Leber Hereditary Optic Neuropathy: Do Folate Pathway Gene Alterations Influence the Expression of Mitochondrial DNA Mutation?

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Abstract
Background: Leber hereditary optic neuropathy (LHON) is an inherited form of bilateral optic atrophy leading to the loss of central vision. The primary cause of vision loss is mutation in the mitochondrial DNA (mtDNA), however, unknown secondary genetic and/or epigenetic risk factors are suggested to influence its neuropathology. In this study folate pathway polymorphisms were examined as a possible LHON secondary genetic risk factor in Iranian patients.

Methods: Common polymorphisms in the MTHFR (C677T and A1298C) and MTRR (A66G) genes were tested in 21 LHON patients and 150 normal controls.

Results: Strong associations were observed between the LHON syndrome and C677T (P = 0.00) and A66G (P = 0.00) polymorphisms. However, no significant association was found between A1298C (P = 0.69) and the LHON syndrome.

Conclusion: This is the first study that shows MTHFR C677T and MTRR A66G polymorphisms play a role in the etiology of the LHON syndrome. This finding may help in the better understanding of mechanisms involved in neural degeneration and vision loss by LHON and hence the better treatment of patients.

Keywords: LHON, MTHFR, MTRR, Folate, Folic acid.

Introduction
Mitochondrial DNA mutations at nucleotide (nt) position 3460, 11778, 14459 and 14484 has been shown to be primary LHON mutations with similar or different phenotypes (1-5). These primary mitochondrial DNA mutations cause energy deficits and have been postulated to lead to neuronal injury via an exocytotoxic mechanism. However, their derangement may be relatively subtle and not sufficient to cause visual loss in LHON (6, 7). Furthermore, the cause for severity of the optic neuropathy and incomplete penetrance of vision loss has been inadequately explained and unknown secondary genetic or environmental factors have been indicated to have a role (8-10). There is a relationship between the mitochondrion and folate derivatives of cell folate metabolism. The folate pathway in the mitochondrion contributes to the entire cellular metabolism of mammals and the mitochondrion has been suggested as a target organelle for the cytotoxic effect of antifolates (11). The active form of folate, tetrahydrofolate (THF), functions to transfer single carbon units that exist in different oxidation states, which is then converted by enzymes located in cytosolic and mitochondrial compartments (12, 13). Two enzymes critical in the folate metabolic pathway are products of methylentetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) genes which are involved in the synthesis of 5-methyltetrahydrofolate and methionine, respectively. 5-Methyltetrahydrofolate (MTHF), the predominant circulating form of folate, acts as the methyl donor for remethylation of homocysteine to methionine by the vitamin B12 dependent enzyme methionine synthase (14, 15). In the case of the MTHFR gene, transition of cytosine (C) to thymidine (T) at nt-677 causes an alanine to valine substitution, leading to an increase in MTHFR thermolability and reduction
of enzyme activity. In the heterozygous C/T and the homozygous T/T genotypes, activities decrease to approximately 35% and 70%, respectively, as compared to that observed for the normal C/C genotype (16, 17). A second common mutation in MTHFR A1298C involves a transversion mutation leading to a glutamate to alanine substitution. A moderate decrease in enzyme activity is observed more frequently in the homozygous mutant (CC) genotype, but with no change in enzyme thermolability (16). Severe MTHFR deficiency is characterized by neurological abnormalities, problems in nerve myelination, vascular disease, neural tube defects and neurodegenerative diseases such as Alzheimer and Parkinson’s (14, 16-19). Methionine synthase reductase (MTRR) keeps methionine synthesis enzyme in an active state for remethylation of homocysteine to methionine. Polymorphism at position A66G leads to replacement of methionine with isoleucine, thus reducing enzyme activity and increasing homocysteine levels (17, 20). Folate gene alteration has been reported to play an important role in neurodegenerative disorders and optic nerve dystrophy such as behcet disease (18-21). To better determine the genetic factors in LHON etiology, folate gene alterations involving the MTHFR C677T, A1298C and MTRR A66G genes of Iranian LHON cases were investigated in this study.

