The genome wide linkage scan using 400 microsatellite markers in nine large Thai LHON pedigrees found the strongest linkage signal at D3S1565 with a Zlr score > 2 ($P < 0.025$) (Phasukkijwatana et al., 2010). Fine mapping of this linkage peak identified rs3749446 and rs1402000 in *PARL* gene, as the strongest modifier of LHON expression (Phasukkijwatana et al., 2010). The follow-up study found 3-SNP haplotypes (rs3749446,

rs1402000 and rs953419) in *PARL* associated with higher risk of being affected by mt.11778G>A mutation (Istikharah et al., 2013).

The nuclear gene *PARL,* approximately 56 kb long with 10 exons and located on chromosome 3q27.1, encodes presenilins-associated rhomboid-like protein (PARL). According to the NCBI Reference Sequence Database (RefSeq), the gene produces two known protein-coding transcript variants by alternative splicing. NM_018622 is full-length transcript with 10 exons, and the other transcript (NM_001037639) is an exon 6 skipping transcript with 9 exons (delta-6 transcript).

Objective of the Study

The objective of the study is to clarify the roles of *PARL* transcripts as a potential LHON modifier. We investigate *PARL* transcript variants extracted from fibroblasts culture of mt.11778G>A carriers, both affected and unaffected with LHON, and normal control samples.

Methodology

Samples

A family including three LHON patients and three unaffected mt.11778G>A carriers were chosen from Siriraj Cohort Study of Leber's Hereditary Optic Neuropathy. The study was approved by Human Subjects Protection Committee of Faculty of Medicine Siriraj Hospital (ethical clearance number 161/2551). Fibroblasts stored in the biorepository or fibroblasts obtained from skin biopsy were used as the source of specimens in this study. The mutation status and mutation load of each sample were determined by restriction fragment length polymorphism method. Seven reference normal control samples from different families were age-matched (within 10 years) with unaffected and affected LHON mutation carriers.

Cell culture of skin fibroblasts

The skin fibroblasts from skin biopsy or from frozen cells stored in liquid nitrogen are cultured in sterile 10% Dulbecco's modified Eagle Medium (DMEM) at 37°C in the 5% CO_2 incubator. DMEM is supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1µg/ml amphotericin B, 100U/ml penicillin in, 100µg/ml streptomycin, 2 mM L-glutamine and 50µg/ml uridine. The culture medium was replaced every 72-96 hours to ensure to be fresh and optimal condition for cell growth. The cell passages less than 6 were used to get higher viability and optimal plating efficiency.

Extraction of RNA from skin fibroblasts

The fibroblasts were harvested for RNA extraction when they reach nearly 90% confluence. Total RNA was extracted using TRIZOL reagent according to the manufacturer's protocol. The RNA pellet was dissolved in RNase-free water and stored at -80°C until further use. The concentration of the extracted RNA is measured by UV spectrum of the NanoPhotometer® (Implen, Munich, Germany). The A260/A280 ratio was used to check the purity of nuclei acids.

RT-PCR amplification

The transcripts of *PARL* gene were amplified by SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase using PTC200 Thermal Cycler. Two sets of primers were used to amplify all exons of *PARL* transcript variants (Figure 1). Primer set 1 including a forward primer (5' AGC TGG GGT TGG TAA GGT C 3') and a reverse

Table 1 Summary of Samples: Affected and unaffected LHON mutation carriers (* Tested only in LHON family members, NA indicated no available data.)

Family ID	Sample	Current Age	Sex	Age of onset (years)	Mutation load*	Family History
1	A1	25	M	15	100%	Yes
1	A2	39	F	11	100%	Yes
1	A3	31	M	19	100%	Yes
1	U1	34	M	Unaffected	100%	Yes
1	U2	41	F	Unaffected	100%	Yes
1	U3	39	M	Unaffected	100%	Yes
2	N1	37	M	Normal Control	NA	No
3	N2	33	M	Normal Control	NA	No
4	N3	37	M	Normal Control	NA	No
5	N4	22	M	Normal Control	NA	No
6	N5	35	M	Normal Control	NA	No
7	N6	34	M	Normal Control	NA	No
8	N7	24	F	Normal Control	NA	No

primer (5' ATA ATA CCT GTC ACA GTC CGC 3') was used to amplify a fragment of exon 1 to 5. Exon 5-10 fragment is amplified by primer set 2 with a forward primer (5' TTC TCT GCA GCG GAC AAT GAT 3') and a reverse primer (5' TGG TCA CTC TAG CCG ATG TC 3'). Synthesis of cDNA was performed at 55°C for 30 min. Amplification of the cDNA was performed by 25 cycles of 94°C for 40 seconds, the annealing temperature (64°C and 60°C for primer set 1 and 2 respectively) for 1 minute and 68°C for 40 seconds.

