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ORIGINAL INVESTIGATION

Genome-wide linkage scan and association study of *PARL* to the expression of LHON families in Thailand

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Abstract Leber hereditary optic neuropathy (LHON) is the most common mitochondrially inherited disease causing blindness, preferentially in young adult males. Most of the patients carry the G11778A mitochondrial DNA (mtDNA) mutation. However, the marked incomplete penetrance and the gender bias indicate some additional genetic and/or environmental factors to disease expression. Herein, we first conducted a genome-wide linkage scan with 400 microsatellite markers in 9 large Thai LHON G11778A pedigrees. Using an affecteds-only nonparametric linkage analysis, 4 regions on chromosomes 3, 12, 13 and 18 showed Zlr scores greater than 2 ($P < 0.025$), which is consistently significant across several linkage statistics. The most suggestive marker D3S1565 (Zlr > 2 in 10 of 16 allele sharing models tested) was then expanded to include the region 3q26.2–3q28 covering *SLC7A14* (3q26.2), *MFN1* (3q26.32), *MRPL47* (3q26.33), *MCCC1* (3q27.1), *PARL* (3q27.1) and *OPA1* (3q28–q29). All of these candidate genes were selected from the Maestro database and had known to be localized in mitochondria. Sixty tag SNPs were genotyped in 86 cases, 211 of their

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relatives and 32 unrelated Thai controls, by multiplex-PCR-based Invader assay. Analyses using a powerful association testing tool that adjusts for relatedness (the M_{QLS} statistic) showed the most evidence of association between two SNPs, rs3749446 and rs1402000 (located in *PARL* presenilins-associated rhomboid-like) and LHON expression (both $P = 8.8 \times 10^{-5}$). The mitochondrial PARL protease has been recently known to play a role with a dynamin-related OPA1 protein in preventing apoptotic events by slowing down the release of cytochrome *c* out of mitochondrial cristae junctions. Moreover, PARL is required to activate the intramembranous proteolyses resulting in the degradation of an accumulated pro-apoptotic protein in the outer mitochondrial membrane. Under these circumstances, variants of *PARL* are suggested to influence cell death by apoptosis which has long been believed to intrigue the neurodegeneration of LHON.

Introduction

Leber hereditary optic neuropathy (LHON) is a maternally inherited disorder of the optic nerves, causing subacute onset of bilateral centro-cecal scotoma and optic atrophy (Nikoskelainen et al. 1987). LHON is one of the most common causes of blindness in young men. The disease is associated with three primary mutations in mitochondrial DNA (mtDNA); G3460A, G11778A and T14484C, in genes coding for the ND1, ND4 and ND6 subunits of the respiratory chain enzyme complex I, respectively (Mackey et al. 1996; Man et al. 2002). Together, these mutations account for about 95% of all LHON cases. The G11778A mutation occurs in approximately 50% of European LHON cases (Man et al. 2003) but in approximately 95% of Japanese cases (Hotta et al. 1995). In Thailand, 98% of the LHON patients detected so far carry the G11778A mutation in their mtDNA (Phasukkijwatana et al. 2006).

Approximately 50% of male and 10% of female cases who harbor the homoplasmic G11778A mtDNA mutation expressed symptoms of optic neuropathy (Harding et al. 1995; Riordan-Eva et al. 1995). Mitochondrial background and environmental factors have been very much debated to be the factors precipitating LHON expression (Hudson et al. 2007; Kirkman et al. 2009; Qu et al. 2006).

In many Thai LHON pedigrees, multiple males of the same generation harbored the same homoplasmic mtDNA mutation and yet there was marked variation in penetrance. This phenomenon was also found in female patients (Phasukkijwatana et al. 2006). It is strongly suggested that their nuclear background plays an important role in the expression of LHON in these families. This study aims to search for novel nuclear susceptibility genes influencing the expression of LHON in our population.

Methods

LHON pedigrees

Samples from 51 LHON pedigrees, of Thai and/or Chinese origin apart from one of Indian origin, with the G11778A mtDNA mutation were included in this study. Pedigrees' subjects consisted of 297 individuals, including 86 cases (affected), 3 people of unknown phenotype, 127 maternal-lineage members who did not show any clinical symptoms of LHON but carried the G11778A mutation (unaffected), 7 individuals of maternal lineage but not carrying the G11778A mutation, and 74 individuals of non-maternal-lineage members. The clinical symptoms of LHON in all families were assessed by one neuro-ophthalmologist (WLC). All blood samples were collected with informed consent approved by Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. For children under 18 years of age, their consents were provided by their parents.

DNA extraction and mitochondrial DNA analysis

DNA was extracted from 5 ml of EDTA blood using the standard protocol. The G11778A mutation was genotyped in all samples, and heteroplasmy of the G11778A mutation was quantified using radioactive restriction analysis modified from Moraes (Moraes et al. 1992). In order to be certain that none of the nine pedigrees subjected to the genome-wide linkage scan have recently shared maternal ancestry, the hypervariable segment 1 (HVS-1) in the mtDNA D-loop (nt 16,024 to nt 16,383) from the proband of each family was sequenced.

