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Identification of Novel Mutations in Complex I Subunit Mtdna in Patients with Leber's Disease

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Abstract

Introduction: Leber Hereditary Optic Neuropathy (LHON) is a rare disease with bilateral optic atrophy, the main cause is mitochondrial genetic mutations associated with dysfunction of the complex I mitochondrial electron transport chain. This study is conducted to determine the spectrum of new mutations in seven subunits of the mitochondrial complex I.

Methodology: The subunit genes of the mitochondrial complex I of 47 Iranian leber patients were sequenced and analyzed with the Sanger method.

Discussion and Results: LHON's unique maternally inherited trait, linked to mitochondrial DNA (mtDNA) point mutations, impacts complex I subunit genes, particularly m.G11778A MTND4, m.G3460A MT-ND1, and m.T14484C MT-ND6 mutations in about 90% of cases. But we found new mutations other than primary mutations that could be important. New variants have been found in the mitochondrial complex I subunit, the frequency of these changes in ND1, ND4, ND5, and ND6 genes were equal to 17%, 12%, 34%, and 37%, respectively. The lately found m.A11775G MTND4 gene with 0.2% of allelic frequency in 2 patients, m.C13540G MTND5 with an allelic frequency of 0.07%in 1 patient, m.T14441A MTND6 with 0.3% of allelic frequency in 4 patients, m.T14503A MTND6 with 0.3% of allelic frequency in 4 patients, and m.A14496T MTND6 with 0.07% of allelic in 1 patient frequency have been reported. The mentioned findings demonstrated the existence of previously unknown mutations associated with LHON, highlighting the importance of ongoing mutation screening to increase our understanding of the genetic variation associated with this disease.

Keywords: mtDNA; LHON; Complex I

Abbreviations

LHON: Leber's Hereditary Optic Neuropathy; ATP: Adenosine Triphosphate; RGCs: Retinal Ganglion Cell; PMB: Papillomacular Bundle; NIGEB: National Institute of Genetic Engineering and Biotechnology; NCBI: National Center for Biotechnology Information.

Introduction

Leber's hereditary optic neuropathy (LHON, OMIM#535000) is a maternally inherited disorder characterized by acute

or subacute central vision loss, leading to central scotoma and blindness [1-3]. The prevalence of LHON is *1:50,000. The peak age of onset in LHON is in the second and third decades of life, with 90% of those who lose their vision doing so before age 50 years [2]. It is also the most common disease caused by mutations in the mtDNA [4]. Incomplete penetrance and male bias of LHON indicates that there must be other factors associated with LHON, such as nuclear gene(s), mtDNA background and environmental factors [5] was first reported by Theodor Leber in 1871 in Germany [6]. Mutations in mitochondrial genes can decrease adenosine triphosphate (ATP) production and increase oxidative stress, causing retinal ganglion cell (RGCs) apoptosis. Maternal transmission of Leber's Hereditary Neuropathy suggests that mutations in mtDNA are involved. So far, more than 30 mutations in mitochondrial DNA have been identified (http://www.mitomap.org/MITOMAP), which are associated with Leber's disease. In most cases, three common mutations in the genes that code subunits of complex I of mitochondrial DNA include 80% to 95% of Leber cases [7,8]. Defects in mitochondrial NADH dehydrogenase complex I lead to vision loss in individuals. These mutations include m.11778G>A (MT-ND4), m.3460G>A (MT-ND1), and m.14484T>C (MT-ND6), which in previous studies were associated with complex I deficiency. It has shown mtDNA respiratory chain [9], oxidative stress, sensitivity to cell death [10], and defects in mitochondrial biogenesis [11].

