Leber Hereditary Optic Neuropathy: A Mitochondrial Disease Unique in Many Ways

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Contents

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Abstract

Leber hereditary optic neuropathy (LHON) was the first mitochondrial disease to be identified as being caused by mutations in the mitochondrial DNA (mtDNA). This disease has been studied extensively in the past two decades, particularly in Brazilian, Chinese and European populations; and many primary mutations have been reported. However, the disease is enigmatic with many unique features, and there still are several important questions to be resolved. The incomplete penetrance, the male-biased disease expression and the prevalence in young adults all defy a proper explanation. It has been reported that the development of LHON is affected by the interaction between mtDNA mutations, mtDNA haplogroup background, nuclear genes, environmental factors and epigenetics. Furthermore, with the help of new animal models for LHON that have been created in recent years, we are continuing to learn more about the mechanism of this disease. The stage has now been reached at which there is a good understanding of both the genetic basis of the disease and its epidemiology, but just how the blindness that follows from the death of cells in the optic nerve can be prevented remains to be a pharmacological challenge. In this chapter, we summarize the progress that has been made in various recent studies on LHON, focusing on the molecular pathogenic mechanisms, clinical features, biochemical effects, the pharmacology and its treatment.

Keywords

Animal model • LHON • mtDNA • Nuclear genes • Therapy

Leber hereditary optic neuropathy (LHON, MIM 535000) is a common cause of acute or subacute central visual loss in young adults, with a high male-to-female ratio for the clinical expression of symptoms (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). LHON was first described by German ophthalmologist Theodore Leber in 1871 (Erickson 1972; Leber 1871) and was later recognized to be a mitochondrial disease (Wallace et al. 1988). In the past two decades, our knowledge about this disease was mainly derived from studies involving patients with European roots; although it is generally considered the disease occurs worldwide. We now know that the majority of LHON cases are caused by the presence of one of three primary mitochondrial DNA (mtDNA) mutations (m.11778G>A, m.14484T>C and m.3460G>A) (Howell et al. 1991; Huoponen et al. 1991; Mackey and Howell 1992; Wallace et al. 1988). From the 1990s, researchers all around the world have carried out large number of studies to characterize the genetic and clinical features of LHON (Carelli et al. 2004; Ji et al. 2008; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009; Yu et al. 2010b; Zhang et al. 2009). These studies have greatly increased our understanding of the pathology and risk factors of LHON. However, the two major features of the disease, incomplete penetrance (not all mutation carriers demonstrate clinical expression of disease) and gender bias, still are the most urgent

challenges for understanding the pathogenesis of this disease (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). Furthermore, medical treatment for this disease has still not been satisfactorily resolved (La Morgia et al. 2014).

1 LHON Clinical Features

The most typical characteristics of patients with LHON are the following: (1) a family history that shows a matrilineal inheritance pattern, although there are a considerable number of sporadic LHON cases; (2) the visual loss is painless, affecting just one eye initially and then involving the other eye and going on to cause a significant loss of vision or even total blindness, within a short time; and (3) more male carriers of the pathogenic mutations develop symptoms of LHON than maternally related female carriers of the same primary LHON mutations. About 50% of the male carriers and 10% of the female carriers will be affected, while males from 15 to 35 years old are the main affected population (Seedorff 1985). The progress of LHON can be divided into an acute phase and a chronic phase based on the clinical features. In the majority of patients, the deterioration of vision is acute and affects the two eyes simultaneously (25%) or sequentially (75%) within about 8 weeks (Harding et al. 1995). This stage of the disease is associated with retinal vascular engorgement, deformation of the optic nerve and oedema of the retina (Yen et al. 2006), while there also are some patients with no pathological changes to be observed (Riordan-Eva and Harding 1995). In addition, the disease progression may present slowly in some patients, lasting for 6 months or more. In this kind of patient, the visual field changes are not significant, thus increasing the difficulty of diagnosis (Nikoskelainen et al. 1996), particularly in the cases which lack the typical family history of LHON. In addition to typical LHON symptoms, the patient may also suffer from other problems, such as muscle atrophy, multiple sclerosis, heart disease and cerebrovascular abnormalities (Jaros et al. 2007; La Morgia et al. 2008; Sorajja et al. 2003).

Because the clinical manifestations of LHON vary widely, patients with complicated symptoms are often not diagnosed with LHON accurately. An important diagnostic marker for LHON is the matrilineal inheritance pattern, but for those patients with the clinical features of LHON but who lack a family history, screening the mtDNA for the three primary mutations (m.3460G>A, m.11778G>A and m.14484T>C) is necessary for an accurate diagnosis (Jia et al. 2006). It is worth noting that there are many suspected LHON patients who have symptoms but no family history and with no known primary mutations, and this suggests other genetic or environmental factors may influence the onset of LHON, or produce similar, and as yet unidentified, diseases (Carelli et al. 2004; Kirkman et al. 2009; Yen et al. 2006).

2 Epidemiology

The reported prevalence of LHON varied among European countries: about 1/25,000 in the north-east of England (Chinnery et al. 2000; Man et al. 2003), 1/39,000 in the Netherlands (Spruijt et al. 2006) and 1/50,000 in Finland (Puomila et al. 2007). But in China and other regions of Asia, there has not been a comprehensive epidemiological survey. In 2008, Elliott et al. screened for LHON primary mutations in the neonatal umbilical cord blood samples of 3,000 normal newborns and identified a relatively high frequency of 0.107% for m.3460G>A (3/2807), 0.108% for m.11778G>A (3/2770) and 0.105% for m.14484T>C (3/2855) (Elliott et al. 2008). This study indicated that the primary LHON mutations do exist in normal populations. We screened for the three LHON primary mutations by using a sensitive allele-specific PCR technique in the general Han Chinese population (1,555 healthy samples), but found no LHON primary mutation carriers, suggesting that the frequency of the LHON primary mutation in Han Chinese general populations is very low (Bi et al. 2010). Large-scale epidemiological surveys for LHON still need to be carried out in Asian populations and populations from other continents to get a complete profile of LHON prevalence.