Genome-wide linkage scan

A genome-wide linkage scan was performed on 91 samples from the 9 largest and most powerful families (Supplementary Fig. 1; Table 1). Forty-five affected samples (from 86 cases) and 46 informative relatives were recruited in order to gain as much information as possible about inherited haplotypes in the affected people. Each sample was genotyped with 400 microsatellite markers in the ABI Prism Linkage Mapping set. Microsatellite genotyping was carried out at the Australian Genome Research Facility (AGRF).

Statistical tests for misspecified relationships were performed on the genome-screen data using PREST (McPeek and Sun 2000). Mendelian genotyping errors were

Table 1 Details of 91 individuals from 9 pedigrees included in the genome-wide linkage scan

Pedigree	Homoplasmic persons		Heteroplasmic persons			Non-maternal lineage
	Affected	Unaffected	Affected	Unaffected	Unknown	
F1	3M	0	0	2F	0	2
F9	2M	1F	0	2M	1F	1
F11	5M, 1F	1M	0	0	0	0
F15	3M	1M, 5F	0	0	0	1
F18	3M, 5F	1M, 2F	0	0	0	0
F19	0	1M	2M, 2F	0	1F	2
F28	4M, 3F	2M, 3F	1M, 1F	1M, 3F	1F	2
F30	4M, 4F	2M, 4F	0	0	0	2
F36	2M	1F	0	0	0	1
Total	39	24	6	8	3	11

M male, *F* female

identified and removed using PedCheck version 1.1 (O'Connell and Weeks 1998). Merlin (Abecasis et al. 2002) was used to detect possibly erroneous genotypes which might be the cause of unlikely inferred double recombinants. Analyses were repeated both with and without these genotypes included.

Nonparametric linkage analysis

Multipoint nonparametric, affecteds-only linkage analysis was performed using Allegro version 1.2 (Gudbjartsson et al. 2000). In the primary analysis, the S_{pairs} statistic [pairs] was used with Kong and Cox's exponential test (exp) (Kong and Cox 1997), and with all families weighted equally (equal). However, given that so little is known about the models under which nuclear modifier genes act on LHON phenotypes, other linkage statistics, tests and weighting schemes were also explored. We used the S_{all} (all), S_{robdom} (robdom) and $S_{mnallele}$ (mnallele) statistics (McPeek 1999), Kong and Cox's linear test (lin), and the default family weighting scheme in Allegro where greater weight is given to contributions from larger families (largefam). Allele frequencies were estimated using genotypes of founders in the pedigrees.

Pedigree F28 was too large for analysis with Merlin and Allegro. For analysis with these programs, it was split into sub-pedigrees F28a (descended from individual II7) and F28b (descended from individual II10) (Supplementary Fig. 1).

Candidate genes and tag SNPs selection

The Maestro database (Calvo et al. 2006) was used to identify proteins which were imported or had been predicted to be imported into mitochondria. This resulted in six mitochondrial-localizing nuclear genes under the entire peak of D3S1565 (3q26.2–3q28), comprising *SLC7A14* (3q26.2), *MFN1* (3q26.32), *MRPL47* (3q26.33), *MCCC1* (3q27.1), *PARL* (3q27.1) and *OPA1* (3q28–q29). They were selected for the association study.

The SNP genotypes of those six candidate genes were downloaded for all SNPs with minor allele frequency (MAF) higher than 0.1 in the HapMap Han Chinese population (CHB). It was reported that the pattern of linkage disequilibrium (LD) of the CHB population was very similar to that of the Thai population with the percentage of coverage of tag SNPs at 93% with pairwise r^2 threshold of 0.8 (Mahasirimongkol et al. 2006). Therefore, the tag SNPs of CHB were useful markers to capture the LD structure for Thais in an indirect association study. The Tagger function implemented in Haploview (Barrett et al. 2005) was used to select tag SNPs for genotyping. More than 90% of alleles were captured by pairwise tagging with MAF greater than 0.1. In addition, we used the dbSNP database (http://www.ncbi.nlm.nih.gov/) to select all non-synonymous SNPs and all other SNPs in both 5′ and 3′ untranslated regions (UTR) with MAF higher than 0.1 and heterozygosity greater than 0.2 in the CHB population.

Multiplex-PCR-based Invader assay

Sixty tag SNPs were genotyped using the multiplex-PCR-based Invader assay (Third Wave Technologies, Madison, WI), a high-throughput SNP genotyping method developed under RIKEN Center for Genomic Medicine (Ohnishi et al. 2001). All primer sequences will be provided upon request to the authors. The sample was extended from 91 samples included in the genome-wide linkage scan to 329 individuals comprising 86 affected and 137 maternal relatives, 74 individuals of non-maternal lineage in the pedigrees, and 32 unrelated controls. Genotype signals were detected by Sequence Detection System (SDS) version 2.1, ABI7900

Sequence Detector (Applied Biosystems, Foster, CA), and the threshold for correcting call rates in quality control was set to 95% or greater.

