Two families with Leber's hereditary optic neuropathy carrying G11778A and T14502C mutations with haplogroup H2a2a1 in mitochondrial DNA

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Received June 22, 2014; Accepted March 18, 2015

DOI: 10.3892/mmr.2015.3714

Abstract. The mitochondrial haplogroup has been reported to affect the clinical expression of Leber's hereditary optic neuropathy (LHON). The present study aimed to investigate the interaction between mutations and the haplogroup of mitochondrial DNA (mtDNA) in families. Two unrelated families with LHON were enrolled in the study, and clinical, genetic and molecular characterizations were determined in the affected and unaffected family members. Polymerase chain reaction direct sequencing was performed using 24 pairs of overlapping primers for whole mtDNA to screen for mutations and haplogroup. Bioinformatics analysis was performed to evaluate the pathogenic effect of these mtDNA mutations and the haplogroup. The G11778A mutation was identified in the two families. In addition, the members of family 2 exhibited the T14502C mutation and those in family 1 exhibited the T3394C and T14502C mutations, which were regarded as secondary mutations. The penetrance of visual loss in families 1 and 2 were 30.8 and 33.3%, respectively. In addition, the two families were found to be in the H2a2a1 haplogroup. In this limited sample size, it was demonstrated that the H2a2a1 haplogroup had a possible protective effect against LHON. Additional modifying factors, including environmental factors, lifestyle, estrogen levels and nuclear genes may also be important in LHON.

Introduction

Leber's hereditary optic neuropathy (LHON; Online Mendelian Inheritance in Man #535000) is characterized by the selective degeneration of retinal ganglion cells, particularly contributing to the papillomacular bundle, which leads to optic atrophy and loss of central vision (1). Typically, the clinical presentation includes acute or subacute central visual loss in one eye, with effects in the other eye 2-4 months later (2), although symptoms occur in the two eyes simultaneously in 25% of patients (3). The disease was first described by Leber in 1871 (4), Wallace *et al* reported the mitochondrial DNA (mtDNA) G11778A point mutation in patients with LHON in 1988 (5). In total >95% of LHON pedigrees are known to exhibit one of three major mtDNA point causative mutations: G3460A (13% of cases), G11778A (69% of cases) and T14484C (14% of cases) (6), which are located in the ND1, ND4 and ND6 mtDNA genes, respectively, and affect the genes encoding complex I subunits of the mitochondrial respiratory chain, which lead to disorders of the oxidative phosphorylation system (7,8). However, the three major mutations are only responsible for 38.3% cases in Chinese individuals with LHON (9) and a number of other pathogenic mtDNA variants have been reported, which are awaiting confirmation for LHON pathogenicity (10).

At present, two key features of LHON require clarification. There is a marked incomplete penetrance and gender bias, with only 50% of male and 10% of female carriers eventually losing their vision (11). This indicates that there are additional genetic or environmental factors in the pathophysiology of the disorders. From then on, the importance of the mitochondrial haplogroup has been considered. A haplogroup is a collection of polymorphisms, which reflect the evolutionary history of the mtDNA molecule (12). In western European, the J2 and J1 haplogroups have been reported to contribute to increased risk of visual failure in families with G11778A and G3460A mutations, respectively (13). In Chilean patients, the Amerindian haplogroup A2 has been associated with delayed onset of LHON and haplogroup C has been associated with improved vision (14). In a previous study, which investigated Chinese patients of G11778A haplogroup F, a protective effect against LHON was observed, whilst haplogroups M7b and M10a were identified as risk factors (15,16). The present study aimed to detect LHON-associated mutations and mitochondrial haplotypes in two families, and investigate the effects of the

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Key words: Leber's hereditary optic neuropathy, mitochondrial DNA mutation, G11778A, haplogroup

Table I. Clinical characteristics of patients carrying the G11778A mutation.

