Mutation analysis of Leber's hereditary optic neuropathy using a multi-gene panel

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Abstract. The present study investigates the spectrum and incidence of mitochondrial DNA (mtDNA) mutations associated with Leber's hereditary optic neuropathy (LHON) in a Han population using a multi-gene panel with 46 LHON-associated mutations among 13 mitochondrial genes. A total of 23 mutations were observed in a cohort of 275 patients and 281 control subjects using multi-gene panel analysis. The causative mutations associated with LHON were identified to be m.11778G>A, m.14484T>C, m.3460 G>A, m.3635G>A, m.3866T>C and m.3733G>A, responsible for 70.55% cases in the patient cohort. The secondary mutations in the Chinese LHON population were m.12811T>C, m.11696 G>A, m.3316G>A, m.3394T>C, m.14502T>C, m.3497C>T, m.3571C>T, m.12338T>C, m.14693A>G, m.4216T>C and m.15951A>G, with incidences of 5.09, 4.36, 4.00, 4.00, 4.00, 2.55, 1.82, 1.82, 1.45, 1.09 and 1.09%, respectively. Besides three hotspot genes, *MT-ND1*, *MT-ND4* and *MT-ND6*, *MT-ND5* also had a high incidence of secondary mutations. Those mutations reported as rare causative mutations in a European LHON population, m.3376G>A, m.3700G>A and m.4171C>A, m.10663T>C, m.13051G>A, m.14482C>G/A, m.14495A>G and m.14568C>T were undetected in the present study. The primary and secondary mutations associated with LHON in the present multi-gene panel will advance the current understanding of the clinical phenotype of LHON, and provide useful information for early diagnosis.

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Abbreviations: LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA

Key words: Leber's hereditary optic neuropathy, mitochondrial DNA, causative mutation, multi-gene panel, gene-target sequencing

Introduction

Leber's hereditary optic neuropathy (LHON; OMIM 535000) is a classic mitochondrial disease, associated with a rapid, painless, acute or sub-acute bilateral visual loss in young adults, predominantly caused by the primary and secondary mutations in mitochondrial DNA (mtDNA). It has been reported that 1:8,500 individuals harbor a primary LHON-causing mutation and 1:31,000 experience visual loss as a result of LHON in the North East of England (1). Few significant improvements in visual acuity are reported following atrophy of the optic discs. LHON typically affects males more frequently than females, with the incomplete and variable penetrance estimated at ~50% in males and 10% in females (2-4). Additionally, certain LHON cases have additional clinical symptoms, such as movement disorders, dystonia, and multiple-sclerosis-like illness, which complicate the diagnosis in the clinical setting (5-7). Although the majority ofcases of LHON transmitted by maternal inheritance have a history of visual loss in families, up to 40% of cases are sporadic (5).

The genetic cause of LHON is mutations in the mitochondrial genome, which is a double-stranded 16,569-nucleotide pair, circular molecule, consisting of one D-Loop region and 37 genes. The three most causative mutations, m.11778G>A (*MT-ND4*), m.14484T>C (*MT-ND6*) and m.3460G>A (*MT-ND1*), have been reported to account for 90% of LHON patients in a Caucasian population, but for only 38.3 and 46.5% of cases in two large cohorts of Chinese Han subjects with LHON (7-10). Our previous studies have shown the spectrum of genes, *MT-ND1*, *MT-ND4* and *MT-ND6*, and the frequency of the three primary mutations in a Chinese LHON population (8-10) using Sanger sequencing. In addition, secondary mutations that contributed to the high penetrance, including m.3394T>C (*MT-ND1*), m.11696G>A (*MT-ND4*), m.12338T>C (*MT-ND5*) and m.15951A>G (*MT-TT*) areusually synergized with m.11778G>A or m.14484T>C or m.3460G>A (11). According to Mitomap (http://www.mitomap.org/), >40 point

mutations in mtDNA are associated with LHON, of which the incidence varies between different ethnic backgrounds.

To further understand the spectrum of mutations associated with LHON in a Chinese population, 46 LHON-associated mutations distributed among 13 mitochondrial genes were selected from Mitomap, and multi-gene target sequencing was performed in 275 cases of LHON as well as in 281 Chinese control subjects to distinguish the most frequent mtDNA mutations associated with LHON in the Han population.