Association analysis

To test for differences in SNP allele frequencies between cases and other members of the Thai population, we used the M_{QLS} (a more powerful quasi-likelihood score) statistic. This method aims to maintain the correct type I error rate by using kinship coefficients to account for relatedness in the sample, while improving the power to detect association relative to conventional family-based tests (Thornton and McPeek 2007). Kinship coefficients and phenotypes (affected or unaffected) were used to assign different weights to different individuals depending on pedigree structure. These weights reflect the fact that: (1) individuals with genotyped relatives in the sample provide less independent information for allele frequency estimation than individuals that have no genotyped relatives, (2) affected individuals with affected relatives are likely to be enriched for susceptibility alleles compared to affected individuals who do not have affected relatives, and (3) individuals with unknown phenotype have a higher expected frequency of disease-susceptibility alleles than individuals who are unaffected.

Individuals with no symptoms of LHON were classed as "definitely unaffected" if they were homoplasmic for the G11778A mutation and older than 30 years (males) or 45 years (females). These ages are one standard deviation above the average ages of onset of LHON in males and females, respectively (Phasukkijwatana et al. 2006). All other individuals with no symptoms of LHON were classed as "unknown". Given the marked sex differences in the penetrance of the G11778A mutation, we also performed subsidiary, sex-specific association analyses. All individuals of the opposite sex were assigned a phenotype of "unknown" in these analyses. For each analysis, a best linear unbiased estimate (BLUE) of allele frequency was calculated for cases and for controls ("definitely unaffected" and "unknown" combined) along with 95% confidence intervals (McPeek et al. 2004).

For significant SNPs ($P < 0.05$) identified in the M_{QLS} analysis, odds ratios were calculated with 95% confidence intervals in R version 2.7.1 (http://cran.r-project.org). A SNP spectral decomposition analysis (SNPSpD) (Nyholt 2004) was used to estimate the number of independent tests being performed and to correct for multiple testing. Merlin (Abecasis et al. 2002) was used to detect Mendelian genotyping errors, and 70 non-maternal-lineage members were used to test for deviations from Hardy–Weinberg equilibrium.

Results

Pedigrees

Details of the pedigrees and samples included in the genome-wide linkage scan are shown in Supplementary Fig. 1 and Table 1. Family 19 had been found to carry two genetic diseases, LHON and FSHD (facioscapulohumeral muscular dystrophy) (Chuenkongkaew et al. 2005). There was no apparent interaction between the two diseases for the clinical and molecular investigation in this family.

Nonparametric linkage analysis

A total of 55 Mendelian inheritance errors were detected using Pedcheck, and a total of 82 genotypes were recoded as missing in order to remove those errors. Including these 82 genotypes, there was a total of 344 missing genotypes (0.94%). A further 11 genotypes were flagged as possibly erroneous by double recombinant analysis with Merlin.

Figure 1 shows a plot of Zlr scores using the exp pairs equal model in Allegro. There were 4 regions where Zlr exceeded 2 ($P < 0.025$): at markers D3S1565 (178 cM), D12S352 (7.6 cM), D13S1265 (114 cM) and D18S68 (89 cM). Several comparisons to other allele sharing models were explored (Table 2). Zlr exceeded 2 at the same 4 regions in more than 5 out of 16 models. Thus, we considered these four regions worthy of further investigation, along with two other following regions. The first was the peak of Zlr > 2 observed at marker D1S207 in chromosome 1 under a number of models. The linkage signal in this region is driven primarily by family F30 (Supplementary Table 1). The second was the peak at marker DXS1227 in the X chromosome, which has long been controversial in male predilection for LHON (Bu and Rotter 1991; Hudson et al. 2005; Vilkki et al. 1991). The removal of 11 likely genotyping errors (causing double recombination) reported by Merlin did not have much effect on the results and there were no additional Zlr scores of more than 2. However, there were 2 markers, D9S287 and DXS8091, where the Zlr dropped to lower than 2 (Table 2). The contribution from each family to the linkage peak in each of the six interesting candidate regions is shown in Supplementary Table 1. As the marker D3S1565 showed high Zlr the most consistently across many models testing, it was regarded as the most suggestive marker to search for nuclear modifiers in this study.

Association analysis

A total of 297 LHON family members (86 LHON affected and 211 of their relatives) together with 32 unrelated individuals were genotyped for 60 tag SNPs of 6 nuclear genes