Patient	Age (years)/gender	Onset age (years)	Visual acuity		Level of visual impairment
			Right eye	Left eye	
Family 1-I-2	75/M	N/A	0.04	0.06	Severe
Family 1-III-5	17/F	17	0.05	0.01	Severe
Family 2-II-1	57/F	17	0.01	HM	Profound
Family 2-III-11	12/F	12	0.05	0.02	Severe

M, male; F, female; N/A, not available; HM, hand motions (ability to visually detect movement at short distances).

interaction between mutations and mitochondrial haplotypes on the phenotypic manifestation of LHON.

Materials and methods

Participants. Individuals from two unrelated families, each containing 22 members, were selected, and certain family members (marked with an asterisk in Fig. 1) were enrolled in the present study. Prior to involvement in the clinical evaluations, informed consent and blood samples were obtained from all the family members involved. Also, informed consent was obtained from family members for minors involved in this study. The present study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China) and was in line with the Declaration of Helsinki. Personal and family medical histories, history of tobacco use and alcohol intake, and details of any other clinical abnormalities were also obtained.

Ophthalmologic evaluations. The ophthalmologic examinations of all patients and available relatives were performed in the department of Ophthalmology at Zhongnan Hospital of Wuhan University (Wuhan, China). The examinations included measuring visual acuity, fundus examination, visual field examination, visual evoked potentials (VEP), fundus photography (TRC-50EX Fundus; Topcon Itabashi-ku Corporation, Tokyo, Japan), and head, orbital and cervical spine magnetic resonance imaging (MRI; Magnetom Trio Tim 3.0 MRI; Siemens, Munich, Germany). The level of visual impairment was defined according to the visual acuity, as follows: Normal, ≥0.3; 0.1≤ mild <0.3; 0.05≤ moderate <0.1; 0.02≤ severe <0.05; and profound, <0.02.

Mutational analysis. Venipuncture was performed on all the family members participated, with the exception of family 1-I-2. Approximately 2 ml EDTA-anticoagulant peripheral blood was drawn from participants for DNA collection. Approximately 0.5 ml blood and 3X Red Blood Cell Lysis Buffer (1 mM, 1.5 ml) were adding to a 10-ml EP tube. The mixture was placed in an ice bath for 30 min prior to centrifugation at 13,700 x g for 10 min at 4˚C (TGL-18R; Hema Medical Instruments Co., Ltd., Zhuhai, China). The upper aqueous component was removed and 3X nucleus pyrolysis liquid (1mM, 1.5 ml), 150 μl of 10% sodium dodecyl sulphate (SDS) and 15 μl of 10 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO, USA) were added, and the mixture was put into the Oven Controlled Crystal Oscillator (IS-RSV1; Crystal Technology and Industries, Inc., Dallas, TX, USA) at 37˚C and 200 x g overnight. Then the digestion solution was gently mixed with equal volume of Tris-phenol (2 mM, pH 8.0). The phases were separated by centrifugation at 13,700 x g for 10 min at 4˚C. The upper aqueous component was retained and equal volume of Phenol/chloroform (volume, 1:1) was added, then the mixture was centrifuged at 13,700 x g for 10 min at 4˚C. The majority of the aqueous phase was transferred into a 1.5 ml EP tube prior to mixing with 1 ml of 70% ethanol. Later the phases were separated by centrifugation at room temperature at 13,700 x g for 10 min twice. The aqueous phase was decanted and the precipitation contained the desired DNA. After draining excess liquid, the precipitation was dissolved with 50 μl TE and stored at 4˚C. While the genomic DNA of family 1-I-2 was extracted using a Chelex-100 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) method from the scapus pili. The mtDNA fragments of ND1, ND4 and ND6, which contain three major mutations (G3460A, G11778A and T14484C), were amplified from the DNA extracted from all family members involved. The PCR products were directly sequenced using one of the PCR primers. The sequences were blasted with the reference sequences to identify the mutations. The amplification of the entire mtDNA of the two probands were performed using previously defined 24 primers, covering overlapped fragments, using polymerase chain reaction (PCR) (17). Each amplification reaction contained 5.0 μl of 10X Ex Taq Buffer, 5.0 μl $MgCl_2$ (25 mM), 2.0 μl of 4X dNTP (2mM), 2.0 μl of 1 mM each primer, 2 units of Taq polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 3.0 μl genomic DNA and double distilled water was added to reach 50 ml reaction volume. The protocol for amplification reactions was as follows: Denaturation at 95˚C for 5 min followed by 35 cycles of a denaturation at 94˚C for 30 sec, an annealing at 52-63˚C for 30 sec and an extension at 72˚C for 45 sec, the terminal extension step at 72˚C for 10 min. The amplification was conducted using a Hema 9600 PCR thermocycler (Hema Medical Instruments Co., Ltd.) and a 3130xl Genetic Analyzer (Applied Biosystems Life Technologies, Carlsbad, CA, USA). The bidirectional sequence results were analyzed and compared with the updated consensus revised Cambridge reference sequence (GenBank accession no. NC_012920). Changes in the mtDNA were compared against a mitochondrial database, including Mitomap (www.mitomap.org/MITOMAP) and mtDB (www.mtdb.igp.uu.se/).