The later stage presents ophthalmoscopic changes, including edema of the retinal nerve fiber layer, tortuosity of the small vessels, and peripapillary microangiopathy [12,13]. More thorough testing may uncover more subtle signs of optic nerve dysfunction. Asymptomatic carriers of LHON mutation 1178 might experience dyschromatopsia with mainly red/green color discrimination and reduced spatial contrast sensitivity, as reported in the literature [14]. "Not all individuals who carry the mutation suffer from visual impairment" [15]. The disease starts with a sudden, painless loss of central vision. About 25% of cases involve both eyes being affected at the same time. Although the disease usually affects both eyes, it does so at different times. Initially, only one eye is affected, and the other eye becomes damaged within a year. Vision loss is painless, but there may be rare discomfort due to inflammation of the optic nerves [16,17]. A sudden onset of blurred and cloudy vision is the most common initial symptom of the acute phase of LHON [12,18-20]. Presymptomatic ophthalmoscopic manifestations may persist throughout life in many patients [12]. Once central vision disappears, new ophthalmoscopic features may appear, such as edema of superior and inferior fiber arcades and axonal loss within the papillomacular bundle (PMB) [12,21,22]. These fundus changes are visible either before or after vision loss [23]. Vision deterioration worsens after four to six weeks, with visual acuity usually at 6/60. Loss of color perception and either central or centrocaecal scotoma is also evident [24,25]. Although this phase typically lasts for several weeks, around 20% to 30% of patients do not exhibit any ophthalmoscopic abnormalities during the acute phase [26]. Later, the edema of the nerve fibers decreases, and the chronic phase is typically reached more than a year after the onset of the disease [27]. Due to the premature failure of the PMB, most of the remaining nerve fibers degenerate, resulting in complete optic atrophy, which is more pronounced on the temporal side [19,28,29]. Although individuals with the 14484 mutation or aged under 20 years may recover spontaneously, vision recovery is typically minimal, and the prognosis for visual recovery is poor [19,30,31]. In the chronic phase, vision loss is typically not progressive, and most patients are considered legally blind [32,33]. Vision loss stabilizes and reaches its lowest point within 6-12 months after the onset of the disease [26,31]. If a patient is diagnosed in the late stages with no maternal history, a brief differential diagnosis is necessary. It takes at least one month to obtain the results of molecular testing.

It is important to conduct thorough testing, including neuroimaging of the retina, optic nerves, chiasm, and optic tracts, without delay to accurately diagnose and treat the condition. Gender and mutation status do not influence the timing or severity of vision loss [12,28]. LHON is a disease that is often misdiagnosed. It is important to correctly identify LHON because it can be mistaken for other conditions such as isolated optic neuritis or neuromyelitis optica. Paying close attention in the clinical setting is crucial for accurately identifying the disease and ruling out other potential causes [34,35]. Molecular genetic testing is conducted to identify LHON by looking for pathogenic variants of mtDNA through a multi-gene panel or complete sequencing of mtDNA. While specific mutations in mtDNA are important, other factors such as medical history, fundus, OCT, and MRI are also relevant for diagnosing the disease [2]. Some studies suggest obtaining an electrocardiogram for all LHON patients due to potential cardiac abnormalities [12,36-38]. The most severe known mutation is 14459GNA, which typically causes a wide range of clinical symptoms, mostly resulting in a LHON plus phenotype (additional neurological features accompanying typical optic nerve atrophy) [39]. However, patients presenting only with classical LHON are also observed [40]. 14459G>A cannot be easily compared to the three aforementioned common mutations. The 3460G>A mutation is considered severe, 11778G>A is intermediate, whereas 14484T>C is rather mild [40]. A minority of cases are attributed to other mtDNA mutations, mostly in ND genes [40]. Some of these mutations have been observed in several independent families, but many potentially pathogenic mutations still need confirmation [40]. Although LHON is a well-studied mitochondrial disorder, the exact mechanism of its pathology is still not fully understood. Since the mitochondrion is the cell's powerhouse, the

most obvious explanation was that the mtDNA defect leads to a significant decrease in energy production and failure in optic nerve function. However, experimental data was confusing, as the rather small decrease in energy production for some mutations could not explain all the observed effects. As a result, alternative explanations such as increased oxidative stress, apoptosis, and altered axonal transport of the organelles have been proposed [40]. The main purpose of this study was to investigate the spectrum of new mutations in seven subunits of complex I mtDNA [11]. Given that the variants of each population are different from the others, investigating mutations in each region is of special importance in treating various diseases [41].