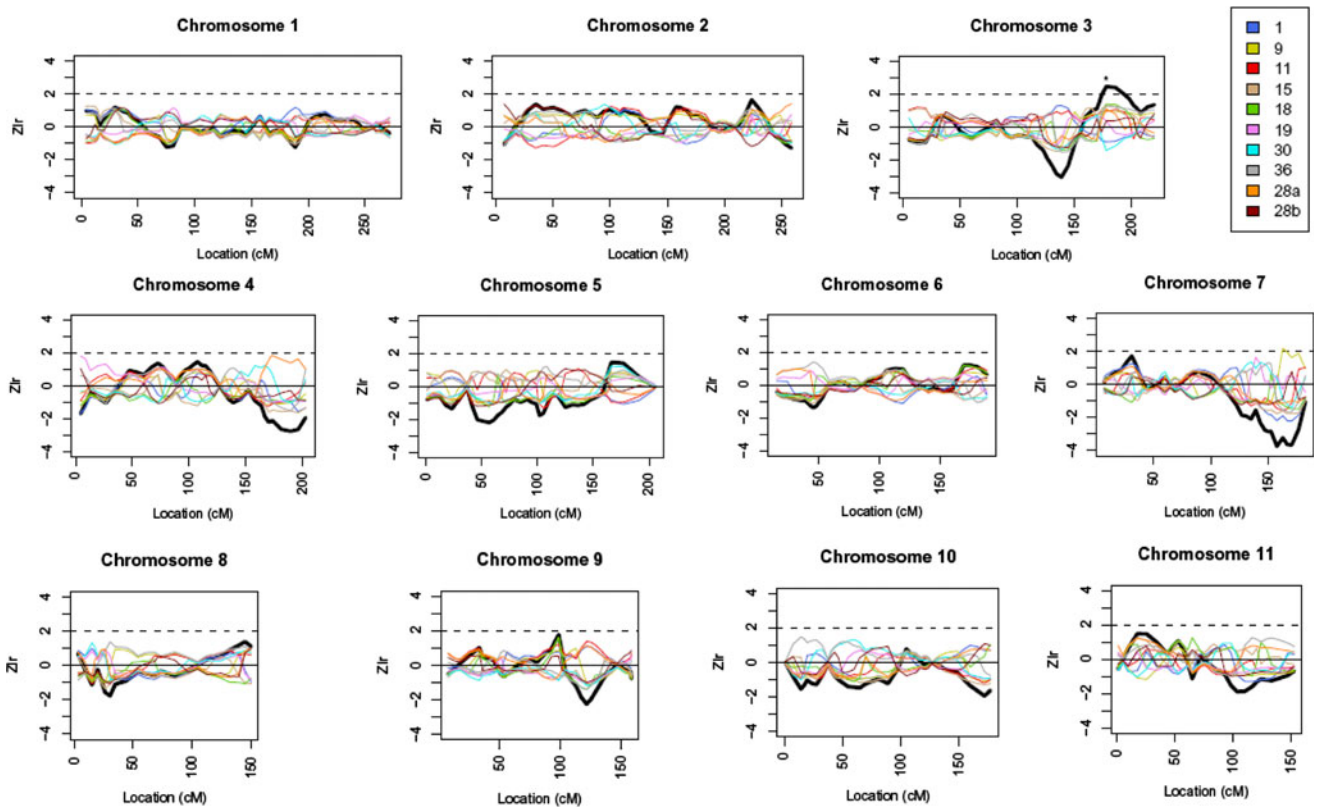

Fig. 1 Zlr curves plotted against location (cM) for each chromosome from the analysis without removing the errors reported by Merlin, using exp pairs equal model. The *asterisks* indicate the positions of the interesting peaks with Zlr of more than 2. The *thick black lines* are Zlr from the overall families. The *thin lines* are family specific Zlr curves as indicated in the legend

comprising; *SLC7A14* (3q26.2), *MFN1* (3q26.32), *MRPL47* (3q26.33), *MCCC1* (3q27.1), *PARL* (3q27.1) and *OPA1* (3q28–q29), that underlie the entire peak of D3S1565 (3q26.2–3q28). All of these candidate genes were selected from the Maestro database and have been confirmed experimentally to be localized in mitochondria. Four subjects (3 affected and 1 maternal lineage) were first excluded due to their problematic occurrences with DNA quality. Therefore, a total of 325 subjects, comprising 83 LHON affected, 210 of their relatives and 32 unrelated individuals were used for further analyses. Three SNPs were discarded from further analysis: rs6767450 (*SLC7A14*) showed a high number of Mendelian inconsistencies (14 erroneous), rs4855096 (*MRPL47*) deviated significantly from HWE ($P = 4 \times 10^{-11}$) and rs13083369 (*MFN1*) was monomorphic in the Thai population. In the remaining 57 SNPs, 6 genotypes (0.04%) were recoded as missing due to Mendelian inconsistencies. The genotyping success rate of multiplex-PCR-based Invader assay was 99.8% (28 out of 16,701 genotypes failed). Details of genotyping tag SNPs were provided in Supplementary Table 2.

Using the M_{QLS} statistic, a strongly significant association was found for two intronic SNPs in *PARL*: rs1402000 and rs3749446 (both $P = 8.8 \times 10^{-5}$). These two SNPs were in linkage disequilibrium (LD) ($D' = 1$, $r^2 = 0.768$). Another nine variants in *SLC7A14*, *MFN1*, *MCCC1* and *PARL* were also nominally associated with LHON by mode of recessive inheritance ($P < 0.05$, Table 3). The significance level under a number of related markers was adjusted by the SNP spectral decomposition analysis (SNPSpD) (Nyholt 2004). While 57 marker loci were tested, the effective number of independent tests was 37.6, thus providing a significance threshold for a 5% type I error rate 0.00136, after adjusting for multiple comparisons. Therefore, only rs1402000 and rs3749446 of *PARL* remained significant after performing multiple corrections ($P < 0.001$). In addition, there was no association for any of the non-synonymous SNPs previously reported in the dbSNP database: rs10513762 (*MRPL47*, H213R), rs2270968 (*MCCC1*, P464H), rs3732581 (*PARL*, L212V) and rs7624750 (*OPA1*, N158S) (data not shown).