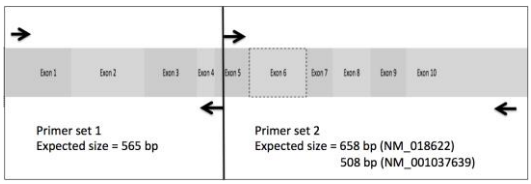

Figure 1 The relative position of primer on *PARL* transcript. The arrows indicated the primer annealing regions.

Visualization of the RT-PCR products

RT-PCR products mixed with loading dye were loaded onto a 3% agarose gel, followed by an electrophoresis at 100V for 90 minutes. The gel was subsequently stained with ethidium bromide and visualized by gel documentation (ImageQuant LAS 4010).

Purification of different RT-PCR products

Each fragment of the RT-PCR products from primer set 2 was purified from agarose gel using GenepHlow™ Gel/PCR (Geneaid Biotech) according to the manufacturer's protocol.

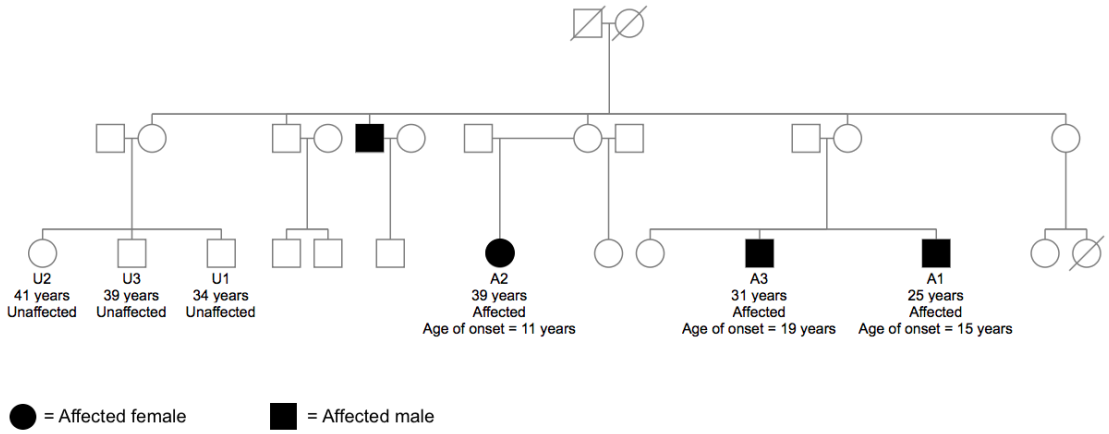

Figure 2 Pedigree of the mt.11778G>A LHON family: selected affected (A1, A2 and A3) and unaffected (U1, U2 and U3) individuals in this study

Sequencing of purified RT-PCR products

Purified RT-PCR product was sequenced using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI Prism Genetic Analyzer 3130 Automated DNA sequencer® (Applied Biosystems) using the same primers as RT-PCR amplification. Both forward and reverse primers were used to confirm the sequence. Sequences were analyzed by using BioEdit Sequence Alignment Editor v7.2.5 (Ibis Biosciences, Carlsbad, CA) to align to NCBI reference sequence (NM_018622).

Results

Mutation load in the LHON samples

Both LHON patients and unaffected carriers harbor homoplasmic (100%) mutation. Any mutation carriers older than the average age of onset are regarded as unaffected. Two male and a female were chosen in both groups (Figure 2).

***PARL* transcripts in normal samples**

Amplification of exon 1 to exon 5 by primer set 1 gave a product at the expected size (565 bp) from all normal samples (Figure 3a). Primer set 2 was used to amplify exon 5 to exon 10, resulting in the full-length and delta-6 amplicons, which were seen at 658 bp and 508 bp, respectively (Figure 3c). In addition, another prominent band between 500 and 600 bp (556 bp) was seen consistently in every normal sample, resulting in a total of three bands from amplification of exon 5 to 10.

***PARL* transcripts in mt.11778G>A mutation carriers**

RT-PCR products from primer sets 1 and 2 obtained from affected and unaffected LHON mutation carriers were shown in figures 3b and 3d. Obviously, there was no difference in the expression pattern of *PARL* transcripts between affected and unaffected LHON mutation carriers. Those transcripts were also consistent with the normal samples.