3 mtDNA Primary Mutations

3.1 Three Well-Known Primary Mutations

In 1988, Wallace and his co-workers were the first to propose an mtDNA mutation as the cause of LHON (Wallace et al. 1988). Since then many mtDNA mutations have been reported as being LHON primary mutations (cf. http://www.mitomap.org) (Fig. 1). Among these mutations, three mutations (m.3460G>A in the *MT-ND1* gene, m.11778G>A in the *MT-ND4* gene and m.14484T>C in the *MT-ND6* gene) are reported to account for more than 95% of the LHON patients (Mackey et al. 1996).

These three primary mutations show considerable variation in their levels of evolutionary conservation. Mutation m.11778A>G is highly conserved, while mutations m.3460G>A and m.14484T>C are less conserved, which suggests that not all pathogenic mutations occur in highly conserved regions. The frequency of these three mutations varies among different geographical regions. In Europe, mutation m.11778G>A is found in 56.6% of patients, and mutations m.3460G>A and m.14484T>C are found in 22.6 and 20.8% of patients, respectively (Hudson et al. 2007b). In Chinese populations, the frequency of m.11778G>A is much higher than that of European populations and populations of European origin and can be up to 90.2%. The frequencies of m.3460G>A and m.14484T>C are much lower than those of European populations and populations of European origin, with a frequency of 1.1 and 8.7%, respectively (Ji et al. 2008; Jia et al. 2006; Yu et al. 2010a,b) (Fig. 2). A founder effect (the fixation of an allele after the migration and population expansion of a small population in a new area)

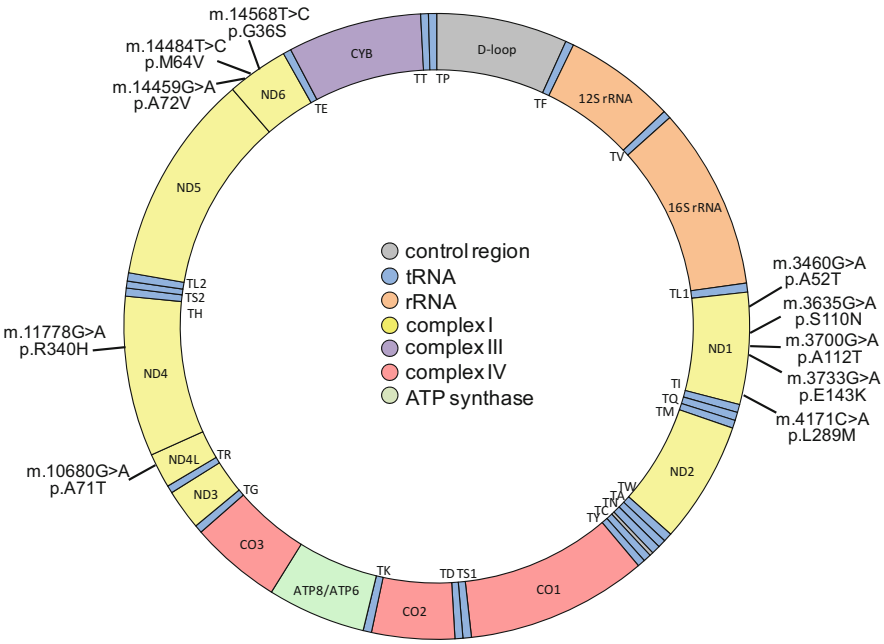

Fig. 1 Schematic profile of LHON mutations. The three primary mutations and the other rare mtDNA mutations that have been reported in at least three independent LHON families, as summarized in MITOMAP, http://www.mitomap.org/bin/view.pl/MITOMAP/MutationsLHON, are shown in their relative positions on the mtDNA molecule (Achilli et al. 2012; Brown et al. 2001b; Bu and Rotter 1991; Fauser et al. 2002a; Fauser et al. 2002b; Gropman et al. 2004; Horvath et al. 2002; Kim et al. 2002; Shoffner et al. 1995; Valentino et al. 2004; Wissinger et al. 1997; Zhang et al. 2012)

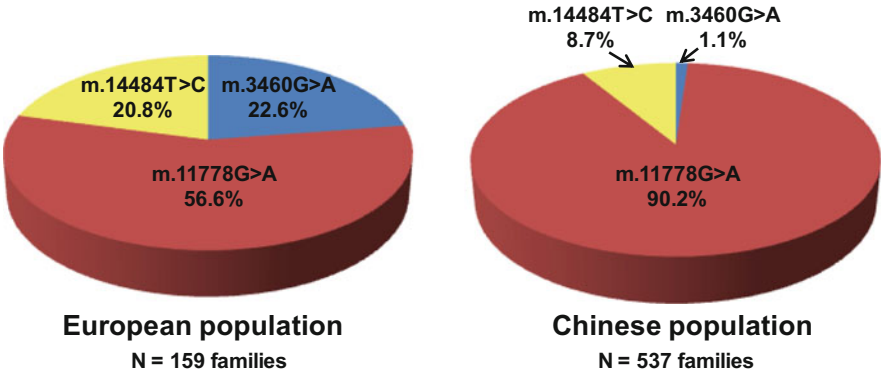

Fig. 2 Mutation spectra of known primary LHON mutations in Chinese and European populations. Frequency of the three LHON primary mutations in European (*left*) (Hudson et al. 2007b) and Han Chinese (*right*) populations (Ji et al. 2008; Jia et al. 2006; Yu et al. 2010a, b)

was observed for m.14484T>C in Canada, which accounts for 87% of the cases and appears to have had an origin in France (Macmillan et al. 2000; Macmillan et al. 1998). However, the best founder effect has been reported from Brazil, where many hundreds of persons can trace their maternal ancestry and the carriage of mutation m.11778G>A, back to an immigrant family that came from Italy (Sadun et al. 2002). The different frequencies of the three primary mutations in different populations may be caused by the past demographic history.