In sex stratified analyses, most of the nominally significant SNPs showed stronger association in males; except for rs6799974 and rs9880460 of *SLC7A14*, which were nominally significant in females (Table 4). The rs1402000 and rs3749446 of *PARL* still showed significant association in the male group after adjusting for multiple testing ($P = 9.3 \times 10^{-4}$). Three additional SNPs (rs2287312, *MFN1*; rs12631031, *PARL* and rs6797542, *OPA1*) were nominally significant in the

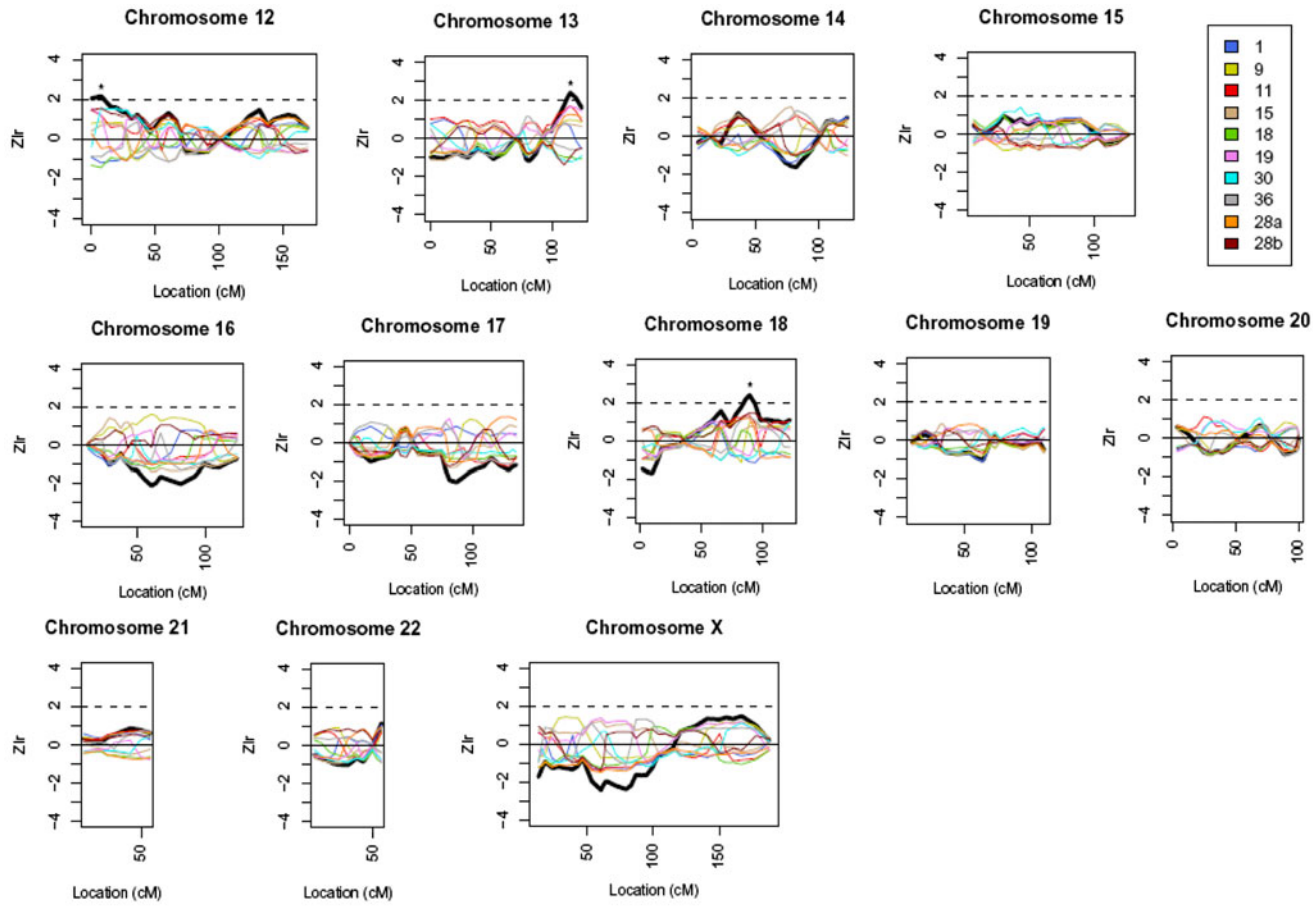

Fig. 1 continued

male group ($P < 0.05$), while 2 SNPs (rs876560, *SLC7A14* and rs11720405, *MFN1*) lost the association in the sex-specific analyses. However, it should be noted that the limited number of subjects could effect this deviation.