Materials and methods

DNA samples, extraction, quantification and quality control. A total of 275 unrelated LHON samples and 281 Chinese control samples were enrolled from the ophthalmology clinics at Zhejiang University School of Medicine (Hangzhou, China) and Wenzhou Medical College (Wenzhou, China) between 2004 and 2015, as described previously (8-10,12), under protocols approved by Zhejiang University and Wenzhou Medical University Ethics Committees. DNA was extracted from 1 ml peripheral blood using a QIAamp DNA Blood Minikit (51106; Qiagen China Co., Ltd., Shanghai, China). The quality and quantity of DNA were assessed using Qubit 3.0 fluorometers (Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA samples with concentration >1.0 ng/μl were employed in the sequencing experiments.

Multi-genepanel design. Multi-gene target sequencing was performed using the VariantPro™ Capture Technology by LC Sciences (Hangzhou, China) as described previously (13). The 46 LHON-associated mutations were selected from Mitomap and previous studies (8-10). As presented in Table I, they were distributed in the following 13 genes: *MT-ND1*, *MT-ND2*, *MT-ATP6*, *MT-CO3*, *MT-ND3*, *MT-ND4L*, *MT-ND4*, *MT-ND5*, *MT-ND6*, *MT-CYB*, *MT-TM*, *MT-TT* and *MT-TE*. Twenty-seven amplicons that covered all 46 mutations were designed by LC Sciences, as described previously (13). All amplicons were pooled into two polymerase chain reaction (PCR) tubes (tube 1 and tube 2) as VariantPro™ PCR mastermix with an average length of 184 nt (range, 167-203 nt).

Library preparation and sequencing. Library generation was performed according to the manufacturer's protocol (LC Sciences). Briefly, 5 ng DNA per pool was amplified in 25 cycles of PCR using probe sequences of 27 library amplicons (Table II) and a VariantPro™ PCR mastermix as described previously (13). PCR runs of 18, 20, 24 and 26 cycles were performed to evaluate the influence of the PCR cycles on the experiment. The correlation coefficient, R^2, ranged from 0.90-1.00 (average, 0.95), implicating that cycles between 18 and 26 had no influence on the experiment outcome. The amplified products were purified using Agencourt AMPure XP beads [Beckman Coulter (UK) Ltd., High Wycombe, UK]. Each library was diluted to 20 pM and sequenced on an Illumina Miseq with a minimum of 2X 150-bp paired-end reads.

Data analysis. Low quality reads (reads containing sequencing adaptors or nucleotides with quality scores <20) were removed before alignment. Cleaned, paired-end sequence reads in paired FASTQ files were aligned using Burrows-Wheeler Alignment version 0.1.19 (14). Variant calling was generated using the Genome Analysis Toolkit version 3.3.0 and its Unified Genotyper module (https://www.broadinstitute.org/gatk/guide/tagged?tag=unifiedgenotyper). A Gaussian mixture model was used to evaluate the confidence score for each putative mutation call and novel potential variants. Sequence reads were aligned to the human mtDNA sequence data relative to the revised Cambridge Reference Sequence (GenBank accession no. NC_012920) (15).

Sanger validation. Thirteen LHON cases associated with m.11778G>A (11) or m.14484T>C (2) mutations and four healthy control samples (available upon request) were selected as the positive and negative controls, respectively, for runs of the panel following validation by Sanger sequencing. Furthermore, 100% correlation was derived from the panel assay and the Sanger sequencing for the positive and negative controls.

Results

Summary of sequencing data. A total of 118 milion reads were obtained, on average 89% of which were mapped to the amplicon targets and resultedin a mean of 7,001 reads to each sample per amplicon (Table III). The mean reads over the 27 amplicons were distrubed with an average uniformity of coverage of 98.0% (amplicon mean coverage, 20%) and an average read-depth of 1,000 X (Fig. 1). A total of 363 variants were distributed in 46 LHON-associated mutations of all samples.

Mutations analysis. A total of 363 variants were identified in the cohort of all 556 samples; 285 variants were detected in LHON cases as an average incidence of 104%, whereas only 78 variants were identified in 281 controls with a mean incidence of 28% (Table IV).