3.2 Rare Primary Mutations

In addition to the three primary mutations, some rare mutations, i.e. m.3635G>A, m.3700G>A, m.14459G>A and so on, as summarized in MITOMAP (http://www.mitomap.org/bin/view.pl/MITOMAP/MutationsLHON) from previous studies (Achilli et al. 2012; Brown et al. 2001b; Fauser et al. 2002b; Gropman et al. 2004; Horvath et al. 2002; Shoffner et al. 1995) have also been reported (Fig. 1). Many of these rare primary mutations were reported in single patients with the typical features of LHON. We have shown how we identified mutations m.3635G>A and m.10680G>A as being pathogenic as follows.

Mutation m.3635G>A was first identified in a Russian LHON family (Brown et al. 2001b) and was confirmed to cause dysfunction of the mitochondrial respiratory chain. Recently, we identified mutation m.3635G>A in a family that had no known primary mutation but had a clear matrilineal inheritance and typical clinical pattern of LHON (Zhang et al. 2009). At about the same time, Yang et al. (2009a) also identified m.3635G>A in two Chinese LHON families. Subsequently, Jia et al. (2010) screened for this mutation in a large sample set of suspected LHON patients who showed the clinical features of LHON but without any of the three primary mutations and identified eight families with the m.3635G>A mutation. These authors further characterized the clinical symptoms of the representative probands with the mutation and provided evidence for a causal association of m.3635G>A with LHON (Jia et al. 2010). We analyzed the complete mtDNA genome sequences of these families and found that m.3635G>A had multiple origins in Chinese (Bi et al. 2012). Furthermore, m.3635G>A is highly conserved in vertebrates, suggesting the importance of this site in the MT-ND1 protein (Zhang et al. 2009). Recently, mutation m.3635G>A was again reported in nine Chinese families and one family from Poland (Kodron et al. 2014; Zhang et al. 2014). Therefore, we can now propose that the mutation m.3635G>A does cause LHON and has a frequency similar to the m.3460G>A mutation (0.57 vs. 0.50%) in Chinese LHON patients (Bi et al. 2012; Jia et al. 2010; Zhang et al. 2009). This mutation should be considered routinely when testing Chinese patients with the symptoms and signs of LHON disease.

Mutation m.10680G>A in the *MT-ND4L* gene can be given as another good example for the identification of additional LHON mutations. This mutation was

first reported in a Chinese family in which all the members had a partial reduction in their vision or had complete blindness, while the mutation was not found in unaffected family members, nor in 100 controls (Yang et al. 2009b). Through complete mitochondrial genome sequencing, they found that the matrilineal family members had the primary mutation m.14484T>C and one novel mutation m.10680G>A. Accordingly, Yang et al. (2009b) suggested that mutation m.10680G>A might be the reason for the complete penetrance of LHON symptoms in this family, although this symptom severity may also be caused by m.14484T>C. Later on, we identified mutation m.10680G>A in one suspected LHON family without any of the three well-known primary mutations and performed an analysis from an evolutionary perspective which suggested a potentially pathogenic role of m.10680G>A in LHON (Zou et al. 2010). Recently, we optimized an allele-specific PCR technique to screen for m.10680G>A in 774 patients with suspected LHON and identified two patients with the mutation (Zhang et al. 2012). The predicted protein structure revealed that mutation m.10680G>A may lead to LHON by changing the structure of the transmembrane region in the MT-ND4L protein (Zhang et al. 2012). However, there remains to be a doubt concerning whether this mutation is significant outside China, as there are eight mitochondrial sequences in the GenBank database from other parts of the world which show this mutation but are not specifically linked to LHON disease (data searched on June 25, 2015).

3.3 Mutation Heteroplasmy and Occurrence of Double Primary LHON Mutations

Depending on its particular type, a cell may contain hundreds or thousands of mitochondria. The presence of a mutation only in some of cells of an individual or in some of the mitochondria within a cell indicates heteroplasmy for that mutation. The existence of wild-type and mutant mtDNA within cells (intracell heteroplasmy) and among cells (intercell heteroplasmy) leads to different levels of mutation load (0–100%) within a cell or tissue (Yao et al. 2015). The heteroplasmy level of a pathogenic mutation may affect the onset of a mitochondrial disease, and there is a threshold effect (DiMauro and Schon 2003). Most families with LHON primary mutation are homoplasmic, but about 10–15% of families do show heteroplasmy (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). However, a high prevalence of LHON family with heteroplasmic m.11778G>A (11 in 30 pedigrees, 37%) was observed in Thai population (Phasukkijwatana et al. 2006). The level of heteroplasmy affects the presence of LHON symptoms, and it is reported that when the level of heteroplasmy is less than 60%, there is a lower probability of disease (Chinnery et al. 2001a). But there are also some reports showing cases with disease symptoms even if the heteroplasmy is at a low level, suggesting a modulation effect from the nuclear genetic background (Black et al. 1996; Jacobi et al. 2001).

In our previous studies, we have found that only a small part of Chinese LHON patients (about 0.2% of patients with m.11778G>A and 5.8% of patients with m.14484T>C) carried heteroplasmic primary mutations, while most of the patients were homoplasmic (Yu et al. 2010b; Zhang et al. 2011b). The pattern of extremely low frequency of heteroplasmic m.11778G>A in Chinese patients appears to be different from that of European patients or patients of European origin. In six LHON families with the mutation m.3460G>A that we analyzed, we only identified one family with a heteroplasmic mutation, and the proband of this family had a heteroplasmic mutation load of about 40%. Interestingly, the penetrance of LHON symptoms in this family was 12.5%, which was significantly lower than the average penetrance of families (25.6%) with a homoplasmic mutation (Yu et al. 2010a). The heteroplasmy status of m.3460G>A may be one of the reasons for the low penetrance of LHON in this family (Yu et al. 2010a), which is consistent with previous findings that only 20% of heteroplasmic carriers, but 47% of homoplasmic carriers, manifested the disease in a Thai population (Phasukkijwatana et al. 2006).

