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The *NDUFB11* gene is not a modifier in Leber hereditary optic neuropathy

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Abstract

Over 95% of Leber hereditary optic neuropathy (LHON) cases are due to mutations in mitochondrial DNA-encoded subunits of NADH:ubiquinone oxidoreductase (E.C.1.6.5.3., complex I). A recessive X-linked susceptibility gene that acts synergistically with the primary mtDNA mutation to produce visual loss is suggested by the high male-to-female ratio among LHON patients.

The ESSS protein is a recently isolated subunit of bovine heart mitochondrial complex I. We revisited the genomic sequence of *NDUFB11*, the human homolog mapping to chromosome Xp11.23, and identified two mRNA isoforms showing different expression profiles in human tissues. Cultured skin fibroblasts from four LHON patients showed a pattern of expression similar to normal controls. Moreover, *NDUFB11* did not seem to influence risk and age at onset of visual loss in a total of 65 individuals from 35 Italian LHON families. Also, the gene was not affected in 11 children with a severe encephalopathy associated with decreased complex I activity in skeletal muscle.

Keywords: Leber hereditary optic neuropathy (LHON); NDUFB11; mtDNA; X chromosome

Leber hereditary optic neuropathy (LHON, MIM 535000) is a maternally-inherited disorder affecting about 1/8500 individuals in the general population [1]. It typically presents subacutely in early adulthood and leads to rapid, painless bilateral loss of vision [2,3]. Since the landmark discovery of the first LHON mutation, the disease has been associated with several variants in the mtDNA, although point mutations in three mitochondrial-encoded complex I subunits (3460G>A in *MTND1*, 11778G>A in *MTND4*, and 14484T>C in *MTND6*) account for over 90% of cases [4]. There is a clear sex bias in LHON with a male-to-female ratio of 4.1:1. Moreover, incomplete penetrance and marked intra- and inter-familial variability are observed. The mutation is almost invariably homoplasmic in affected individuals but similar mutant loads might be observed in unaffected or oligosymptomatic maternal relatives, a condition rarely encountered in mtDNA-related disorders [5]. Nearly 50% of men and 90% of women harboring the "primary" LHON-associated mtDNA mutations [6] do not develop visual failure indicating that additional genetic or environmental factors are needed for full penetrance. Segregation analyses are consistent with a nuclear-encoded X-linked susceptibility allele in some pedigrees [7,8] and an X-chromosomal haplotype that interacts with specific mt*ND* mutations to cause visual failure in LHON has

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recently been defined [9]. However, no modifier gene able to account for variable severity, age at onset, and progression of visual impairment has yet been identified [10].

The ESSS protein is a recently identified subunit of bovine heart mitochondrial complex I that is encoded by an X-linked gene (*NDUFB11*) in humans [11,12]. We revisited the structure of *NDUFB11*, analyzed its splicing and expression patterns in several human tissues including skin fibroblasts from four LHON patients, and investigated whether nucleotide variants in *NDUFB11* influenced the individual risk of visual loss or the age at disease onset in 35 LHON pedigrees. We also investigated the possible disease-causing role of *NDUFB11* in a sample of 11 children with a severe mitochondrial encephalopathy presenting various degrees of complex I reduction in skeletal muscle.

Materials and methods

A total of 65 individuals (53 affected and 12 unaffected relatives, 38 men and 27 women, median age 42 ± 12 yr) were recruited from 35 Italian families with LHON. The clinical phenotype was determined by expert neuro-ophthalmologists. Affected individuals mostly presented with acute or subacute painless sequential or bilateral visual failure in the presence of an established pathogenic mtDNA mutation, and in the absence of any other identified inflammatory, metabolic, or structural cause. Direct sequencing of the mt*ND* genes or reported mutation specific PCR-RFLP methods [13], or both, were used to confirm the diagnosis. Levels of heteroplasmy were determined as previously reported [14,15]. Healthy relatives had neither visual symptoms nor other neurological involvement, were older than 31 yr (the median age at onset of LHON), and harbored one of the three major LHON-associated mutations in the mt*ND* genes.