Discussion

Using nine large Thai families, we have performed the first genome-wide linkage scan for LHON. We found a number of linkage peaks (Zlr > 2, corresponding to $P < 0.025$), and proposed that the six most promising regions worthy of follow-up are those on chromosomes 1, 3, 12, 13, 18, and X, as indicated more precisely in Table 2. None of the peaks attained the thresholds proposed by Lander and Kruglyak (1995) for declaring significant or suggestive linkage. However, those authors also supported the reporting of all regions with *P* values less than 0.05 in genome-wide linkage scans for complex diseases. Evidence from this study and previous studies suggest that the nuclear genetic architecture of LHON susceptibility is complex (Brown 1999; Howell 1998).

An X-linked susceptibility locus was first identified at the microsatellite DXS7 (Xp11.3) in Finnish pedigrees (Vilkki et al. 1991). Subsequent studies in different populations identified other susceptibility loci on the X chromosome, suggesting genetic heterogeneity between different ethnic groups (Hudson et al. 2005; Shankar et al. 2008). Interestingly, our X-linked locus study showed some linkage to the marker DXS1227 (Zlr > 2), which is close to the novel susceptibility locus for LHON reported recently in a large Brazilian pedigree (Shankar et al. 2008). However, the two closest genes, *CDR1* and *LDOC1*, did not show evidence of association with LHON.

The most significant linkage peak was at the microsatellite marker D3S1565, making this region a high priority for follow-up in a screen of nuclear modifier genes. We hypothesized that the most likely candidates in this region were six genes encoding proteins which are imported into mitochondria. Sixty SNPs were selected to tag common variation in these six genes, and the SNPs were genotyped

Table 2 Chromosomal regions showing Zlr > 2 in 16 allele sharing models

Allele sharing models			Zlr in the chromosomal region where Zlr > 2																						
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
			D1S207	D2S126	D3S1565	D4S406			D7S507		D9S287			D12S352	D13S1265	D14S70	D15S1007			D18S68					DXS1227
			107 cM	233 cM	178 cM	115 cM			32 cM		99 cM			7.6 cM	114 cM	36 cM	29 cM			89 cM					150 cM
exp	pairs	0.5			2.09						2.01[a]			2.2						2.06					
exp	all	0.5	2.32																						
exp	robdom	0.5	2.51																						
exp	mnallele	0.5			2.61									2.3	2.34					2.18					2.07[a,b]
exp	pairs	equal			2.47									2.16	2.4					2.4					
exp	all	equal													2.2										
exp	robdom	equal																							
exp	mnallele	equal			2.24									2.87	2.11										2.42[a,b]
lin	pairs	0.5			2.11															2.17					
lin	all	0.5															2.26								
lin	robdom	0.5	2.01														2.3								
lin	mnallele	0.5			2.2										2.08										2.2
lin	pairs	equal			2.42				2.04						2.37					2.45					
lin	all	equal		2.03	2.3									2.06	2.48					2.45					
lin	robdom	equal		2.18	2.01	2.07									2.51	2.02				2.28					
lin	mnallele	equal			2.28										2.2					2.04					2.13

[a] Zlr dropped below 2 when 11 most likely errors detected by Merlin were removed

[b] Peak at DXS 8091 (167 cM)

Table 3 Association of nominally significant SNPs analyzed by M_{QLS} statistic in G11778A LHON

RefSNP ID	Location	All cases (affected = 83, unaffected = 36, unknown = 206)[a]			Odds ratio (95% CI)	
		$\hat{P}_{case}$ (95%CI)	$\hat{P}_{control}$ (95%CI)	M_{QLS} *P* value	No. of risk allele	
					1	2
SLC7A14	**3q26.2**					
rs6799974	intron2	0.48 (0.39, 0.57)	0.37 (0.22, 0.51)	0.033232	1.01 (0.56, 1.82)	2.69 (1.34, 5.39)
rs876560	intron1	0.74 (0.66, 0.82)	0.64 (0.49, 0.78)	0.039067	2.43 (0.68, 8.75)	2.16 (0.61, 7.64)
rs9880460	intron1	0.64 (0.55, 0.72)	0.50 (0.35, 0.65)	0.00913	1.48 (0.68, 3.22)	2.04 (0.93, 4.50)
MFN1	**3q26.32**					
rs2287312[b]	intron3	–	–	NS	0.60 (0.25,1.43)	1.11 (0.47, 2.60)
rs6804758	intron3	0.63 (0.55, 0.72)	0.51 (0.36, 0.66)	0.013569	1.36 (0.65, 2.82)	2.15 (1.02, 4.52)
rs11720405	3′ near gene	0.32 (0.24, 0.41)	0.29 (0.15, 0.43)	0.036046	1.76 (1.03, 2.99)	1.76 (0.73, 4.21)
MCCC1	**3q27.1**					
rs10513790	intron1	0.54 (0.45, 0.63)	0.42 (0.27, 0.57)	0.007733	0.84 (0.44, 1.60)	1.23 (0.63, 2.40)
rs937652	exon1 (5′UTR)	0.32 (0.23, 0.40)	0.22 (0.09, 0.35)	0.002466	1.13 (0.65, 1.95)	1.93 (0.93, 4.03)
PARL	**3q27.1**					
rs12631031[b]	intron4	–	–	NS	1.13 (0.66, 1.96)	1.49 (0.72, 3.07)
rs1402000	intron2	0.86 (0.80, 0.93)	0.76 (0.63, 0.89)	**0.000088**[c]	0.81 (0.20, 3.19)	3.19 (0.91, 11.15)
rs953419	intron1	0.58 (0.49, 0.67)	0.43 (0.28, 0.58)	0.007532	1.65 (0.84, 3.26)	2.78 (1.34, 5.75)
rs3749446	intron1	0.86 (0.80, 0.93)	0.76 (0.63, 0.89)	**0.000088**[c]	1.33 (0.27, 6.45)	4.07 (0.91, 18.12)
rs3792588	5′ near gene	0.57 (0.48, 0.66)	0.43 (0.28, 0.58)	0.013974	1.78 (0.90, 3.49)	2.63 (1.26, 5.47)
OPA1	**3q28–q29**					
rs6797542[b]	intron29	–	–	NS	2.10 (1.23, 3.56)	0.92 (0.19, 4.50)