In addition, there are sporadic reports of the presence of two LHON mutations in one family. Brown et al. (2001a) reported a 9-year-old Caucasian girl carrying both the m.11778G>A and m.14484T>C mutations, with m.11778G>A in a heteroplasmic status. Despite carrying two pathogenic mutations, the patient's mother was apparently normal (Brown et al. 2001a). Tonska et al. (2008) reported a Polish LHON family with both the m.11778G>A and m.3460G>A mutations, with m.3460G>A in a heteroplasmic status. Except for typical LHON symptoms, no other nervous system disorders were found in this family, and all the female members were unaffected (Tonska et al. 2008). These studies seem to indicate that heteroplasmic mutations may be linked to fewer manifestations of LHON.

4 Effect of Secondary Mutations and mtDNA Genetic Background

According to the clinical observation, different families present a variable penetrance of LHON symptoms, ranging from 10 to 100%, albeit harbouring the same primary mutation, as shown by the two families with m.11778G>A in Han Chinese (Wang et al. 2008). Secondary mtDNA mutations and mtDNA haplogroup genetic background appear to have some effect on the penetrance of LHON. These two factors may not lead to LHON directly, but can act in synergy with primary mutations to affect the onset of LHON.

4.1 The Secondary Mutations May Act in Synergy with the Primary Mutations

Most of the secondary mutations are common variants. For instance, variant m.12811T>C, which is the defining variants of haplogroup M7b1′2 and has a

high frequency in the general population (Kong et al. 2006), was considered to be one of the secondary mutations of LHON (Huoponen et al. 1993). We have obtained consistent results to show that haplogroup M7b1′2 is an important risk factor for the development of LHON in Han Chinese patients with m.11778G>A mutation (Ji et al. 2008; Zhang et al. 2011b). Intriguingly, m.12811T>C also defined a clade of H3h in European populations or populations of European origin, which also shows an increased prevalence of LHON (Bandelt H-J et al. unpublished data). All these results suggested m.12811T>C as being a secondary mutation for LHON, both in Chinese and European populations. Also the mutation m.593T>C is a common variant found in the *MT-tRNA*^{Phe} gene. Our recent study revealed that this variant may lead to altered structure of *MT-tRNA*^{Phe} and thus acts in synergy with m.11778G>A to increase the penetrance of LHON (Zhang et al. 2011a). Note that many of the secondary mutations of LHON are haplogroup-defining variants and have a much higher frequency than the primary LHON mutations. Just why some of these secondary mutations are associated with LHON only in some populations, but not others, is unresolved.

4.2 mtDNA Haplogroup Background Effect

Because of high mutation rate and lack of recombination, mtDNA accumulates mutations in a time order within a relatively short span of time and forms different haplotypes (Pakendorf and Stoneking 2005). In the process of human evolution and migration, descendants of an original mtDNA haplotype would sequentially accumulate additional mutations to generate a group of related haplotypes that was termed as a haplogroup (Achilli et al. 2008; Kong et al. 2006; Torroni et al. 2000). The current global mtDNA phylogenetic tree contains over 4,000 different haplogroups, which form a region-specific distribution pattern (Achilli et al. 2008; Kong et al. 2010; Kong et al. 2006; van Oven and Kayser 2009). mtDNA haplogroups have become important genetic markers for different populations. In the past decades, mtDNA haplogroups have also been identified to be associated with many different metabolic and neurodegenerative diseases. As early as in 1992, Brown et al. (1992) found that mtDNA haplotype (5244-13708-15257-15812) was associated with significantly increased risk for the onset of LHON. Later, Torroni et al. (1997) analyzed mtDNA haplogroup in Italian LHON patients and identified haplogroup J as a risk factor for patients with m.11778G>A and m.14484T>C. Shafa Shariat Panahi et al. (2006) found that haplogroups J and W increased disease risk in Iranian LHON patients carrying m.11778G>A and m.3460G>A, respectively. However, the sample size of these studies was too small to obtain a firm conclusion. In recent years, further studies have conducted to investigate the association between mtDNA haplogroup and LHON. One of the most comprehensive studies was carried out by Hudson et al. (2007b), who analyzed mtDNA haplogroups in 3,613 individuals from 159 - European LHON families and found that haplogroups J2, J1 and K increased the LHON risk in patients with m.11778G>A, m.14484T>C and m.3460G>A,

respectively, while haplogroup H was a protective factor for patients with m.11778G>A. Phylogenetic analysis indicated that the nonsynonymous variants located in the root of haplogroups J and K may account for the association between these haplogroups and LHON (Hudson et al. 2007b).

Because the genetic structure is different between Asian and European populations, we have performed a comprehensive study, with the largest number of LHON patients in the Han Chinese population including 1,859 individuals from 182 Chinese families (including one from Cambodia) with the m.11778G>A mutation, to investigate whether mtDNA haplogroups affect the penetrance of LHON in East Asian families. Haplogroup M7b1′2 was identified to be significantly associated with visual loss, whereas haplogroup M8a showed a protective effect (Ji et al. 2008). Further analyses of the complete mtDNA sequences from LHON families with m.11778G>A suggested that variants m.12811T>C (p.Y159H) in the *MT-ND5* gene and m.8584G>A – m.8684C>T (p.A20T – p.T53I) in the *MT-ATP6* gene, which are haplogroup-defining variants for haplogroups M7b1′2 and M8a, respectively, might account for the association of these two haplogroups with LHON (Ji et al. 2008). Subsequently, we analyzed the matrilineal structure of further 304 Chinese patients with m.11778G>A and 843 patients with suspected LHON, to determine the effect of the mtDNA genetic background on the onset of disease (Zhang et al. 2011b). The enlarged sample size of LHON patients confirmed the results that were obtained in our previous study based on 182 families (Ji et al. 2008). Specifically, the LHON samples differed from the general Chinese or samples with suspected LHON by harbouring fewer lineages belonging to haplogroup F and more lineages belonging to M7b and D4. In contrast, the matrilineal structure of the suspected LHON population resembled that of the general Chinese population, suggesting no role of mtDNA haplogroup in these suspected LHON patients (Zhang et al. 2011b). In Southeast Asian population, haplogroup B5a1 was identified to be significantly associated with m.11778G>A and appeared to modify the risk of visual loss (Kaewsutthi et al. 2011).