Material

The study included a sample of 47 individuals with LHON disease, whose diagnosis was confirmed by a relevant physician. With individual consent, approximately 2 cc of blood was collected from the antecubital vein while the participant was sitting. The blood was then transferred to a tube containing EDTA [42].

DNA Extraction

Total DNA was isolated from peripheral blood using the National Institute of Genetic Engineering and Biotechnology (NIGEB) kit. The quality and quantity of the extracted DNA was checked using the nanodrop device [43]. The A260/A280 ratio was used to determine the qualitative characteristics of the DNA by measuring the amount of light absorption at a wavelength of 260 nm.

Polymerase Chain Reaction (PCR)

The seven ND mtDNA genes were amplified in 7 segments and were screened for mutations in the patients. Primer sequences for these fragments are as described in Table 1. A total volume of 50 µl, containing 50-100 ng/µl mitochondrial DNA, 7 pmol of each primer, 0.5 mM dNTP, 1.2 mM MgCl2, and 1.5 units of Taq polymerase (Fermentas), was used in a thermocycler (Astec, PC818, Japan). The cycling thermal conditions were as follows: 94°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 10 seconds, 72°C for 1 minute and 40 seconds, and finally 72°C for 10 minutes (Table 1).

Length (bp)	Reverse Primer	Forward Primer
1463	ONP65:5'GGAAATACTTGATGGCAGCT3'	ONP82:5'CTCAACTTAGTATTATACCC3'
1529	ONP67: 5'GGCTCGAATAAGGAGGCTTA3	ONP64:5'GTCATCTACTCTACCTACTT3'
731	ONP70: 5'AGTATTATTCCTTCTAGGCA3'	ONP91: 5'CACTATCTGCTTCATCCGCC3'
214	ONP13:5'GAGGTTAGCGAGGCTTGCTA3'	ONP12: 5'TCGTAGTAACAGCCATTCTC3'
519	ONP74:5'GGTTGACCTGTTAGGGTGAG3'	ONP71:5'TGCTAGTAACCACGTTCTCC3'
1341	ONP2.9:5'GAATTTTGGGGGAGGTTATA3'	ONP2: 5'GCAGTCTGCGCCCTTACACA3'
1076	ONP76: 5'TGTCTACTGAGTAGCCTCCT3'	ONP2.8:5'CACCAACAAACAATGCTGAA3'

Table 1: Sequence of forward and reverse primers for MT-ND gene.

DNA Sequencing

PCR products were checked for specificity utilizing 1.5% agarose gel electrophoresis. Automated double-stranded sequencing was also performed using an ABI 3100 sequencing machine (Applied Biosystems, Kavash Fanavran Kosar, Iran). All fragments were sequenced in both forward and reverse directions to confirm any nucleotide changes [42]. Sequence variants of LHON patients were analyzed by Finch TV (a chromatogram display that shows DNA sequence traces) and compared with the human mitochondrial reference sequence NC_012920 provided by the National Center for Biotechnology Information (NCBI) [44]. The MITOMAP database was searched to determine the sequence types [45,46]. Subsequently, using Polyphen, the possible pathogenicity of the mutations was checked (Table 2).