NS not significant ($P > 0.05$), $\hat{P}_{case}$ is the best linear unbiased estimate (BLUE) for allele frequency in cases (affected), with 95% confidence intervals (95% CI), $\hat{P}_{control}$ is the BLUE for allele frequency in controls ("definitely unaffected" and "unknown" combined)

[a] Totally 325 individuals were included in the M_{QLS} analysis. There were 83 affected (64 males and 19 females), 36 unaffected (13 males and 23 females) and 206 unknown phenotypes. The "unaffected" phenotype was asymptomatic individual who was homoplasmic for G11778A and was older than 30 years for male or 45 years for female (definitely unaffected); otherwise, an asymptomatic individual was classified as "unknown" phenotype (see "Association analysis" in "Methods")

[b] M_{QLS} statistic was nominally significant ($P < 0.05$) when the analysis was subsidiary by sex (see results in Table 4)

[c] *P* value was still significant after adjusting for multiple testing on related markers by SNPSpD (threshold significant *P* value = 0.001)

as described in the results section. Two intronic SNPs, rs3749446 and rs1402000, in the presenilins-associated rhomboid-like (*PARL*) gene showed strongly significant association: rs3749446 located 157 nucleotides upstream of exon 2, and rs1402000 located 112 nucleotides downstream of exon 2. Using the Tagger function in Haploview with pairwise r^2 threshold of 0.5, 3 tag SNPs were identified in the same LD block as the two associated SNPs: rs11918588 (intron 1), rs6782942 (intron 1) and rs12634358 (intron 4). The bioinformatics tools, Transcription Element Search System (TESS) (Schug 2003) and RegRNA (http://regrna.mbc.nctu.edu.tw/html/prediction.html), were used to predict whether any of the SNPs affected transcriptional regulatory sites. Single base changes from C to A of rs11918588 and from G to T of rs6782942 resulted in loss of predicted binding sites for the Nova-1 protein. This protein is a neuronal-specific RNA binding protein (RBP) which is involved in alternative RNA splicing. RNA splicing abnormalities caused by this protein have been implicated in several neurological disorders (Dredge and Darnell 2003; Dredge et al. 2001). There was no predicted functional effect of the rs3749446 SNP. However, the SNPs rs1402000 (C/T) and rs12634358 (A/G) alter the TTT sequence which is known to act as a *cis*-acting element for neuron-specific splicing of human amyloid precursor protein (APP) (Shibata et al. 1996). These data suggest that these intronic SNPs may play a role in neuron-specific splicing. However, more evidence is needed to implicate them in the neurodegeneration associated with LHON.

Evidence showing the correlation between mitochondrial fusion dynamics and apoptosis suggests that the mechanism of cell death signaling from mitochondria exists (Chan 2006; Cipolat et al. 2006; Frezza et al. 2006; Gottlieb 2006; Pellegrini and Scorrano 2007). Mitochondrial GTPases mitofusin 1 (MFN1) and optic atrophy 1 (OPA1) proteins played a role in mitochondrial fusion machinery (Zhang and Chan 2007). OPA1 could inhibit