Although association studies have indicated that the onset of LHON is influenced by mtDNA haplogroup, functional characterization of this effect is a daunting task. There have been several attempts that have increased our knowledge regarding the background effect. For instance, Suissa et al. (2009) found that ancient mtDNA variants of haplogroup J reduced the replication and stability of mtDNA. Using cybrid cells with different mtDNA haplogroup backgrounds, Ghelli et al. (2009) found that haplogroup J increases the sensitivity of cells to 2,5-hexanedione toxicity, indicating that the mtDNA haplogroup background affects the development of LHON in combination with environmental factors. However, tests of respiratory function in cybrid cell lines carrying European mtDNA haplogroups (including J) that were demonstrated to be associated with LHON showed no detectable differences in multiple parameters, suggesting that the effect of the mtDNA haplogroup may be more complex than what we had thought (Carelli et al. 2002).

Note that a search of the 30,000 mtDNA sequences (data searched on June 25, 2015) held by the GenBank database shows that many of the European sequences

containing a primary LHON mutation do come from the subgroups of haplogroup U, a feature which as yet has not raised the attention of researchers.

4.3 Occurrence of mtDNA Pathogenic Mutations with the Primary Mutations

Besides common variants, there were some pathogenic mutations for other mitochondrial diseases coexisting with the LHON primary mutations. Mimaki et al. (2003) reported one 51-year-old Japanese patient carrying both m.11778G>A and m.12192G>A (a pathogenic mutation for cardiomyopathy). The initial symptom of this patient was rapid reduction of weight, with acute visual loss developing 6 months later. We reported a LHON family with two pathogenic mutations m.11778G>A and m.1555A>G, which may account for the high penetrance (78.6%) of LHON in this family (Zhang et al. 2008). Mutation m.1555A>G is the major cause of aminoglycoside-induced and non-syndromic hearing loss (Guan 2011; Kokotas et al. 2007; Petit et al. 2001). However, none of the matrilineal relatives showed problems with their hearing, and we therefore inferred that the mutation m.1555A>G in this family may act in synergy with m.11778G>A to significantly increase the penetrance of LHON (Zhang et al. 2008). Interestingly, the co-occurrence of m.1555A>G and m.11778G>A was also observed in two Indian families recently, with one family showing a high penetrance of LHON (62.5%) (Khan et al. 2013).

4.4 mtDNA Mutational Hotspots in Suspected LHON Patients

Though most of the LHON patients have been identified to carry known mtDNA primary mutations, there still are many patients with typical clinical features of LHON but without known mtDNA primary mutations. These patients have not been fully investigated, and genetic analysis has revealed that there might be mtDNA mutation hotspots in these patients. Studies in European populations revealed that the *MT-ND1* and *MT-ND6* genes were mutational hotspots for nonsynonymous mutations (Chinnery et al. 2001b; Valentino et al. 2004), whereas in our study, albeit with a limited number of Han Chinese families, we showed that the *MT-ND1* and *MT-ND5* genes are mutational hotspots in families with suspected LHON (Zou et al. 2010). However, it remains to be answered why the mutations occur mainly in these gene regions, how these mutations affect the onset of LHON and if there are any other nuclear modifiers to these mtDNA mutations (Yen et al. 2006).

5 Effect of Nuclear Genes on LHON

5.1 Chromosome X

The gender bias of LHON suggests the chromosome X genes may be involved in this disease (Bu and Rotter 1991, 1992). According to the proposed two-locus mitochondrial and X-chromosome-linked nuclear gene model (Bu and Rotter 1991, 1992), LHON symptoms occur only when the mtDNA pathogenic mutations and chromosome X-sensitive gene(s) are present at the same time and the onset in female carriers was dependent on the homozygous status of susceptible gene(s) in chromosome X. This model is a reasonable explanation of the gender bias of LHON, and different studies have identified several regions in chromosome X showing linkage with LHON. The pioneer linkage study in six LHON families performed by Vilkki et al. (1991) found that the onset of LHON is determined by genes linked to the DXS7 region of chromosome X. The similar study performed by Hudson et al. (2005) in 100 European LHON families identified that the DXS8090 (166)-DXS1068 (258) region of chromosome X is linked to the onset of LHON. Also a study in a Brazilian LHON family found that region Xq25-Xp27.2 of chromosome X confers susceptibility to LHON (Shankar et al. 2008). There were relatively few studies performed in Chinese patients. Recently, Ji et al. (2010) analyzed 12 microsatellite markers and 4 single nucleotide polymorphisms (SNPs) in chromosome X in Chinese male LHON patients and controls and identified two microsatellites (DXS6803 and DXS984) that are associated with LHON. However, there were negative results reporting no association between chromosome X and LHON (Man et al. 2002a; Oostra et al. 1996; Pegoraro et al. 1996; Petruzzella et al. 2007). How genes in chromosome X affect LHON is still a mystery. The recent study performed by Hudson et al. (2007a) indicated that the oestrogen receptor-related genes in chromosome X may be susceptible genes for LHON. These results need to be validated, and further functional characterization should be carried out to elucidate the cause of the male-biased prevalence of LHON.