As 46 mutations of 13 mitochondrial genes were selected from all populations of the world, variants from LHON cases in the current study were deposited in 8 mitochondrial genes, *MT-ND1*, *MT-CO3*, *MT-ND4*, *MT-ND5*, *MT-ND6*, *MT-CYB*, *MT-TT* and *MT-TE*, with frequencies of 16.14, 0.35, 61.40, 6.67, 12.63, 0.35, 1.05 and 1.40%, respectively. Consistent with our previous reports (8-10), *MT-ND1*, *MT-ND4* and *MT-ND6* were the hotspots associated with LHON, and almost cover 90% of variants in the present study.

Twenty-three out of the 46 LHON-associated mutations were detected in all subjects. These were as follows: 3316G>A, 3394T>C, 3460G>A, 3497C>T, 3571C>T, 3635G>A, 3733G>A, 3866T>C, 4025C>T and 4216T>C mutations in *MT-ND1*, 9804G>A in *MT-CO3*, 11253T>C, 11696G>A and 11778G>A in *MT-ND4*, m.12338T>C and 12811T>C in *MT-ND5*, 14325T>C, 14484T>C and 14502T>C in *MT-ND6*, 14831G>A and 15812G>A in *MT-CYB*, 15951A>G in *MT-TT* and 14693A>G in *MT-TE*. The incidence of these three common mutations m.11778G>A, m.14484T>C and m.3460G>A in this Chinese cohort were 58.90, 9.10 and 0.73%, respectively. In addition, two causative mutations were detected; m.3635G>A in 1 patient and m.3866T>C in 4 patients (one case carrying both m.11778G>A and m.3866T>C mutations), as reported

Table I. Mutations in the multi-gene panel (n=46).

Index	Gene name	Var start	Var end	Ref allele	Var allele	Amino acid change
1	*MT-ND1*	3316	3316	G	A	A-T
2	*MT-ND1*	3376	3376	G	A	E-K
3	*MT-ND1*	3394	3394	T	C	Y-H
4	*MT-ND1*	3460	3460	G	A	A-T
5	*MT-ND1*	3497	3497	C	T	A-V
6	*MT-ND1*	3571	3571	C	T	L-F
7	*MT-ND1*	3635	3635	G	A	S-N
8	*MT-ND1*	3700	3700	G	A	A-T
9	*MT-ND1*	3733	3733	G	A	E-K
10	*MT-ND1*	3866	3866	T	C	I-T
11	*MT-ND1*	4025	4025	C	T	T-M
12	*MT-ND1*	4171	4171	C	A	L-M
13	*MT-ND1*	4216	4216	T	C	Y-H
14	*MT-ND2*	4640	4640	C	A	I-M
15	*MT-ND2*	5244	5244	G	A	G-S
16	*MT-ATP6*	9101	9101	T	C	I-T
17	*MT-CO3*	9804	9804	G	A	A-T
18	*MT-ND3*	10237	10237	T	C	I-T
19	*MT-ND4L*	10663	10663	T	C	V-A
20	*MT-ND4L*	10680	10680	G	A	A-T
21	*MT-ND4*	11253	11253	T	C	I-T
22	*MT-ND4*	11696	11696	G	A	V-I
23	*MT-ND4*	11778	11778	G	A	R-H
24	*MT-ND5*	12338	12338	T	C	M-T
25	*MT-ND5*	12811	12811	T	C	Y-H
26	*MT-ND5*	12848	12848	C	T	A-V
27	*MT-ND5*	13051	13051	G	A	G-S
28	*MT-ND5*	13528	13528	A	G	T-A
29	*MT-ND5*	13637	13637	A	G	Q-R
30	*MT-ND5*	13730	13730	G	A	G-E
31	*MT-ND6*	14279	14279	G	A	S-L
32	*MT-ND6*	14325	14325	T	C	N-D
33	*MT-ND6*	14482	14482	C	A	M-I
34	*MT-ND6*	14482	14482	C	G	M-I
35	*MT-ND6*	14484	14484	T	C	M-V
36	*MT-ND6*	14495	14495	A	G	L-S
37	*MT-ND6*	14498	14498	T	C	Y-C
38	*MT-ND6*	14502	14502	T	C	I-V
39	*MT-ND6*	14568	14568	C	T	G-S
40	*MT-ND6*	14596	14596	A	T	I-M
41	*MT-CYB*	14831	14831	G	A	A-T
42	*MT-CYB*	15812	15812	G	A	V-M
43	*MT-TM*	4435	4435	A	G	tRNAMet
44	*MT-TT*	15951	15951	A	G	tRNAThr
45	*MT-TE*	14693	14693	A	G	tRNAGlu
46	*MT-TE*	14727	14727	T	C	tRNAGlu

Var start, start site of variant; var end, end site of variant; ref allele, referenced allele; Var allele, variant allele.