Genomic DNA purification from peripheral blood or skeletal muscle was performed. PCR amplification and direct sequence analysis of the *NDUFB11* gene were carried out using intronic primers Ex 1-F (5′-TGGA GAAAAGGAGTAGCTATTA), Ex 1-R (5′-AAATTCCCAAGTCTTCT AGGC-3′), Ex 2–3 F (5′-ATGGACCAAGTTATGCA-3′), and Ex 2–3 R (5′-GTGCTC TGAGAAGAGGTCAGA-3′), and the optimized PCR conditions were as follows: 35 cycles of 30 s at 95 °C, 1 min at 53/57 °C, and 1 min at 72 °C, followed by a final 7-min elongation step at 72 °C. Sequencing reactions were performed using BigDye 3.1 chemistry on an ABI 3130XL (Applied Biosystems, Foster City, CA). The traces were assembled into contigs and analyzed using the STADEN software package.

Aliquots of total RNA samples from a panel of human tissues (Ambion Inc., Austin, TX) and cultured skin fibroblasts from three normal controls and four LHON patients (three 11778G>A and one 3460G>A) were reversely transcribed using oligo-dT primer from the AMV Reverse Transcriptase RNAseH minus kit (AMV, Finnzymes, Finland). The PCRs were performed in a total volume of 25 μl, containing 200 ng of each cDNA, and 7.5 pmol of the specific primers. Using oligonucleotide primers MrnaESSS-F1 (5′-ACCACACCGTGGCAAGAG GA-3′) and MrnaESSS-R1 (5′-GTGCTCTGAGAAGAGGTCAGA-3′), we co-amplified the "long" (AY359056) and the "short" (NM_019056) isoforms. The relative amplicons (414 nt and 384 nt, respectively) correspond to the two isoforms which differ in 30 nucleotides at the 3′-end of exon 2. The PCR conditions were: 35 cycles of 45 s at 94 °C, 45 s at 59 °C, and 45 s at 72 °C, followed by a final elongation step at 72 °C for 7 min. For the amplification of the endogenous gene (*GAPDH*), used as internal reference, we used primers F2 (5′-CTTCCATCAGTCGGATACAC-3′) and R2 (5′-TAGGAGGCTGCATCATCGT-3′) and specific PCR conditions consisting of 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C. Each sample was run in triplicate and the results reported are the mean values of three separate experiments. To determine whether our PCR conditions were able to detect quantitative variations of the two transcripts, we performed preliminary experiments mixing known amounts of amplicons obtained from each *NDUFB11* mRNA isoforms. To produce independent amplification of the two isoforms in normal cultured skin fibroblast we used the oligonucleotides S/L-ESSS-1F (5′-A CTTGTATGAGAAGAACCCAGA-3′) and LongESSS-1R (5′-ACGC TCTTGGACACCCTGTGC-3′), to amplify only the "long" isoform, and S/L-ESSS-1F and ShortESSS-1R (5′-CTTTCATCCTGTAGTCAGGC-3′) to amplify only the "short" isoform. PCR conditions of 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C were used. The amplicons corresponding to the two isoforms were then mixed in known amounts (1:0, 1:1, 1:2) and used in the aforementioned PCR conditions for co-amplification. The results were analyzed using the Bio-Rad Versa Doc Imaging System and "Volume Analysis Tools" of the Quantity One software (Bio-Rad, Hercules, CA) and expressed as the ratio of the signal intensities of the *NDUFB11* bands to those of the *GAPDH* band for each analyzed sample.

Prediction of the secondary structure of the two alternative proteins was performed using the PSIPRED software [16].

Results

The *NDUFB11* gene (Gene ID #54539) on chromosome Xp11.23 encodes the human homolog of the bovine ESSS protein and it is made up of three putative exons spanning 3289 bp of the genomic clone CTD-2522E6. Combining database search and direct sequencing strategies, we confirmed the coding sequence of *NDUFB11* and its untranslated regions, and identified two transcripts representing the products of alternative splicing. One is a 953-bp long cDNA sequence (GenBank Accession No. NM_019056), with a coding region of 492 bp encoding a protein of 163 aa (NP_061929) [12,17]. This transcript belongs to the UniGene cluster *Hs.521969*. A longer transcript of 1095 bp (AY359056), with a 462 bp coding region predicting a 153 aa-long-protein (AAQ89415), is present in the ESTs database. The two transcripts differ in the last 30 nucleotides of exon 2 and are the products of an alternative splicing. The 3′-UTR contains an atypical polyadenylation signal (ATTAAA). The insertion of 10 amino acids at position 111 of the short isoform predicts a dramatic alteration of the secondary structure [16]: a first α-helix (81–89 aa) of the shorter isoform is disrupted and a second α-helix domain (90–108 aa) is shortened. In the 80–110 aa region the structure appears to change from a "helix-turn-helix" to a "strand-helix" motif (Fig. 1).