Materials and Methods

**Studied Leber patients and sample collection**

One hundred and forty patients diagnosed with acute visual loss were referred to the Genetic Diagnostic Unit of the National institute of Genetic Engineering and Biotechnology, Tehran, Iran, for screening of mtDNA mutations associated with Leber hereditary optic neuropathy (LHON). Informed consent was obtained from all participants who consisted of both cases and controls and the study was approved by the ethics committee of the institute. Twenty one cases were diagnosed with LHON. Total DNA was isolated from blood lymphocytes using the standard salting out method and stored at -20º C prior to the genotype analysis. Mutational analysis of mtDNA was performed according to the protocols have been previously described (20, 22-24). Four mutations 11778, 3460, 14484 and 14459 located in subunits 1, 4 and 6 of the NADH dehydrogenase gene were examined by PCR-RFLP.

**Analysis of MTHFR and MTRR gene polymorphisms**

Twenty one LHON patients and 150 normal controls were tested for common MTHFR C677T, A1298C and MTRR A66G polymorphisms. A pair of primers was designed to amplify the 254 bp fragment of the MTHFR gene containing codon 677. Forward and reverse primers were (5’gcc tct cct gac tgt cat cc3’) and (5’gga gct tat ggg ctc tcc tg3’), respectively. Primers for PCR amplification of fragments containing the MTHFR A1298C and MTRR A66G mutations were obtained respectively from (25). Homozygous and heterozygous mutant samples used in the preliminary experiments were obtained form Dr. Jill James, National Center for Toxicological Research (USA). PCR thermal cycle started with an initial denaturation of 95º C for 5 min, and 32 cycles consisting of denaturation at 95º C for 30 seconds, annealing at 60º C for 1 min and extension at 72º C for 30 seconds, followed by a final extension at 72º C for 10 min. To test the MTHFR C677T mutation, the PCR product was digested with HinfI (Roche, Germany), according to the method by (26). The presence of the C677T mutation within the MTHFR gene creates a HinfI restriction site which is detected by the appearance of 147 and 108 bp fragments (Fig. 1).

The reaction mixtures and PCR conditions for the MTHFR A1298C and MTRR A66G mutations were the same as that for the C677T mutation, except for the annealing temperature of the A1298C primer pair which was set as 55º C. The amplified fragment of A1298C was 163 bp in size and was digested with MboII (Fermentas, Vilna, Lithuania). The A to C mutation abolishes one of the four MboII restriction sites that are detected by the appearance of four fragments...
(30, 84, 31 and 18 bp) in the individual with the homozygous mutation and five fragments (30, 56, 28, 31 and 18 bp) in the normal homozygous control (Fig. 2).

To test MTRR A66G polymorphism the 154 bp amplified fragment was digested with NdeI endonuclease (Fermentas, Vilna, Lithuania). The A66G mutation leads to the appearance of the NdeI restriction site which can be detected by the generation of two fragments of 24 and 130 bp in the homozygous mutant instead of the 154 bp fragment observed in the normal homozygous individual (20) (Fig. 3). The fragments were analyzed on 8% PAGE and visualized using silver staining method. To determine the identity of amplified PCR products related to MTHFR and MTRR genes all three amplified products were sequenced and their similarities to human sequences were determined using the NCBI-BLAST homology search.

Statistical analysis
Allele frequencies were calculated for each genotype by the allele counting method. Comparisons of allele frequencies between the case and control groups were determined using the Pearson $\chi^2$ test and calculation of the corresponding P-values were carried out by using the statistical software SPSS for windows version 12.0 (Chicago, Illinois).