5.2 Other Nuclear Factors

A nuclear genetic effect has been thought to account for the puzzle posed by the incomplete penetrance of LHON, and identification of nuclear modifier(s) has received wide attention from researchers in the field. A previous study focusing on oxidative stress and apoptosis pathways identified *EPHX1* and *TP53* genes as candidate nuclear genes associated with earlier onset of LHON (Ishikawa et al. 2005). Recent genome-wide expression and genome-wide linkage studies have further explored the potential nuclear genes that may play a modifying role in LHON. In the genome-wide linkage study performed by Phasukkijwatana et al. (2010) in patients from Thailand, chromosome region 3q26.2-3q28 was found to be linked to LHON. This genomic region contains six genes, including *PARL*, *OPA1*

and others. Further analysis of SNPs in the *PARL* gene indicated that SNPs rs3749446 and rs1402000 conferred a susceptibility to LHON (Phasukkijwatana et al. 2010). However, the association between *PARL* SNPs and LHON could not be replicated in the Han Chinese population in our validation study (Zhang et al. 2010). The *OPA1* gene is a pathogenic gene for autosomal dominant optic atrophy (ADOA), which presents many overlapping clinical features with LHON (Carelli et al. 2004). Previous studies have revealed that the OPA1 protein is located in the mitochondrial inner membrane and plays important roles in mitochondrial fusion and apoptosis (Olichon et al. 2003). Interestingly, PARL is also a mitochondrial protein and was recently found to regulate OPA1 in the apoptosis pathway (Cipolat et al. 2006). In the genome-wide expression assay in LHON patients and controls performed by Amero-Abu et al. (2010), 137 up-regulated and 152 down-regulated genes were identified, in which the *OPA1* gene was found to be significantly down-regulated in LHON patients. Whether the OPA1-PARL pathway plays a key role in the onset of optic atrophy, from either LHON or ADOA, is an important and interesting question remaining to be answered.

A recent profiling of the mitochondrial proteome of fibroblasts from LHON patients with m.11778G>A showed a down-regulation of bioenergetics and mitochondrial protein quality control pathways (Tun et al. 2014). Coincidentally, Giordano et al. (2014) found that unaffected LHON mutation carriers had a significantly higher mtDNA copy number and mitochondrial mass and higher capacity for activating mitochondrial biogenesis under metabolic demand compared with their affected maternally related relatives and healthy individuals. These findings suggest that increased mitochondrial biogenesis in LHON mutation carriers may overcome some of the pathogenic effect of the LHON mutation, and this may account for the incomplete penetrance in LHON (Giordano et al. 2014). These efforts aiming at identifying nuclear modifiers in LHON have provided some helpful information for uncovering the puzzle of LHON, although we suggest that more studies are essential to define the modifier genes and their regulatory mechanisms in biochemical pathways.

6 Environmental Factors

The gender bias and incomplete penetrance of LHON strongly suggest that environmental factors which are more specific to males than to females, such as smoking and alcohol, may contribute to the increased disease risk of LHON in males (Charlmers and Harding 1996; Kerrison et al. 2000; Kirkman et al. 2009). A study by Charlmers and Harding (1996) showed that alcohol, but not smoking, may significantly increase the penetrance of LHON. However, different observations were obtained in a recent large-scale epidemiological study by Kirkman et al. (2009), which showed that smoking is significantly linked to the penetrance of LHON, independent of gender and alcohol intake, and excessive drinking would lead to increased risk of LHON. Furthermore, a recent study found that oestrogens could rescue the mitochondrial dysfunction caused by LHON primary mutations

(Giordano et al. 2011), which may underline the potential reason for the gender bias of LHON. There are some anecdotal reports for the effect of exposure to toxic substances or medicines, such as carbon monoxide poisoning (Hwang and Park 1996) and anti-tuberculosis medication including ethambutol (Seo et al. 2010), to increase the clinical expression of LHON.

7 Biochemical Effects of mtDNA Mutations and Their Implication for LHON Treatment

7.1 Biochemical Effect of mtDNA Mutations

It is of note that almost all the primary mutations and mutational hotspots for LHON are located in genes that encode the subunits of complex I in the mitochondrial respiration chain. For instance, mutations m.3460G>A (p.A52T) and m.3635G>A (p.S110N) occur in the MT-ND1 subunit, mutation m.11778G>A (p.R340H) is located in the MT-ND4 subunit and mutation m.14484T>C (p.M64V) is located in the MT-ND6 subunit, whereas mutation m.10680G>A (p.A71T) is located in the MT-ND4L subunit. All these mutations change the amino acids in different complex I subunits and were considered to cause LHON through impairing the function of complex I (Fig. 3) (Yen et al. 2006). Mitochondrial complex I, also known as NADH dehydrogenase, is crucial for cellular metabolism and ATP production (Hirst 2013). In addition, complex I is the major source of reactive oxygen species (ROS) (Hirst 2013). A large number of studies have showed significantly decreased complex I activity and respiration rate, as well as ATP level, in cybrids, fibroblasts or lymphoblasts with LHON pathogenic mutations (Baracca et al. 2005; Brown et al. 2000; Cock et al. 1999; Hirst 2013; Hofhaus et al. 1996; Lin et al. 2012; Majander et al. 1996; Wong et al. 2002). It is suggested that the increased oxidative stress caused by complex I dysfunction (Beretta et al. 2004; Wong et al. 2002) leads to a series of mitochondrial defects and further triggers the clearance of damaged mitochondria, which is also known as mitophagy (Melser et al. 2015). The excess of mitophagy might be the major reason for the decreased level of mitochondrial mass, as well as ATP production observed in LHON (Giordano et al. 2014). In this way complex I dysfunction may eventually lead to increased apoptosis and cell death (Hofhaus et al. 1996; Wong et al. 2002; Zanna et al. 2003) and be the potential reason for the optic neuron loss observed in LHON patients and animal models (Fig. 3). Consistent with this hypothesis, the chemically induced mouse model initiated by the use of rotenone, an inhibitor of complex I, presented similar degeneration of retinal ganglion cells as in LHON (Zhang et al. 2002). Therefore, complex I dysfunction induced by LHON primary mutations may well be a key pathogenic cause for the onset of LHON symptoms.