Table II. PCR amplicons.

Index	Gene name	Tgt start	Tgt end	Prb strand	Prb start	Prb end	Var start	Var end	Prb length (bp)	Amp length (bp)
1	*MT-ND1*	3,316	3,866	-	3,269	3,439	3,288	3,425	171	303
2	*MT-ND1*	3,316	3,866	+	3,402	3,585	3,415	3,571	184	316
3	*MT-ND1*	3,316	3,866	-	3,526	3,699	3,543	3,684	174	306
4	*MT-ND1*	3,316	3,866	+	3,643	3,812	3,661	3,794	170	302
5	*MT-ND1*	3,316	3,866	-	3,745	3,936	3,767	3,918	192	324
6	*MT-ND1*	4,025	4,025	-	3,952	4,140	3,962	4,126	189	321
7	*MT-ND1*	4,171	4,216	+	4,132	4,334	4,145	4,315	203	335
8	*MT-TM*	4,435	4,435	-	4,363	4,529	4,380	4,514	167	299
9	*MT-ND2*	4,640	4,640	-	4,482	4,667	4,494	4,655	186	318
10	*MT-ND2*	5,244	5,244	+	5,140	5,317	5,154	5,302	178	310
11	*MT-ATP6*	9,101	9,101	-	9,026	9,223	9,041	9,208	198	330
12	*MT-CO3*	9,804	9,804	-	9,639	9,839	9,653	9,824	201	333
13	*MT-ND3*	10,237	10,237	-	10,161	10,331	10,178	10,310	171	303
14	*MT-ND4L*	10,663	10,680	-	10,541	10,710	10,560	10,692	170	302
15	*MT-ND4*	11,253	11,253	+	11,226	11,404	11,239	11,386	179	311
16	*MT-ND4*	11,696	11,778	+	11,642	11,833	11,660	11,813	192	324
17	*MT-ND5*	12,338	12,338	-	12,213	12,399	12,230	12,385	187	319
18	*MT-ND5*	12,811	12,848	+	12,764	12,941	12,776	12,924	178	310
19	*MT-ND5*	13,051	13,051	-	12,967	13,146	12,984	13,128	180	312
20	*MT-ND5*	13,528	13,730	+	13,488	13,682	13,503	13,665	195	327
21	*MT-ND5*	13,528	13,730	+	13,577	13,774	13,590	13,760	198	330
22	*MT-ND6*	14,279	14,325	-	14,251	14,421	14,267	14,408	171	303
23	*MT-TE*; *MT-ND6*; *MT-CYB*	14,482	14,831	+	14,444	14,645	14,460	14,628	202	334
24	*MT-TE*; *MT-ND6*; *MT-CYB*	14,482	14,831	-	14,587	14,762	14,610	14,748	176	308
25	*MT-TE*; *MT-ND6*; *MT-CYB*	14,482	14,831	+	14,699	14,866	14,720	14,857	168	300
26	*MT-CYB*; *MT-TT*	15,812	15,951	+	15,780	15,966	15,801	15,945	187	319
27	*MT-CYB*; *MT-TT*	15,812	15,951	-	15,792	15,989	15,809	15,974	198	330

PCR, polymerase chain reaction; Tgt, target; prb, probe; var, variation; +, forward primer; -, antisense primer.

previously (16). Notably, three mutations, m.3733G>A, m.4025C>T and m.11253T>C, were observed in one LHON case each, but absent in the control cohort. Whereas, m.4025C>T and m.11253T>C were observed in the control population in our recent studies (8,9).

Thirteen secondary mutations were identified in 30.55% patients. The incidence of these known secondary mutations, m.12811T>C, m.11696G>A, m.3316G>A, m.3394T>C, m.14502T>C, m.3497C>T, m.3571C>T, m.12338T>C, m.14693A>G, m.4216T>C, m.15951A>G, m.14831G>A and m.9804G>A were 5.09, 4.36, 4.00, 4.00, 4.00, 2.55, 1.82, 1.82, 1.45, 1.09, 1.09, 0.36 and 0.36%, respectively. A total of 88 variations from these 13 mutations were observed in patients. Among these, 67 variations were concurrent with either m.11778G>A (59 variations, except 1 from the m.3866T>C mutation) or m.14484T>C (8 variations). Besides the hotspots of *MT-ND1*, *MT-ND4* and *MT-ND6*, the *MT-ND5* gene was frequently accumulated in the distribution of secondary mutations with an incidence of 6.91% in the patients. In addition, the incidence of *MT-ND1*, *MT-ND4* and

Table III. Summary of sequencing data in the panel for 556 samples.