Table 1A summarizes the clinical and molecular data relating to the LHON patients and Table 1B the main clinical and biochemical data from a separate sample of 11 children with a clinical condition characterized by a severe mitochondrial encephalopathy of as yet unknown etiology. In all, 47 LHON cases (28 men and 19 women) harbored the 11778G>A mutation, while 10 individuals (7 men and 3 women) had the 3460A>G mutation, and eight the 14484T>C mutation (3 men, 5 women). Whereas the patients consistently carried the specific mutation in homoplasmy, in the unaffected individuals levels of mutant mtDNAs ranged from 76 to 93% (11778G>A mutation) and from 39 to ⩾95% (3460G>A and 14484T>C mutations). Of the 65 individuals bearing LHON-associated

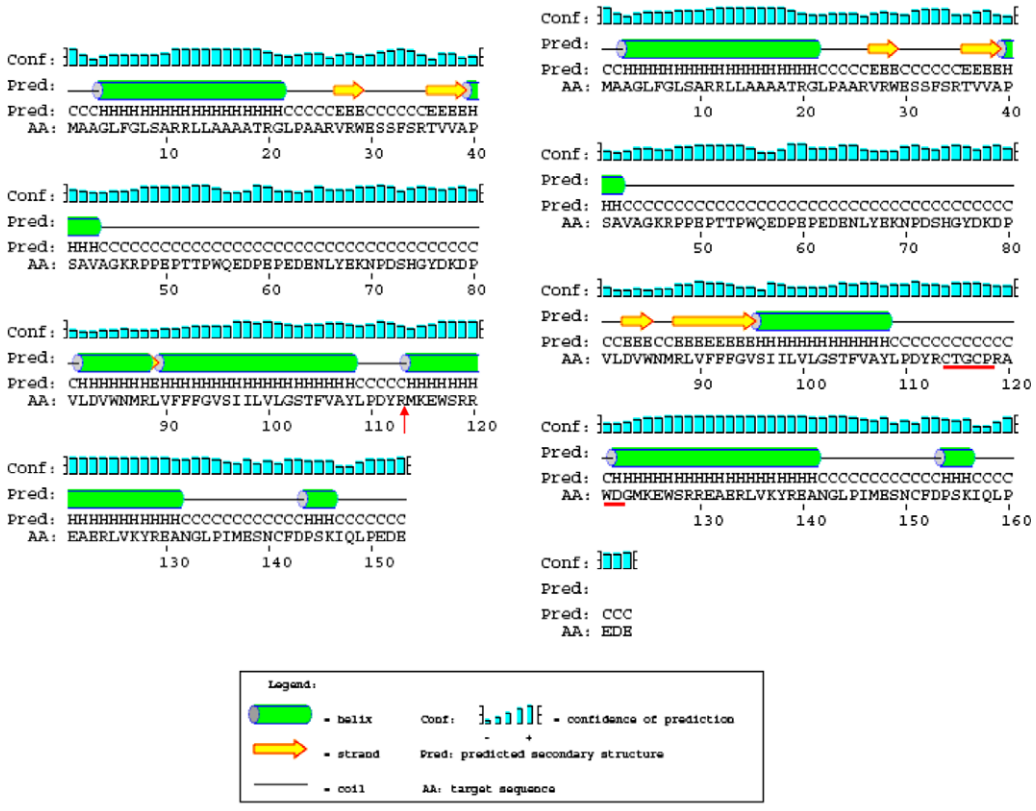

Fig. 1. Schematic representation of secondary structures of the "short" and "long" *NDUFB11* isoform as predicted by PSIPRED software analysis [16]. Symbols legend is boxed.

Table 1A
Clinical and molecular data in patients with Leber hereditary optic neuropathy (LHON)

	LHON patients	LHON relatives
Median age ± SD	31 ± 14	35 ± 11
Sex	34M/19F	4M/8F
Onset of impaired vision (median age, yr)	17 ± 4	NA
Age when legally blind (median age, yr)	25 ± 8	NA
Toxic habit (cigarette smoking/Etoh intake)	Yes (21%)	Yes (25%)
Associated neurological features		
Migraine with or without aura	Yes (15%)	Yes (22%)
Dystonia	Yes (3%)	No
Peripheral Neuropathy	Yes (1%)	No
Cardiac conduction defects	Yes (5%)	Yes (10%)
Epilepsy	No	No
Strokes/transient ischemic Attacks	No	No
Others		Muscle weakness (10%)
Abnormal brain CT or MRI scan	Yes (12%)	
mtDNA variant (% mutant genomes, range)		
11778G>A	100%	76–93%
3460A>G	100%	39–>95%
14484T>C	100%	43–100%