Novel *PARL* transcript variant

Each band of RT-PCR product was sequenced and aligned with a consensus sequence reported in the NCBI RefSeq (NM_018622). The

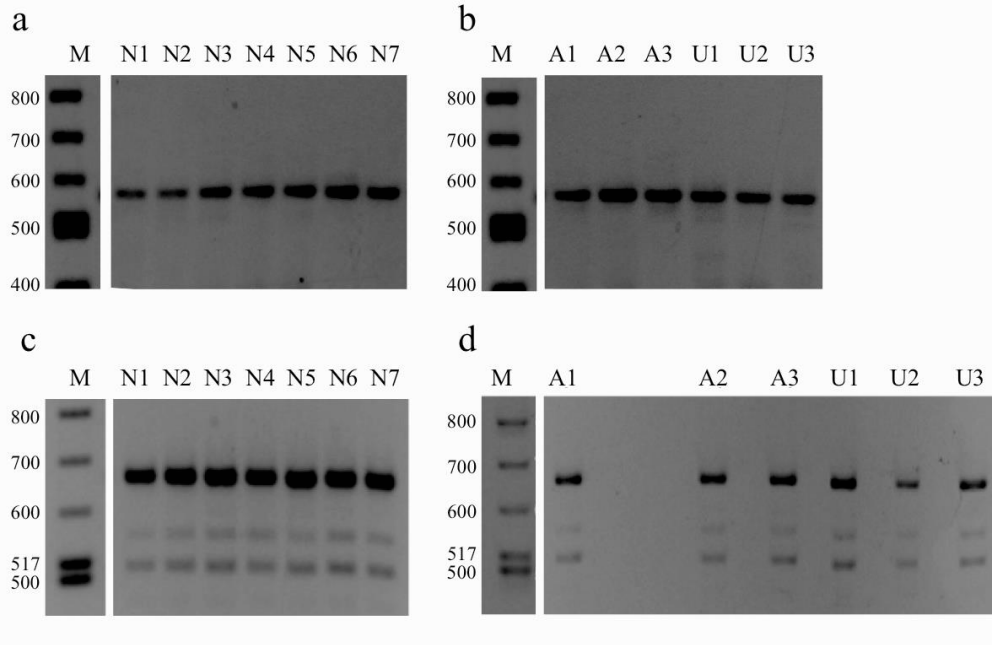

Figure 3 RT-PCR products from amplification of *PARL* transcripts of control individuals, and affected and unaffected LHON mutation carriers by primer set 1 (a, b) and primer set 2 (c, d) (M = molecular weight marker (100 bp), N = normal control, A = affected, U = unaffected).