Variable	Outcome
Total no. of reads	118,156,518
Reads mapped to the amplicons (forward primer)	110,440,487
Reads mapped to the amplicons (reverse primer)	108,812,285
Reads mapped to the amplicon targets	105,107,193
Reads mapped to each amplicon (average)	3,892,859
Reads mapped to each sample per amplicon (mean)	7,001.5
Reads enrichment to the targets,% (average)	89
Uniformity of coverage, % (20% mean)	98
Total no. of variants among 46 point mutations	363

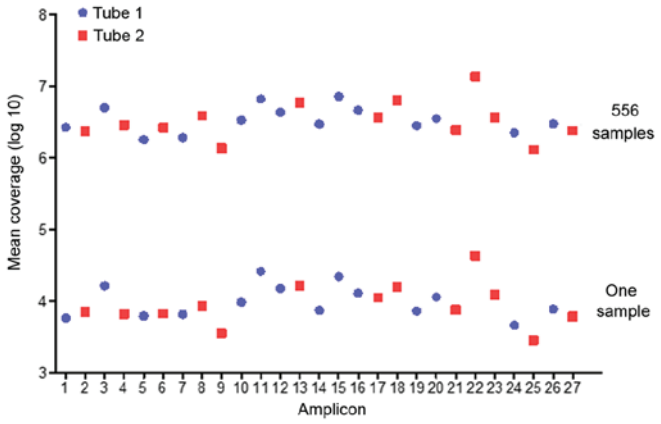

Figure 1. Mean coverage of 27 amplicons. Upper: Average read coverages (log 10) for each amplicon (556 samples). Lower: Read coverages (log10) for each amplicon from one sample.

MT-ND6 for secondary mutations, were 13.45, 4.36 and 4%, respectively. The secondary mutations were predominantly present in the patient and control populations. Of those, mutations m.12338T>C m.4216T>C, m.3571C>T and m.14831 G>A had higher incidences in the control cohort than in the patients. Two LHON-associated mutations (m.14325T>C and m.15812G>A) only arose in the control population in the present study.

Discussion

The present study evaluated the distribution of mitochondrial genes and mutations among 275 Chinese LHON patients, in parallel with a control cohort of 281 subjects, using a multi-gene panel. Mutations of the multi-gene panel were designed from the Mitomap database, previous reports and our previous study in a Chinese Han population (8-10). Twenty-seven amplicons, the specific primers for the point mutations, covered those of 46 mutations, as well as other substitutions in the amplicons, such as pathogenic mutations, m.3697G>A, m.10197G>A and m.14459G>A in the fragments of amplicon 4, 13 and 23, respectively. None of these three rare causative mutations were recorded among subjects in the test. Of the 46 mutations, the m.14727T>C variant in the *MT-TE* gene reported in encephalomyopathy patients (17), was set as a negative control variant in the panel and was absent in all of the subjects. The panel with high-throughput sequencing makes it possible to screen multi-genes or multi-single nucleotide polymorphisms (SNPs) for subjects in a run, and provide more information than traditional Sanger sequencing. Certainly, sequencing the whole mtDNA genome is an optional selection using next generation sequencing. The information from the primary and secondary mutations may be indicative regarding the incomplete penetrance and other clinical symptoms.