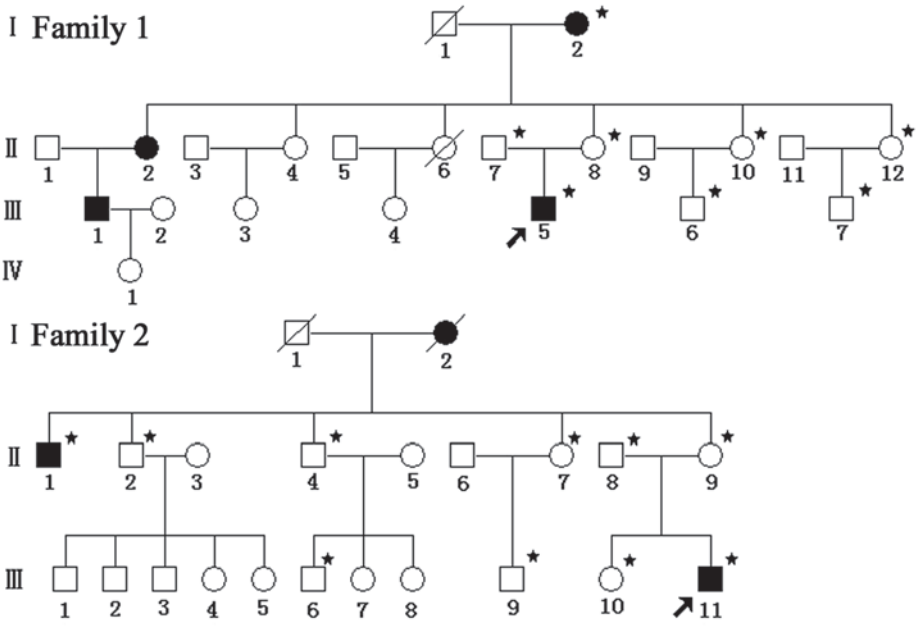

Figure 1. Families with LHON. Squares indicate males and circles indicate females. The black and white symbols indicate the patients with LHON and unaffected individuals, respectively. The slash through the symbol represents deceased. The black arrows indicate the proband and the asterisk denotes the members involved in the present study. LHON, Leber's hereditary optic neuropathy.

Phylogenetic analysis. Conservative amino acid analyses of missense mutations were identified in the Mitochondrial Single Nucleotide Polymorphism public database (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml), which compared the human amino acid variants with those of 60 vertebrates, including *Artibeus jamaicensis, Didelphis virginiana and Hippopotamus amphibius*. The degree of conservation is defined as the percentage of species in the 60 vertebrates with the same amino acid in the same position as in humans (18).