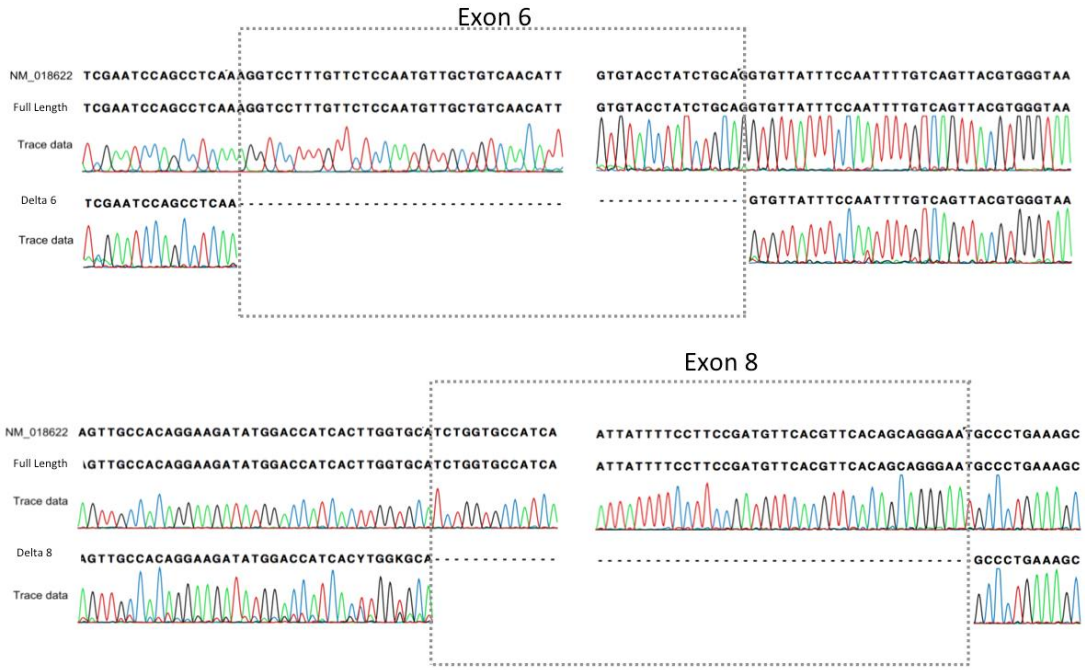

Figure 4 Sequence alignment of RT-PCR products from primer set 2 to NCBI RefSeq NM_018622. The upper panel shows sequence alignment between NM_018622, the full-length (568 bp), and delta-6 (508 bp) amplicons. The lower panel shows sequence alignment between NM_018622, the full-length (568 bp) and delta-8 (556 bp)

Table 2 Transcript variants with highest similarity to delta 8 transcript from NCBI BLASTN 2.2.32

Description	Max score	Total score	Query cover	E value	Identity	Accession
PREDICTED: H.sapiens *PARL*, transcript variant X2, mRNA	924	924	97%	0.0	99%	XM_005247584.3
PREDICTED: P.vampyrus *PARL*, transcript variant X2, mRNA	725	725	90%	0.0	94%	XM_011382590.1
PREDICTED: D.novemcinctus *PARL*, transcript variant X2, mRNA	678	678	92%	0.0	91%	XM_012527595.1
PREDICTED: H.sapiens *PARL*, transcript variant X1, mRNA	477	659	68%	2e-131	98%	XM_005247582.3
PREDICTED: P. troglodytes *PARL*, transcript variant X1, mRNA	477	925	97%	2e-131	98%	XM_001135897.3

bands at the expected sizes (565 bp fragment from primer set 1, and 508 bp and 658 bp fragments from primer set 2) were confirmed as *PARL* transcripts (Figure 4). However, the unexpected band (556 bp), from primer set 2, was identified as another *PARL* transcript variant with exon 8 skipping (Figure 4). Using NCBI BLASTN 2.2.32 (Altschul et al., 1997) to search for available information of this transcript revealed the best match to be XM_005247584.3, a predicted *PARL* transcript variant X2 mRNA (Table 2). This new variant was designated as a delta 8 variant.

Discussion and Conclusion

The underlying pathology of blindness in LHON is the apoptosis of RGCs and the atrophy of the optic nerve. The specific involvement of only RGCs is the greatest challenge for researchers to obtain the affected tissues for an experimental model. In this study, we used the skin fibroblast as our model to study LHON since both RGCs and fibroblast are developed from ectoderm.

In our study, we analyzed the association between transcript variation of *PARL* gene and LHON expression since *PARL* has been shown to be a potential nuclear modifier gene for LHON (Istikharah et al., 2013; Phasukkijwatana et al., 2010). PARL is a transmembrane protease in the inner mitochondrial membrane with 7 transmembrane helixes (TMH) naming TMHA and TMH 1-6 (Hill and Pellegrini, 2010). The proper spatial ordering and orientation of amino acids S277 on TMH-4 and H335 on TMH-6 are required for the protease activity of PARL (Jeyaraju et al., 2011). The short isoform of PARL lacks exon 6 resulting in an in-frame shorter protein with a deletion of 50 amino acids (Leu205 to Val254). At present, there is no published literature comparing the functional difference between the two isoforms of PARL; however, the full-length protein is assumed to be fully functional than the shorter protein.

We did not find any difference in isoforms of *PARL* transcripts between affected and unaffected

mt.11778G>A mutation carriers. The included family members might also have similar *PARL* haplotype, therefore the transcript variants identified were same between affected and unaffected mutation carriers. Since we did not find the evidence that *PARL* transcript variants could modify the disease expression, other genetic or environmental modifiers might play a role in controlling LHON expression in the family included in this study.

We unexpectedly identified a novel transcript variant of *PARL*, which lacks exon 8. Exon 8 contains one of the active sites of PARL (Ser277), which is a part of TMH 4. The best matching transcript, XM_005247584.3 is predicted to encode XP_005247641.1, PARL protein isoform X2, which is not yet validated.

This study emphasized only on the splice variants of *PARL* gene and its effect on LHON expression. We still have no evidence linking the total or individual expression level of these variants in affected and unaffected LHON mutation carriers. Further studies are needed to identify the genotype of *PARL* and the level of transcript variants in LHON expression.

Noteworthy, this study included only one family of 11778G>A mutation. The result could then be family specific and has limited generalizability. We would gain more insight into a role of *PARL* in LHON expression when more families were included in the study.

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