It is generally accepted that LHON-associated mutations and their incidence are varied in populations with different ethnic backgrounds (18). Consistent results were confirmed in our multi-gene panel screening. Twenty-three mutations in the panels were absent in the patients and control cohort in the current study. Those mutations reported as rare causative mutations in a European LHON population, m.3376G>A, m.3700G>A and m.4171C>A in the *MT-ND1* gene (19-21), m.10663T>C in the *MT-ND4L* (20), m.13051G>A in the *MT-ND5* gene (22), m.14482C>G/A, m.14495A>G and m.14568C>T in the *MT-ND6* gene (8,20,23), were undetected in the present study. However, 6 causative mutations, m.11778G>A, m.14484T>C, m.3460G>A, m.3635G>A, m.3866T>C and m.3733G>A were observed in 194 LHON cases with their contribution of 83.51, 12.89, 1.04, 0.52, 2.06 and 0.52. Of these, the incidence of m.3460G>A was markedly lower in this cohort than that in a Caucasian population reported by Mackey *et al* (24). Additionally, the spectrum of secondary mutations associated with LHON was also dependent on their ethnic background, and were distinct between the Chinese and Caucasian cohorts. This panel screening demonstrated that the secondary mutations, m.12811T>C, m.11696G>A, m.3394T>C, m.3316G>A, m.14502T>C and m.12338T>C had higher frequencies in the patient cohort, as these mutations were assigned to Asian mtDNA lineage, including the macro-haplo group of M and N. Certainly, m.12811T>C is considered to be a polymorphic variant in sub-haplo groups of M7. While, mutations, m.3394T>C and m.11696G>A are categorized as haplo group-specific variants of M9a and D4j, respectively (25,26). Congruent results were obtained in our previous reports (27).

Usually, secondary mutations, proposed to increase the penetrance of LHON (25-27), are observed in LHON cases associated with m.11778G>A or m.14484T>C mutations. In the present study, 77.27% of variations of secondary mutations were coexistent with one of the primary mutations, m.11778G>A and m.14484T>C. Their detailed distribution was illustrated in Table V. Secondary mutations m.12811T>C, m.11696G>A, m.14502T>C, m.3394T>C and m.3316G>A exhibited the most co-occurrence with m.11778G>A. Meanwhile, three LHON cases carried m.14484T>C and m.14502T>C together. Notably, the m.14502T>C mutation was evidenced as a modifier in the phenotypic manifestation of LHON (28), although it was reported as a causative mutation elsewhere (https://www.mitomap.org/foswiki).

Table IV. Summary information of 46-point mutations in the cohort.

Index[a]	Gene	SNP	Total variants	Patients (n=275)	Primer mutations			Controls (n=281)	Incidence (%)	
					11778	14484	3460		Patients	Controls
1	*MT-ND1*	3316G>A	15	11	7	1	0	4	4.00	1.42
2		3376G>A	0	0	0	0	0	0	0.00	0.00
3		3394T>C	19	11	7	0	0	8	4.00	2.85
4		**3460G>A**	2	2	0	0	**2**	0	0.73	0.00
5		3497C>T	14	7	4	0	0	7	2.55	2.50
6		3571C>T	11	5	3	0	0	6	1.82	2.14
7		3635G>A	1	1	0	0	0	0	0.36	0.00
8		3700G>A	0	0	0	0	0	0	0.00	0.00
9		3733G>A	1	1	0	0	0	0	0.36	0.00
10		3866T>C	4	4	1	0	0	0	1.45	0.00
11		4025C>T	1	1	0	0	0	0	0.36	0.00
12		4171C>A	0	0	0	0	0	0	0.00	0.00
13		4216T>C	11	3	0	0	0	8	1.09	2.85
14	*MT-ND2*	4640C>A	0	0	0	0	0	0	0.00	0.00
15		5244G>A	0	0	0	0	0	0	0.00	0.00
16	*MT-ATP6*	9101T>C	0	0	0	0	0	0	0.00	0.00
17	*MT-CO3*	9804G>A	2	1	1	0	0	1	0.36	0.36
18	*MT-ND3*	10237T>C	0	0	0	0	0	0	0.00	0.00
19	*MT-ND4L*	10663T>C	0	0	0	0	0	0	0.00	0.00
20		10680G>A	0	0	0	0	0	0	0.00	0.00
21	*MT-ND4*	11253T>C	1	1	1	0	0	0	0.36	0.00
22		11696G>A	18	12	10	1	0	6	4.36	2.14
23		**11778G>A**	162	162	**162**	0	0	0	58.90	0.00
24	*MT-ND5*	12338T>C	19	5	2	0	0	14	1.82	4.98
25		12811T>C	21	14	12	0	0	7	5.09	2.49
26		12848C>T	0	0	0	0	0	0	0.00	0.00
27		13051G>A	0	0	0	0	0	0	0.00	0.00
28		13528A>G	0	0	0	0	0	0	0.00	0.00
29		13637A>G	0	0	0	0	0	0	0.00	0.00
30		13730G>A	0	0	0	0	0	0	0.00	0.00
31	*MT-ND6*	14279G>A	0	0	0	0	0	0	0.00	0.00
32		14325T>C	2	0	0	0	0	2	0.00	0.71
33		14482C>A	0	0	0	0	0	0	0.00	0.00
34		14482C>G	0	0	0	0	0	0	0.00	0.00
35		**14484T>C**	25	25	0	**25**	0	0	9.10	0.00
36		14495A>G	0	0	0	0	0	0	0.00	0.00
37		14498T>C	0	0	0	0	0	0	0.00	0.00
38		14502T>C	18	11	8	3	0	7	4.00	2.49
39		14568C>T	0	0	0	0	0	0	0.00	0.00
40		14596A>T	0	0	0	0	0	0	0.00	0.00
41	*MT-CYB*	14831G>A	5	1	0	0	0	4	0.36	1.42
42		15812G>A	1	0	0	0	0	1	0.00	0.36
43	*MT-TM*	4435A>G	0	0	0	0	0	0	0.00	0.00
44	*MT-TT*	15951A>G	5	3	1	2	0	2	1.09	0.71
45	*MT-TE*	14693A>G	5	4	3	1	0	1	1.45	0.36
46		14727T>C	0	0	0	0	0	0	0.00	0.00
		Total variants	363	285	162[b] (60)	25[c] (8)	2	78	103.64	27.76