Haplogroup analyses. The entire mtDNA sequence of the probands was assigned to mitochondrial haplogroups using the nomenclature of the mitochondrial haplogroups (19), according to PhyloTree (http://www.phylotree.org/) (20).

Results

In family 1, the proband (III-5) was a 17 year old male from Henan, China. This individual developed impaired vision in the right eye, followed four days later by impaired vision in the left eye, with no clear cause. As presented in Table I, III-5 presented with 0.05 visual acuity in the right eye and 0.01 visual acuity in the left eye, with dense central scotomas and optic disk pallor bilaterally (21). Visual field analysis revealed large visual field defects connected to the physiological blind spot in the right and left eye. The flash VEP demonstrated bilateral delays in the amplitudes of the latency. No other clinical abnormalities were observed on the head, orbital and cervical spine MRI. In addition, III-5 had a history of tobacco and alcohol use for almost two years. In the matrilineal relatives, there were 2 affected males and 2 affected females; thus, the ratio of affected males to females was 1:1. However, in total there were 4 males and 9 females in this family. Thus, the morbidity of males and females was 2/4 and 2/9, respectively. The ratio of 2/4 to 2/9 was the incidence between the males and females, which equaled 2.25:1, and the penetrance of visual loss (affected matrilineal relatives/total matrilineal relatives) was 30.8%.

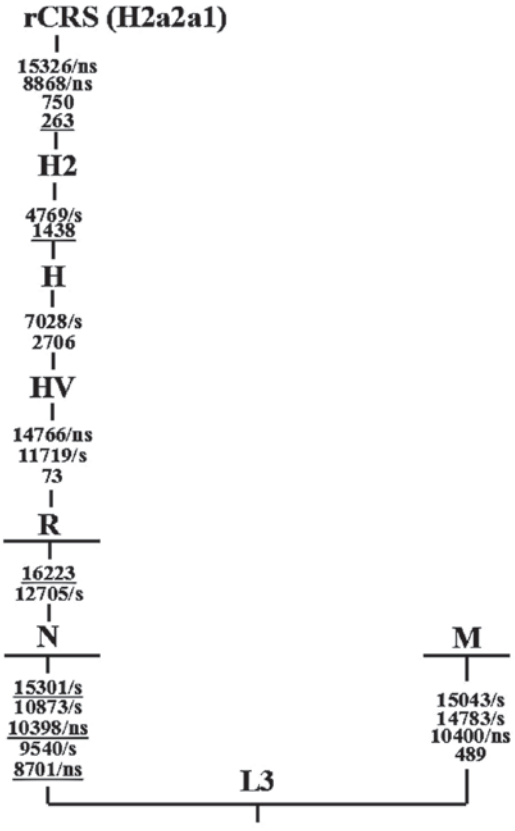

Figure 2. rCRS (12,13). The rCRS represents the nodal H2a2a1. haplogroup Recurrent mutations are underlined. The synonymous and non-synonymous coding-region variants in the mitochondrial DNA sequences are denoted by '/s' and '/ns', respectively. rCRS, revised Cambridge reference sequence.

Table II. List of the mtDNA variants identified in the two families containing individuals diagnosed with Leber's hereditary optic neuropathy.