Locus	Allele	A.A.C	Patient ID	N.C	Percentage of Allelic Abundance	Pathogenicity (Polyphen)
ND1	A4159C	q284P	27	A/C	0.1	PROBABLY DAMAGING 0.999
	A4157C		41			
	C3716G	A137G	10,29	C/G	0.1	PROBABLY DAMAGING 1.000
	G3984A	E227P	46	G/A	0.1	PROBABLY DAMAGING 1.000
		E227N	41			
	T4216A	Y304N	3	T/A	0.1	BENIGN 0.001
	T4217A	Y304M	36	T/A		POSSIBLY DAMAGING 0.839
ND4	A11847C	S363R	9	A/C	0.07	POSSIBLY DAMAGING 0.617
		S363S		A/A		
	G11846A	S363D	26,46	G/A	0.1	Benign (0.250)
	A11845G	S363D	26,46	A/G	0.1	Benign (0.250)
	G11848A	S363D	43,36,48	G/A	0.2	Benign (0.250)
	A11775G	S339x	9, 11	A/G	0.2	PROBABLY DAMAGING (1.000)
		S339G	24	A/G		PROBABLY DAMAGING (0.989)
ND5	A13383G	N349S	6	A/G	0.1	PROBABLY DAMAGING 0.996
		H348R	32			PROBABLY DAMAGING 0.994
	A12469T	I46L	8	A/T	0.1	BENIGN 0.131
		I45F	43			POSSIBLY DAMAGING 0.481
	T13095G	V254G	27	T/G	0.1	PROBABLY DAMAGING 0.997
		M252S	32			PROBABLY DAMAGING 0.967
	C13151G	L272V	18	C/G	0.1	BENIGN 0.452
		P271A	32			BENIGN 0.092
	A13226G	N296R	32	A/G	0.1	PROBABLY DAMAGING 0.998
		I298R	40			PROBABLY DAMAGING 0.998
	A13290T	I317V	32	A/T	0.1	POSSIBLY DAMAGING 0.925
		I319S	43			PROBABLY DAMAGING 0.994
	G13137A	A267Y	33	G/A	0.1	PROBABLY DAMAGING 0.970
		E268K	43			PROBABLY DAMAGING 0.996
	A13045C	M237P	40	A/C	0.1	PROBABLY DAMAGING 0.986
		M237L	43			POSSIBLY DAMAGING 0.698
	C13113A	L260Y	40	C/A	0.1	PROBABLY DAMAGING 1.000
		L260S	43			PROBABLY DAMAGING 1.000
	G13138A	E268K	46,40	G/A	0.1	PROBABLY DAMAGING 0.996
	A13229T	I298S	46,40	A/T	0.1	PROBABLY DAMAGING 0.994
	T13242A	I301N	40	T/A	0.1	PROBABLY DAMAGING 0.998
		V302K	42			PROBABLY DAMAGING 0.999
	G12770A	E145K	42,46	G/A	0.1	PROBABLY DAMAGING 0.996
	C13438T	L368F	9	C/T	0.07	PROBABLY DAMAGING 1.000
	C13540G	S402W	2	C/G	0.07	PROBABLY DAMAGING 1.000

ND6	A14480C	M64G	42	A/C	0.07	PROBABLY DAMAGING 0.997
	A14315C	S120R	27	A/C	0.1	BENIGN0.306
		S120G	42			BENIGN0.084
	T14347A	Y135I	29,46	T/A	0.1	POSSIBLY DAMAGING0.635
	A14330C	V116G	36	A/C	0.1	POSSIBLY DAMAGING0.876
		V115W	37			PROBABLY DAMAGING 0.973
	G14435A	A86V	36	G/A	0.1	BENIGN 0.001
		A85G	40			POSSIBLY DAMAGING 0.921
	A14496T	M63K	36	A/T	0.07	PROBABLY DAMAGING 0.997
	C14355T	V92G	37	C/T	0.2	POSSIBLY DAMAGING 0.666
		V94G	46			POSSIBLY DAMAGING 0.500
		V94I	43			BENIGN 0.003
	T14432G	A79P	37	T/G	0.1	PROBABLY DAMAGING 1.000
		A81G	42			POSSIBLY DAMAGING 0.906
	A14457C	M73G	37,42	A/C	0.1	PROBABLY DAMAGING 0.997
	C14252T	G141M	40	C/T	0.07	PROBABLY DAMAGING 1.000
	C14252A		9	C/A	0.07	
	T14350A	Y109F	42,40	T/A	0.1	BENIGN 0.001
	T14441A	Y78F	16,36,37,40	T/A	0.3	PROBABLY DAMAGING 0.985
	T14503A	L57F	27,37,40,46	T/A	0.3	PROBABLY DAMAGING 0.988
	A14496T	L60W	40	A/T	0.07	PROBABLY DAMAGING 1.000
	C14352A	E132R	40,42	C/A	0.1	POSSIBLY DAMAGING 0.529

Table 2: Analysis of new mutations in MT-ND genes with their pathogenicity percentage.