[a]The index number is according to Table I; [b]162 subjects carrying the m.11778G>A mutation, of which 60 subjects were carrying the m.11778G>A and one of the secondary variants; [c]25 subjects carrying the m.14484T>C mutation, of which 8 subjects were carrying the m.14484T>C and a secondary variant. The three common mutations for LHON are emboldened. SNP, single nucleotide polymorphism.

Table V. Distribution of secondary mutations with m.11778 G>A and m.14484 T>C.

Primary mutation	Secondary mutation (cases harboring successive secondary mutations, n)			Samples, n
	1	2	3	
	12811T>C	-	-	12
	11696G>A	3394 T>C (1)	-	10
	14502T>C	14693 A>G (1)	9804 G>A (1)	8
		3866 T>C (1)	-	
	3316G>A	-	-	7
11778 G>A	3394T>C	11696 G>A (1)	-	7
	3497C>T	3571 C>T (3)	-	4
	14693A>G	14502 T>C (1)	-	3
	3571C>T	3497 C>T (3)	-	3
	12338T>C	-	-	2
	9804G>A	14502 T>C (1)	14693 A>G (1)	1
	11253T>C	-	-	1
	15951A>G	-	-	1
14484 T>C	14502T>C	-	-	3
	15951A>G	-	-	2
	11696G>A	-	-	1
	3316G>A	-	-	1
	14693A>G	-	-	1

Furthermore, more than one secondary mutation co-occurred with m.11778G>A, but not with m.14484T>C in this panel. m.3497C>T and m.3571C>T, which belong to the haplo group variants of B4c1, arose in three cases associated with m.11778G>A. In addition, m.9804G>A and m.14831G>A, reported as LHON-associated mutations in Caucasian cases, were common in the present control cohort according to the panel analysis. It was confirmed that the spectrum of mutations varied between ethnic backgrounds and indicated that the selected SNPs of the panel would be optimized for a Han population in the future.

In conclusion, the current data indicates that the spectrum and incidence of mtDNA mutation-associated LHON cases in the Han population are different to those in a Caucasian population. Here, the causative mutations associated with LHON, m.11778G>A, m.14484T>C, m.3460G>A, m.3635G>A, m.3866T>C and m.3733G>A, were observed in 70.55% of the patient cohort. The common secondary mutations in the Chinese LHON population were m.12811T>C, m.11696G>A, m.3394T>C, m.3316G>A, m.14502T>C and m.12338T>C. Furthermore, besides the three hotspots genes *MT-ND1*, *MT-ND4* and *MT-ND6*, *MT-ND5* also had a high incidence of secondary mutations including m.12811T>C and m.12338T>C; this finding was comparable with a previous study (29). The primary and secondary mutation-associated LHON cases in the present multi-gene panel will advance current understanding of the clinical phenotype of LHON, and offer valuable information for the early diagnosis and subsequent options of intervention for mitigating risk of additional vision loss in LHON patients.

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