NA, not applicable; CT, computerized tomography; MRI, magnetic resonance imaging.

mutations and the 15 normal controls, no subject showed sequence variants in the exonic sequences of *NDUFB11,* or in the intronic regions and the 5′- and 3′-UTRs. Similarly, we did not detect differences from the reference sequence in the 11 children whose neurological condition was associated with a decreased activity of complex I in skeletal muscle. In these patients, the absence of mutations in the seven mtDNA-encoded subunits of complex I increases the likelihood of a mutation in one of the known remaining 38 nDNA-encoded subunits of the complex.

Table 1B
Clinical features of 11 children with deficient activity of the respiratory chain complex I in skeletal muscle

Case #	Age at onset/sex	Family history	Symptom at onset	Lactic acidosis (blood/CSF)	Brain MRI	Residual Complex I	Diagnosis
1	3 mo/M	+	Ftt/hypotonia	+/+	BG, Pons	45%	Leigh syndrome
2	1 yr/M	+	Dev.delay	+/+	BG	33%	Leigh-like syndrome
3	11 mo/F	–	ftt	+/+	BG, cerebellum	37%	Leigh syndrome
4	4.3 mo/M	–	Cardiomyopathy	nd	nd	56%	HCM
5	5 yr/M	–	Ataxia	–/+	PWM changes	52%	Leukoencephalopathy
6	4.2 mo/F	–	ftt	+/+	BG	43%	Leigh-like syndrome
7	2.2 mo/M	+	ftt	+/nd	BG	32%	Leigh syndrome
8	1.5 yr/F	–	Psychomot.delay	+/+	BG	31%	Leigh syndrome
9	8 yr/M	–	ftt	–/+	Pons, Medulla	32%	Encephalomyopathy
10	10 yr/M	–	Anemia	–	nd	33%	Encephalomyopathy
11	6.4 mo	+	ftt	+/+	PWM changes	31%	Leukoencephalopathy

Ftt, failure to thrive; dev. delay, developmental delay; MRI, magnetic resonance imaging; BG, basal ganglia; PWM, periventricular white matter; HCM, hypertrophic cardiomyopathy; nd, not done.

Fig. 2A compares the levels of expression of the two *NDUFB11* mRNA isoforms in four LHON fibroblasts and three normal controls relative to *GAPDH* mRNA, measured on the same samples and used as internal control for RNA integrity and loading. Fig. 2B shows a representative gel. Fig. 3 reports the *NDUFB11* mRNA patterns in several human tissues. Consistent with the mitochondrial localization of the protein, tissues with high oxidative metabolism, such as brain, heart, skeletal muscle, and liver, showed the highest levels of both *NDUFB11* mRNAs. Regardless of the tissue investigated, the shortest ESSS isoform was consistently more abundant (on average 1.5X) than the longest one.

Discussion

Of the 45 identified subunits of bovine complex I [18], MWFE and ESSS are the only two encoded by a gene on the X chromosome in humans. MWFE and ESSS are small integral membrane proteins localized, respectively, in the so-called Iα and Iβ subcomplexes [12,19,20]. The MWFE subunit is encoded by the human *NDUFA1* gene on Xq24 and it is believed—at least on the basis of evidence in rodent cells [21]—to affect both assembly and activity of complex I. The human protein ESSS was initially identified through its homology to mouse Np15.6, the neuro-