Mutation Analysis with Polyphen Phenotyping

The pathogenicity of the mutations was determined using polymorphism phenotyping, which is a bioinformatic tool for predicting the effect of amino acid substitution on the structure and function of human proteins [47] (Table 2).

Results and Discussion

Seven mitochondrial complex I subunit genes (mtDNA) from 47 patients with LHON were examined. In one patient out of 47, no new or old mutations were observed. In total, 41 new variants were identified in mitochondrial complex I ND genes and the frequency of each was equal to ND1=17, ND4=12, ND5=34, and ND6=37%, respectively. in this study, 41 new mutations may be involved in the disease LHON. The most changes of 41 variants respectively in ND6 gene include: m.14441T>A with Tyr 78 Phe change in four patients (#16,36,37,40) and pathogenicity 0.98 which has been reported in the clinvar area close to this m.14444T>C mutation in Leigh syndrome The significance of this is still unknown , m.14503T>A with Leu 57 Phe change in 4 patients (#27,37,40,46) and pathogenicity 0.98 is the closest mutation site of m.14502T>C, which is found in diseases of auditory neuropathy spectrum disorder(Pathogenic), Leigh syndrome(Benign), Leber optic atrophy(risk factor), m.14496A>T with Leu 60 X (change to stop codon) and in one patient with 100% pathogenicity The closest mutation to this region is m.14495A>G in ClinVar, which has been reported in Leber optic atrophy (Pathogenic) and Mitochondrial disease (Likely pathogenic (14252C>T with Gly141Met amino acid change was in one patient(#40) with 100% pathogenicity (Table 2).

ND5 includes: m.13540C>G with Ser402X conversion (change to Stop codon) in one patient (#2) with 100% pathogenicity , 13113C>A with change of leucine to two amino acids tyrosine and serine with allelic frequency of 0.1 in two patients and 100% pathogenicity, m.13112T>C The closest locus to this region has been identified in Leigh syndrome with uncertain significance, 13045A> C by changing the amino acid methionine to proline and leucine and the pathogenicity of methionine to proline was 98% and methionine to leucine was 68%, The region m.13046T>C was the closest point in Juvenile myopathy, encephalopathy, lactic acidosis and stroke with the possibility of Likely pathogenic It was reported in a 12-year-old girl with sudden and

painless loss of vision, which shows the overlapping of two diseases LHON/MELAS [48]. ND1 gene mutation 3716C>G with Ala137Gly amino acid change in 2 patients (#10,29) and 100% pathogenicity, The closest locus this mutation was m.3715G>A in Leigh syndrome but Uncertain significance, 3984G>A mutation with glycine to proline and asparagine conversion was observed in 2 patients (#46,41) with 100% pathogenicity.

ND4 gene includes: 11775A>G with the conversion of serine to STOP codon and glycine in two patients (#9,11), serine to stop codon with 100% pathogenicity and serine to glycine 98% were heteroplasmic, and primary mutations were not observed in these patients (Table 2). In this study, the range of mutations of seven subunits of a mitochondrial genome assembly was examined in which 53% of mutations have been found. In similar studies, Lu Q, et al. [49] found a significant G11778A mutation in the ND4 gene by Sanger sequencing in which eight of eleven patients lost their vision. Among the main mutations of Leber patients is G11778A, which was also observed in the study of Lu Q, et al. [49] The similarity of our study with Qian Lu is that mutation A11775G is close to this region, and the corresponding nucleotide change is also like this. This finding shows that it can be of great importance due to the change in the type of amino acid that has become a stop codon and also has this position. In a groundbreaking study on Leber Hereditary Optic Neuropathy (LHON), researchers identified a new homoplasmic mutation, m.3395A>G in the ND1 gene. This mutation alters a highly conserved amino acid at codon 30, previously linked to LHON, causing a complex I defect [50].