Table 4 Sex-specific association analyzed by M_{QLS} statistic

RefSNP ID	Location	Male cases only			Female cases only		
		(affected = 64, unaffected = 13, unknown = 248)[a]			(affected = 19, unaffected = 23, unknown = 283)[a]		
		$\hat{P}_{case}$ (95%CI)	$\hat{P}_{control}$ (95%CI)	M_{QLS} *P* value	$\hat{P}_{case}$ (95%CI)	$\hat{P}_{control}$ (95%CI)	M_{QLS} *P* value
SLC7A14	**3q26.2**						
rs6799974	intron2	–	–	NS	0.60 (0.40, 0.79)	0.35 (0.18, 0.52)	0.03264
rs9880460	intron1	–	–	NS	0.71 (0.52, 0.89)	0.45 (0.28, 0.63)	0.017669
MFN1	**3q26.32**						
rs2287312	intron3	0.70 (0.61, 0.78)	0.52 (0.30, 0.74)	0.017158	–	–	NS
rs6804758	intron3	0.62 (0.53, 0.71)	0.43 (0.21, 0.65)	0.029351	–	–	NS
MCCC1	**3q27.1**						
rs10513790	intron1	0.55 (0.45, 0.64)	0.48 (0.26, 0.70)	0.030467	–	–	NS
rs937652	exon1 (5′UTR)	0.32 (0.23, 0.40)	0.27 (0.07, 0.46)	0.020662	–	–	NS
PARL	**3q27.1**						
rs12631031	intron4	0.41 (0.32, 0.51)	0.22 (0.04, 0.40)	0.019508	–	–	NS
rs1402000	intron2	0.85 (0.78, 0.92)	0.68 (0.47, 0.88)	**0.000934**[b]	–	–	NA
rs953419	intron1	0.58 (0.49, 0.68)	0.36 (0.15, 0.57)	0.002675	–	–	NS
rs3749446	intron1	0.85 (0.78, 0.92)	0.68 (0.47, 0.88)	**0.000934**[b]	–	–	NA
rs3792588	5′ near gene	0.57 (0.48, 0.66)	0.36 (0.15, 0.57)	0.00708	–	–	NS
OPA1	**3q28–q29**						
rs6797542	intron29	0.20 (0.12, 0.28)	0.12 (−0.02, 0.27)	0.048443	–	–	NS

NS not significant ($P > 0.05$), *NA* M_{QLS} test was not appropriate since a low risk allele did not occur in female cases, $\hat{P}_{case}$ is the best linear unbiased estimate (BLUE) for allele frequency in cases (affected), with 95% confidence intervals (95% CI), $\hat{P}_{control}$ is the BLUE for allele frequency in controls ("definitely unaffected" and "unknown" combined)

[a] For subsidiary sex-specific association analyses by M_{QLS} in "Male cases only", of 325 individuals, there were 64 affected, 13 unaffected and 248 unknown phenotypes. In "Female cases only", of 325 individuals, there were 19 affected, 23 unaffected and 283 unknown phenotypes. The "unaffected" phenotypes of either male or female were assigned as described in Table 3. In each sex-specific analysis, the individuals of the opposite sex were classified as "unknown" (see "Association analysis" in "Methods")

[b] *P* value was still significant after adjusting for multiple testing on related markers by SNPSpD (threshold significant *P* value = 0.001)

apoptosis through the mitochondrial membrane protease activity of PARL. To be more precise, the short soluble form of OPA1 is released by cleavage of PARL in the inner membrane, which would slow down the release of cytochrome *c* and thus lessen the apoptotic signals in the cells (Cipolat et al. 2006; Jeyaraju et al. 2006). *OPA1* has recently been identified as a disease gene for autosomal dominant optic atrophy (ADOA). The disease has a very similar clinical phenotype to LHON and is specifically expressed in retinal ganglion and nerve cells (Heiduschka et al. 2010; Ju et al. 2005; Pesch et al. 2004). While there have been limited studies of *PARL* expression in the retina, expression of Rhomboid-7 (*rho-7*) (an ortholog of human *PARL*) has been shown to cause severe neurodegeneration and reduced the lifespan in mutant *D. melanogaster* (Lessing and Bonini 2009; McQuibban et al. 2006).

Another action of PARL is to prevent the induction of apoptosis when cytokines are limiting in lymphocytes and neurons (Chao et al. 2008). Together with Hax1, a Bcl-2 family-related protein, PARL could activate HtrA2 protease to prevent the accumulation of the pro-apoptotic Bax through an unknown mechanism. The anti-apoptotic role of mitochondrial protease PARL suggests a new keeper lid of mitochondrial apoptotic cell death. Variants of PARL might modify sensitivity of retinal ganglion cells to apoptosis in LHON progression. Recently, published data suggested that the *PARL* SNP rs3792589 affects mitochondrial content (Curran et al. 2009). In relation to this, an increase of mtDNA content has been observed in both G11778A patients and asymptomatic carriers (Yen et al. 2002) but increased up to certain levels in T14484A carriers (Nishioka et al. 2004). This provides indirect supportive evidence of an association between PARL and LHON. However, the SNP rs3792589 is not polymorphic in either the Han Chinese or our population. Instead we found suggestive evidence for association between LHON and rs3792588 (Tables 3, 4) which is located in the promoter region and only six base pairs away from rs3792589.

This study has highlighted the importance of PARL in mitochondrial function as well as in the pathophysiology of mitochondrial diseases. We hypothesize that variation in PARL may disturb the normal function of mitochondria

which could favor the apoptosis of retinal ganglion cells and lead to the neurodegeneration in LHON. Our present data led us to suggest that PARL would be one of the nuclear modifier(s) for the expression of LHON. Although this study has provided the first evidence of autosomal nuclear modifiers in LHON, a replication study or a functional study of PARL in LHON is needed in order to confirm these results.

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Appendix

For association testing using related individuals with the M_{QLS} statistics refer http://www.stat.uchicago.edu/~mcpeek/software/MQLS/index.html. For bioinformatics tools to predict the transcription element binding sites refer http://www.cbil.upenn.edu/cgi-bin/tess/tess; http://regrna.mbc.nctu.edu.tw/html/prediction.html.

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