Gene	Position	Replacement	CRS	Family 1	Family 2	Conservative degree[a] (%)
D-loop	**73**	**A>G**	**A**	**G**	**G**	
	146	T>C	T	C	C	
	152	T>C	T	C	C	
	204	T>C	T	-	C	
	263	**A>G**	**A**	**G**	**G**	
	272	A>G	A	-	G	
	489	A>G	T	-	C	
	16093	T>C	T	C	C	
	16193	C>T	C	T	T	
	16223	**C>T**	**C**	**T**	**T**	
	16311	T>C	T	-	C	
	16357	T>C	T	C	C	
	16497	A>G	A	G	G	
	16519	T>C	T	C	-	
12SrRNA	709	G>A	G	A	A	
	750	**A>G**	**A**	**G**	**G**	
	1438	**A>G**	**A**	**G**	**G**	
16SrRNA	2706	A>G	A	G	G	
NDI	3394	T>C (Ty83His)	T	C	-	96.7
	4140	C>T	C	T	T	
ND2	**4769**	**A>G**	**A**	**G**	**G**	
COI	6965	T>G	T	G	-	
	7028	**C>T**	**C**	**T**	**T**	
	7250	A>G	A	G	G	
ATP6	**8701**	**A>G (Thr59Ala)**	**A**	**G**	**G**	28.3
	8764	A>G (Ala80Thr)	G	-	A	26.7
	8793	T>C	T	C	C	
	8856	G>A	G	A	A	
	8860	**A>G (Thr112Ala)**	**A**	**G**	**G**	66.7
COIII	**9540**	**T>C**	**T**	**C**	**C**	
ND3	**10398**	**A>G (Thr114Ala)**	**A**	**G**	**G**	43.3
	10400	C>T	C	T	T	
ND4L	10529	A>G	A	-	G	
	10646	G>A	G	A	A	
ND4	**10873**	**T>C**	**T**	**C**	**C**	
	10203	C>T	C	-	T	
	11674	C>T	C	-	T	
	11719	**G>A**	**G**	**A**	**A**	
	11778	G>A (Arg340His)	G	A	A	98.3
	12034	C>T	C	T	-	
ND5	12549	C>T	C	T	T	
	12705	**C>T**	**C**	**T**	**T**	
	13135	G>A (Ala267Thr)	G	A	-	5.0
	13152	A>G	A	G	G	
	13774	A>G (Thr480Ala)	A	-	G	-
	14097	C>T	C	T	-	
	14110	T>C	T	-	C	
ND6	14502	T>C (Ile58Val)	T	C	C	26.7

Table II. Continued.

Gene	Position	Replacement	CRS	Family 1	Family 2	Conservative degree[a] (%)
CytB	**14766**	**C>T (Thr7Ile)**	**C**	**T**	**T**	50.0
	14783	T>C	T	C	C	
	15043	G>A	G	A	-	
	15301	**G>A**	**G**	**A**	**A**	
	15326	**A>G**	**A**	**G**	**G**	
	15526	C>T	C	-	T	

Conservative degree[a] was determined by comparing the *Homo sapiens* mtDNA variants with 60 other vertebrates. The variations used to determine the haplogoup affliation of mtDNA are highlighted in bold. mtDNA, mitochondrial DNA; CRS, Cambridge reference sequence.

In family 2, the proband (III-11) was a 12 year old male from Hubei, China, who exhibited painless, progressive deterioration of the left eye, followed by rapid vision loss in his right eye 11 months later. Visual acuity was 0.05 and 0.02 in the right and left eyes, respectively. No other clinical abnormalities of the eye were observed. The ratio between the number of affected males and females was 2:1 and the incidence in males and females was 2/5 and 1/4 respectively. Therefore, the males:female incidence ratio was 1.6:1 and the penetrance of visual loss was 33.3%.

In order to elucidate the molecular basis of LHON, mutational screening of three major mutations, G3460A, G11778A and T14484C, was performed in the two families using PCR direct bidirectional sequencing. The results revealed the presence of G11778A in the two families, but absence of the G3460A and T14484C mutations.

To determine the role of the haplogroup in the phenotypic expression of the G11778A mutation, the entire mitochondrial genome was analyzed using PCR direct sequencing, as shown in Table II. These missense variants were further evaluated by phylogenetic analysis among 60 types of vertebrate, particularly the T3394C and T14502C mutations, which are considered to be secondary mutations in the expression of LHON (18,21,22). The analyses revealed that T3394C was highly conserved in 58/60 mammalian species and the conservative degree was 96.7%. G11778A was highly conserved in 59/60 mammalian species, with a conservative degree of 98.3%. The degree of conservation of T14502C was 72.13%, which suggested it as a potential polymorphism. The analyses of the complete mtDNA sequences indicated that the two families exhibited the H2a2a1 haplogroup (Fig. 2).