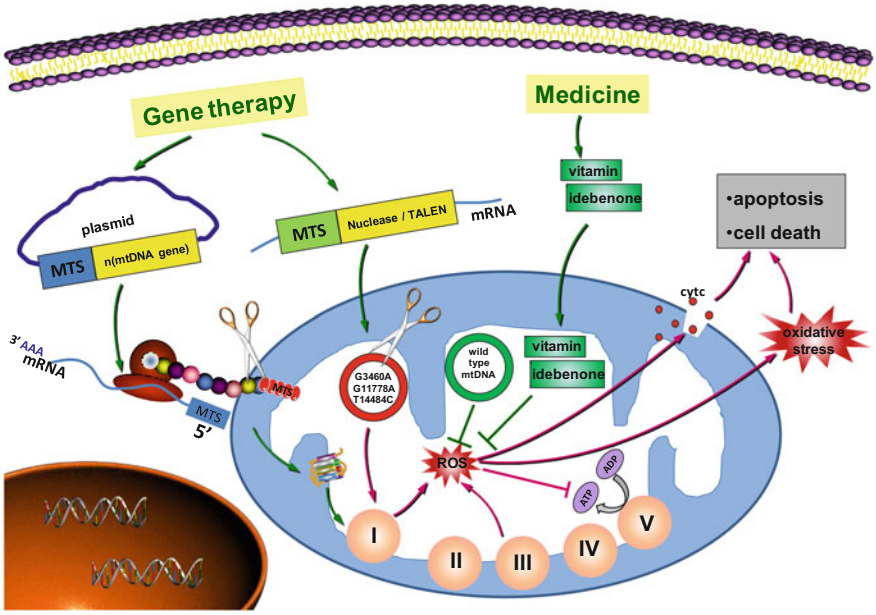

Fig. 3 Potential pathogenic role of LHON mutations in the cell and treatment strategy. The dysfunction of complex I due to LHON mutations leads to the overproduction of ROS, and the significantly increased oxidative stress triggers a series of mitochondrial-related dysfunctions and increased apoptosis and cell death (Yen et al. 2006). Antioxidants like idebenone might therefore be potentially effective medicines (Mashima et al. 2000). Gene therapy through allotopic expression of mitochondrial genes (Guy et al. 2002) or genome-editing technique using mitochondria-targeted restriction endonucleases or TALENs (Reddy et al. 2015) may also increase the percentage of wild-type mtDNA and prevent the germline transmission of pathogenic mtDNA mutations and is, therefore, another promising treatment for LHON symptoms

7.2 Functional Characterization of the LHON Primary Mutations

The pathogenicity of the LHON primary mutations needs to be proven by the use of functional assays (Bandelt et al. 2009). One feasible approach is to develop cell cybrids using blood platelets as these cells do not have nuclei (King and Attardi 1989), and this is now the most frequently used method to study the direct association between mtDNA variants and alterations in mitochondrial function. Early studies of the cybrids with LHON primary mutation m.11778G>A showed a significantly decreased oxygen consumption, demonstrating a pathogenic role of this mutation in impairing cell respiration (Vergani et al. 1995). A later study, however, showed mutations m.3460G>A and m.11778G>A could result in complex I defects, whereas m.14484T>C caused only a much milder biochemical defect (Brown et al. 2000). In addition, LHON primary mutations were found to disrupt glutamate transport in cybrid cell lines (Beretta et al. 2004).

Besides the trans-mitochondrial cybrids, the allotopic expression system, in which the mtDNA gene was converted to a nuclear-encoded version and the protein

was first expressed in the cytosol and then imported into mitochondria with the direction of mitochondrial targeting peptide (Fig. 3) (Manfredi et al. 2002), has also been demonstrated to be efficient for the functional characterization of certain mtDNA mutation(s) in our recent study (Bi et al. 2015) and others (Guy et al. 2002; Manfredi et al. 2002). Through allotopic expression of wild-type *ND4* gene in cybrids with m.11778G>A, Guy et al. (2002) found that this method could efficiently rescue the defective complex I-dependent ATP production and cell growth in galactose medium. However, because of the high hydrophobicity of mtDNA-encoded protein, the efficiency of this method was questioned in many studies (Figueroa-Martinez et al. 2010; Oca-Cossio et al. 2003). Kaltimbacher et al. (2006) optimized the allotopic expression system, in which the mRNA was first targeted to the mitochondrial surface and then the protein translation was coupled with protein transport. Allotopic expression of wild-type *ND1* and *ND4* genes in fibroblasts containing m.3460G>A and m.11778G>A using this optimized method successfully restored the complex I activity and mitochondrial dysfunction induced by the LHON primary mutations (Bonnet et al. 2008). Using the same method, Ellouze et al. (2008) introduced the *ND4* gene harbouring the m.11778G>A mutation into rat eyes by in vivo electroporation and developed an animal model that mimics the essential aspects of LHON, including degeneration of retinal ganglion cells and decline in visual performance. Further overexpression of the wild-type *ND4* gene in these LHON models could improve these signs.

7.3 New Avenues of Research: LHON Gene Therapy and Genome Editing

In order to accomplish a more efficient gene delivery, the allotopic expression method was further developed by injecting adeno-associated virus (AAV) with mtDNA genes into eyes (Chadderton et al. 2013; Koilkonda et al. 2014; Yu et al. 2012). Koilkonda et al. (2014) estimated the safety and effect of this AAV mediate allotopic expression system in non-human primate and ex vivo human eyes and found that the allotopic expression was efficient and long lasting with no serious adverse reactions in most retinal ganglion cells. These studies indicated that the gene therapy through allotopic expression of mitochondrial genes may be a promising method in the treatment of LHON (Fig. 3). Also, Iyer et al. (2012) had success by using healthy donor mtDNAs complexed with recombinant human mitochondrial transcription factor A (TFAM) to improve respiration and biogenesis in a LHON cell line. Recently, further work of this type has been reported from France (Cwerman-Thibault et al. 2014; Cwerman-Thibault et al. 2015), and a trial in carriers of the mutation m.11778G>A has been initiated. However, evidently, there is still a long way to go before this method is fully accepted as a therapy.