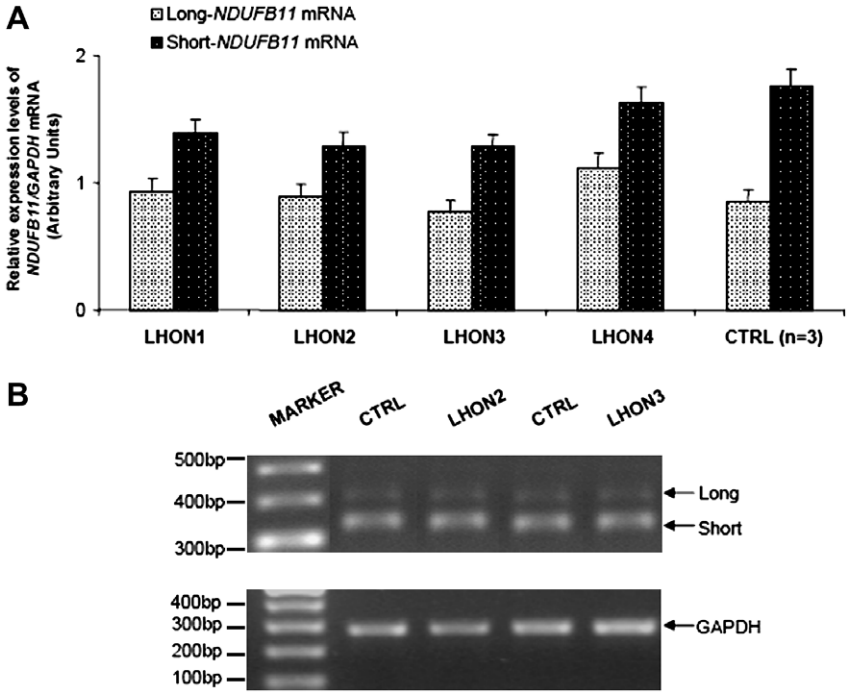

Fig. 2. Relative expression levels of the two *NDUFB11* isoforms in normal controls and LHON patients. (A) Histogram showing the relative expression levels of the "long" and "short" *NDUFB11* isoforms in three normal controls and four LHON patients (three 11778G>A and one 3460G>A) with respect to *GAPDH* mRNA, used as internal control. Data represent means ± SD of three different determinations. (B) Representative agarose gel showing the results of RT-PCR co-amplification of the two *NDUFB11* isoforms and GAPDH mRNA, used as internal control.

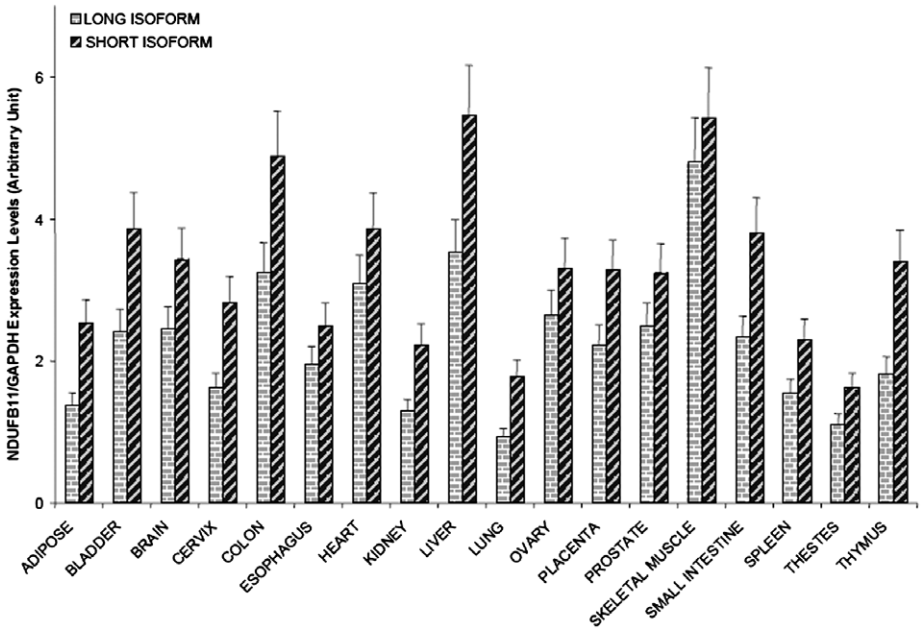

Fig. 3. Histogram representing the relative expression levels of the "long" and "short" *NDUFB11* mRNA isoforms in several human tissues after normalization for the expression of *GAPDH* mRNA. Data represent means ± SD of three different determinations.

nal-specific homolog of the *Drosophila* retinal degeneration B gene [22], and termed neuronal protein 17.3 (Np17.3) [17]. Subsequent work showed that the bovine heart protein is an integral protein of the mitochondrial membrane and that it is phosphorylated at residue Ser 20 [23]. Recent experimental data in murine tissues showed that the *NDUFB11* gene encoding Np15.6 is expressed at all stages of embryonic development and that it appears to be involved in the growth, maintenance, and survival of neurons [24]. Although its function is still unclear, the ESSS subunit seems to be needed equally for the assembly and activity of complex I [25].