Discussion

In the present study, according to the typical clinical signs, symptoms and family histories, the probands were diagnosed as having LHON (22,23). Genetic and molecular characterization of the matrilineal relatives was subsequently conducted. The G11778A mutations were observed in all maternal members in the two families and were in the H2a2a1 haplogroup.

In previous reports, the average penetrance of visual loss was 19.2% in 11 families, who were carrying only the G11778A mutation, while the average penetrance of visual loss in four families carrying the G11778A and T14502C mutations was 38.8% (24,25-27). In addition, the penetrances of visual loss in families carrying T3394C and G11778A mutations have been reported as 38, 38, 44 and 56% (28). In the present study, the penetrances of visual loss in family 1 and family 2 were 30.8 and 33.3%, respectively. This suggested that the G11778A mutation itself was not sufficient to induce visual loss, while the second mutations were. Subsequently, conservative analysis of the missense mutations, which were identified in the genotype analysis of the two families, was performed. The T3394C mutation was highly conserved, while T14502C and the other missense variants were potential polymorphisms. Therefore, the T3394C mutation may cooperate with the G11778A variant to contribute to the penetrance of LHON. Although the T14502C variant was identified as a potential polymorphism, it has been previously reported to act synergistically with the T11778C mutation (24).

The penetrances of visual loss were 30.8 and 33.3% in the two families, which were lower than those carrying either the T14502C or T3394C second mutation. It was suggested that the H2a2a1 haplogroup had a protective effect on the phenotypic manifestation of LHON, similar to a previous observation, in which haplogroup H had a protective effect in families carrying the G11778A mutation (13). However, it is not possible to exclude the small sample size as a cause for the deviation.

When comparing the probands of families 1 and 2, age-at-onset was <20 years old, severe visual impairment was present, and the G11778A mutation and H2a2a1 haplogroup were identified in both families. In family 1, the proband carrying T3394C and T14502C mutations, presented initially with loss of vision in one eye. Loss occurred in the other eye shortly after, indicating the rapid development of the disease in this patient. However, in family 2 the proband only carried the T14502C mutation, and vision loss began in his second eye 11 months following loss of vision loss in his first eye. This indicates a slower development of the disease compared with that in family 1. Therefore, we hypothesized that carrying a second mutation increased the rate of visual impairment. It was suggested that individuals with the two second mutations in family 1 were more likely to have shorter intervals between the loss of vision in the right and left eyes. It is generally accepted that possessing more second mutations may increase the penetrance of LHON (29), however, this was not observed in the present study. Additional modifying factors including environmental factors, lifestyle, estrogen levels and nuclear genes may be important in

LHON (30,31). Cytoplasmic hybrid (cybrid) cell model experiments have previously demonstrated that estrogen may reduce the production of reactive oxygen species in complex I-defective LHON cybrids, improve cybrid energetic competence and lead to coordinated activation of mitochondrial biogenesis (32). Additionally, estrogen receptor β has been observed to localize to the human retinal ganglion cells and the retinal nerve fibre layer (32). A previous study observed that a female with Perrault syndrome, which leads to a reduction in estrogen, also suffered a severe manifestation of LHON due to the G11778A mtDNA mutation (33), which further suggested that estrogen has an important function in LHON. The variation in the *PARL* gene of LHON also suggested that it may be involved in the penetrance of the disease (34).

In conclusion, in the small sample of patients examined in the present study, patients with LHON who carry the G11778A mutation were observed to carry two second mutations, T14502C and T3394C, and were more likely to exhibit shorter intervals between visual loss in the right and left eyes. In addition, the H2a2a1 haplogroup may have a protective effect on the phenotypic manifestation of LHON.

Acknowledgements

The authors would like to thank the patients and family members who were involved in and Dr Changzheng Chen at Renmin Hospital of Wuhan University (Wuhan, China) for his cooperation.

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