Another promising approach for LHON therapy is mitochondrial replacement, which involves spindle, pronuclear or polar body genome transfer into healthy enucleated donor oocytes or embryos, and this approach has been successfully used to eliminate pathogenic mtDNA variant(s) and to prevent the transmission of

mtDNA disease (Craven et al. 2010; Paull et al. 2013; Tachibana et al. 2009, 2013; Wang et al. 2014). More recently, Reddy and colleagues (2015) took advantage of the recently advanced genome-editing technique and selectively prevented the germline transmission of a mtDNA haplotype using either mitochondria-targeted restriction endonucleases or transcription activator-like effector nucleases (TALENs). They successfully reduced LHON mutation level in mammalian oocytes using mitochondria-targeted TALENs (mito-TALENs). We believe that the most recently available CRISPR/Cas system, which is very powerful for genome editing (Cong et al. 2013), will also be a workable method for mtDNA genome editing in the future. The safety and efficacy of this mtDNA genome-editing approach open a new avenue for LHON therapy (Fig. 3). However, there are also potential concerns about the use of these techniques in a clinical setting (Craven et al. 2011). Besides the complex technical issues, all these techniques still need to be confirmed as being safe for use in humans.

7.4 Implications for Medical Therapy for LHON

There is currently no effective treatment that can be proven to prevent, or restore, the visual loss in LHON (La Morgia et al. 2014; Yu-Wai-Man et al. 2014). But several different approaches and therapies have been suggested. LHON mutation carriers may have their symptoms precipitated by vitamin B12 deficiency, and known carriers should have an adequate dietary intake of vitamin B12 (Pott and Wong 2006). Spontaneous visual recovery is observed in some LHON patients, and idebenone in combination with vitamin B2 and vitamin C has been suggested to speed up this recovery (Fig. 3) (Mashima et al. 2000). Idebenone is described as an antioxidant (Mordente et al. 1998) and transports electrons bypassing complex I to complex III and thereby partly restores ATP production in cases where there is a complex I defect (Giorgio et al. 2011; Haefeli et al. 2011). Pharmacological study in a LHON mouse model with complex I dysfunction induced by rotenone indicated that idebenone can restore the loss of retinal ganglion cells and vision defects in a LHON mouse model (Heitz et al. 2012). In recent years, more evidence has shown that LHON patients might benefit from idebenone treatment (Carelli et al. 2011; Klopstock et al. 2011, 2013), suggesting that agents active against complex I dysfunction and oxidative stress may be an efficient treatment for LHON in the future (Fig. 3). However, the question of whether or not a new LHON patient should be offered treatment with idebenone is still unproven.

Another approach to therapy for LHON is promising but still very much at the theoretical stage and involves considering how to control the balance between mitochondrial biogenesis and clearance (mitophagy). Mitochondrial biogenesis is regulated by a complex system of transcriptional regulator networks of mitochondrial proteins (Vega et al. 2015). Furthermore, it was reported that the mitochondrial biogenesis and mitophagy could be regulated through the post-translational modification of mitochondrial proteins (i.e. lysine acetylation), which was found to be modified by the nutritional homeostasis (Webster et al. 2014). There are many

agents, chemicals and medicines that are known to reduce mitochondrial biogenesis, such as antibiotics targeting mitochondria like erythromycin, tetracycline, chloramphenicol and glycylcyclines (Lamb et al. 2015). Recent studies have identified several potentially therapeutic agents that could stimulate key regulators of mitochondrial biogenesis (Sanchis-Gomar et al. 2014; Valero 2014), including activators of the PPAR-PGC1α axis, bezafibrate, thiazolidinediones, pioglitazone, rosiglitazone and fenofibrate (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Yadav et al. 2014; Zamora and Villena 2014); agonists of AMPK, resveratrol, AICAR and metformin (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Zamora and Villena 2014); triggering factors of SIRT1, SRT1720, quercetin, resveratrol and several isoflavone-derived compounds (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Zamora and Villena 2014); and the agonists of Nrf2, triterpenoids and *Bacopa monnieri* (Yadav et al. 2014). However, the hypothesis to increase ATP production at times of stress through increasing mitochondrial mass has not been clinically proved. But it is an interesting line of research that may be of significance to carriers of LHON mutations, as the possibility of taking a simple medication to prevent the onset of symptoms is a pleasing prospect.

8 Conclusion and Future Perspective

LHON, the most extensively studied mitochondrial disease, predominantly causes blindness in young males and brings a lot of suffering to the patients and their families. Although many primary mtDNA mutations have been identified, the phenotypic expression of these primary mutations is very complex. Incomplete disease penetrance, male-biased onset, involvement of additional genetic factors (secondary mutations, mtDNA background, nuclear genetic modifiers, epigenetic changes) or environmental factors (smoking, alcohol, toxic substance) are all involved in the pathogenesis of the disorder; but their relevance has not been fully understood. Currently, there is no effective treatment for LHON. Studies in the past two decades have shown us that complex I dysfunction is the main biochemical effect of the pathogenic mutations and gene therapy or medicine aimed against complex I dysfunction are promising treatments for LHON. The new techniques of genome editing also offer the possibility of a successful treatment for LHON. Further studies focusing on the underlying mechanism of LHON will be of importance in elucidating the pathophysiology of retinal neuron loss as well as in searching for new clues that may finally prevent the visual loss caused by LHON.

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