The localization, on chromosome Xp11.23, of *NDUFB11*, which encodes the human ESSS protein, together with the suggestion of a partial complex I defect in the cells of LHON patients [26], prompted us to investigate its possible role as a modifier of the clinical phenotype of this disease. Of note, the gene is located on the chromosomal interval (Xp11.3–Xp11.23) believed to be associated with a number of human diseases affecting the eye, including Aland Island eye disease, X-linked optic atrophy, X-linked congenital nystagmus, and X-linked retinitis pigmentosa 2 [27–30]. Furthermore, *NDUFB11* lies in close proximity to the DXS1219–DXS8016 region found to interact with LHON-associated *MTND* mutations in white kindred [9]. However, we did not find any intronic or exonic sequence variations in patients, unaffected family members, or ethnically-matched normal controls when compared to the *NDUFB11* reference sequence. Moreover, sequence changes were not detected in 11 children with a proven biochemical defect of complex I. These findings argue against a role for the gene as a modifier or cause of the disease, at least in the cohort of patients we studied. A similar neutral effect on disease penetrance in LHON has been reported for *NDUFA1*, the other gene located on chromosome X encoding a complex I subunit [31].

We also showed that the *NDUFB11* gene produces two mRNAs, the result of an alternative splicing, differing only in 30 nt and predicting two proteins differing in 10 aa in the middle part of the protein (Fig. 1). This difference presumably brings about a change in protein domain architecture. The highest levels of expression of the two isoforms were observed in brain, skeletal muscle, and liver (Fig. 3) but it is known that mitochondrial proteins express mostly in tissues that have the greatest energy demand.

The identification of two *NDUFB11* transcripts fits in well with growing evidence that alternative splicing is a ubiquitous and fundamental mechanism for generating protein diversity from a single gene. It is possible that there is some fine tuning of *NDUFB11* expression in certain tissues, in the developmental stage, or in diseased as opposed to normal tissue [32]. When tested semi-quantitatively by RT-PCR, both transcripts were co-expressed with a similar pattern and the levels of the "short "prevailed over the "long" isoform in all the tissues examined. Cultured skin fibroblasts from four LHON patients (three 11778G>A and one 3460G>A) showed similar levels of expression of the two isoforms when compared to three normal controls (Fig. 2A). Although our findings run counter to the hypothesis of a pathological role for one or both of the two isoforms, we cannot exclude entirely the possibly of their having a finer regulatory role in other tissues, including the optic nerve.

The reason for the sex bias and variable penetrance observed only in LHON, and not in other mtDNA-related disorders, thus remains unclear; unanswered, too, is the question of whether genetic or epigenetic mechanisms are

implicated. A recent multicenter study of a large cohort of European patients [9] defined an X-chromosomal haplotype interacting with specific mt*ND* mutations and showed an independent effect of the mtDNA genetic background, thereby offering an explanation for the variable penetrance and the male predominance that characterize LHON. The common haplotype—defined between markers DXS8090 and DXS1068—seems to increase greatly the lifetime risk of visual failure. In the above mentioned study, an as yet unidentified nuclear modifier, likely an ancient genetic variant that may be highly frequent in the population, was hypothesized. The fact that the authors of another study, a multigenerational Brazilian kindred with the 11778G>A LHON-associated mutation, seemed to reach similar conclusions, reinforces the importance of stepping up the search for disease-modifying factors [33].

Another mtDNA-related disorder—namely, aminoglycoside-induced and nonsyndromic sensorineural hearing loss (AISNHL) associated with the 1555A>G mutation in the 12S rRNA gene—shows 100% mutant mtDNA genomes both in patients and in healthy relatives, probably reflecting the effects of one or more modifier genes or environmental factors similar to those observed in LHON [34–36]. The observation of no sex bias in AISNHL led to the identification of a new locus on chromosome 8p23.1 interacting with the 1555A>G mutation in the 12S rRNA gene [37], but this finding was not replicated in a later Finnish study [38]. Recently, sequence variants in the mt-tRNAs discovered in affected Chinese pedigrees [39] and, even more recently, a polymorphic variant (p.Ala10Ser) identified in the *TRMU* gene in Arab-Israeli and European kindred [40], were suggested to influence the phenotypic manifestation of deafness-associated mtDNA mutations. Further research will be needed to clarify whether additional X-linked genes, other than *NDUFB11* and *NDUFA1*, encoding proteins necessary for correct functioning of complex I in neural tissues, including the eye, or ancillary proteins necessary for complex I assembly or regulatory mechanisms can interact with the primary mtDNA mutations.

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