In a study by Martínez-Romero I, et al. [51] a patient with typical clinical features of Leber Hereditary Optic Neuropathy but none of the three mitochondrial DNA mutations was identified. Genetic analysis showed that the m.3472T>C transition in the MT-ND1 gene changes phenylalanine at position 56 to leucine. This mutation expands the spectrum of deleterious changes in mtDNA-encoded complex I polypeptides that are associated with this pathology and highlights the difficulties in assigning pathogenicity to new incompletely penetrant homoplasmic mutations in Leber hereditary neuropathy patients. In this study, three variants (m.G3984A, m.C3716G, and m.A4159C) were found in the ND1 gene, all of which were associated with amino acid changes (Table 2). The ND1 gene is known to be a mutational hotspot in LHON, and this subunit is considered one of the most important and conserved subunits [52]. In a study which is conducted by Rezvani Z, et al. [42] sequencing of MT-ND genes from 35 LHON patients showed a total of 44 nucleotide changes. Among these, fifteen specific changes were observed in 27 LHON patients, including A14020G, A13663G, C10399T, C4932A, C3893G, C10557A, C12012A, C12012A, and C120 12A, C149T, C12012A, C14934T, C12012A, C120134, T 4941A, T13255A, T14353C and del A4513 [42].

LHON may be caused not only by mtDNA mutations but also by mutations in nuclear encoded genes. Therefore, other factors, such as mtDNA background, heteroplasmy level of the mtDNA mutation, nuclear genes and environmental factors, have been shown to play critical roles in the phenotypic expression of LHON [42,53,54]. Several missense mutations have been dentified in patients with LHON. Heredity in this disease has incomplete penetration and men are more affected. Houshmand M, et al. [55] investigated mitochondrial mutations in LHON and found that 78% of patients have G11778A mutation and 7% have G3460A mutation. They also reported that 7% of patients had the T14484C mutation and another 7% had the G14459A mutation. They found no association between major Iranian LHON point mutations and haplogroups J and M. This haplogroup polymorphism may affect the activity of the respiratory chain and play a role in the occurrence of the disease. However, Panahi MSS, et al. [55] 90 found that Iranian patients with G11778A mutation are associated with haplogroup J of D ring. They also revealed an association between haplogroup W and the G3460A mutation. The researchers hypothesize that mutations in haplogroups J and W increase the risk of blindness because of the possibility of synergism in intensifying the penetrance of the G11778A and G3460A mutations [55].

Houshmand M, et al. [56] also identified a new alternative in a subgroup of patients with LHON lacking four main mutations (G3460A, G11788A, T14484C and G14459A). G14290A nucleotide substitution has been reported in the ND6 gene, which is a mitochondrial gene and encodes subunit 6 of complex I of the respiratory chain. They found that most of the mutations in this gene occur in a region containing less than 50 amino acids at the end of the amino chain; The part that is the most permanent part of the ND6 gene. In other words, a substitution at this site results in a mutation, not a polymorphism. The stability of this part of the chain during evolution indicates its vital function. Furthermore, mutations in this region can be deleterious [56].

According to previous studies that an unknown percentage of mutations have not yet been identified in some patients, this study was conducted only to identify new mutations. In this study, a total of 41 new variants were found in 47 patients with Leber's disease. Based on whether the nucleotide change leads to an amino acid change and whether these changes are pathogenic or not, as well as the number of repeats in more patients are reported. Based on the new mutations that were found, we reported that the most changes were observed in T14441A, T14503A ND6 and these mutations are of a new type, and no primary mutation was observed in these patients. Also, by referring to related articles, we

showed that in other patients, there are mutations in areas close to the original mutations, not absolutely at one point, but this shows that this spectrum of mutations is related to certain genes. We also showed that novel mutations in Leber patients may be associated with different diseases or perhaps only specific to Leber disease. These mutations can aggravate or ameliorate the disease and show the importance of understanding the function of these mutations.

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