Results
Twenty one cases were diagnosed to be positive for mtDNA mutations and confirmed to have LHON according to their clinical and paraclinical features. MtDNA mutations were found at nt-11778 in 16 cases, nt-3460 in 3 cases, nt-14484 and nt-14459 in the remaining 2 cases (Table 1). Patients were unrelated and resided in various provinces of Iran.

All 21 LHON cases and 150 normal controls were genotyped for common C677T, A1298C and A66G polymorphisms. The allele frequencies of the C677T, A1298C and A66G among the chromosomes of cases were 45.0% (19/42), 40% (17/42) and 62.0% (26/42), respectively while the corresponding frequencies among chromosomes of the control group were 22% (66/300), 34% (102/300) and 37.6% (113/300), respectively (Table 2). The frequencies of the C/C, C/T, and T/T genotypes at the 677 MTHFR locus in the cases were 33.3%, 42.9% and 23.8%, respectively, whereas the corresponding frequencies among the control group were 61.3%, 33.3% and 5.3%, respectively. In the cases, genotyping result at the 66 MTRR locus displayed frequencies of 4.8%, 66.7% and 28.6% for the A/A, A/G, and G/G genotypes whereas the corresponding frequencies among the control group were 31.3%, 62% and 6.7%, respectively. As indicated the frequencies of the T/T genotype at the 677 MTHFR and G/G genotype at the MTRR locus are approximately 4 times more common among LHON patients compared to those observed in the normal control.

Throughout statistical analysis significant associations were interpreted for C677T MTHFR ($P=0.00$) and A66G MTRR ($P=0.00$). However, no significant association was obtained at the A1298C MTHFR ($P=0.69$) locus. Simultaneous occurrence of the TT/CT alleles at the C677T MTHFR locus and GG/AG alleles at the A66G MTRR were significantly ($P=0.00$) higher among cases (61.9%), than those observed among the control group (26%). This result is consistent with previous reports in that C677T MTHFR and A66G MTRR polymorphisms can significantly increase the blood homocysteine levels, however MTHFR A1298C has no independent effect on total plasma homocysteine concentrations.
Table 1: mtDNA mutations and MTHFR C677T, MTHFR A1298C and MTRR A66G polymorphisms in 21 LHON cases

| Case | mtDNA | C677T | A1298C | A66G |
|------|-------|-------|--------|------|
| 1    | 11778 | TT    | CC     | GG   |
| 2    | 11778 | TT    | CA     | GA   |
| 3    | 11778 | TT    | CA     | GA   |
| 4    | 11778 | TC    | CA     | GA   |
| 5    | 11778 | TC    | CA     | GA   |
| 6    | 11778 | TC    | AA     | GA   |
| 7    | 11778 | CC    | AA     | GA   |
| 8    | 11778 | CC    | CA     | GA   |
| 9    | 11778 | CC    | CA     | GG   |
| 10   | 11778 | TT    | AA     | GG   |
| 11   | 11778 | CC    | AA     | GA   |
| 12   | 11778 | TC    | AA     | GA   |
| 13   | 11778 | TC    | AA     | AA   |
| 14   | 11778 | TC    | CA     | GA   |
| 15   | 11778 | TT    | AA     | GA   |
| 16   | 11778 | CC    | CC     | GA   |
| 17   | 3460  | TC    | CC     | GG   |
| 18   | 3460  | CC    | CA     | GA   |
| 19   | 3460  | TC    | AA     | GG   |
| 20   | 14484 | CC    | CC     | GG   |
| 21   | 14459 | TC    | CA     | GA   |

Table 2: Specific genotype number and their frequencies (%) between case and normal control groups and their P and chi square value obtained after chi square analysis to determine association between different genotypes of MTHFR C677T, MTHFR A1298C and MTRR A66G polymorphisms and LHON.

| polymorphism | case | control | P (X²) |
|--------------|------|---------|--------|
| MTHFR 677 CC | 7 (33.3) | 92 (61.3) |        |
| MTHFR 677 CT | 9 (42.9) | 50 (33.3) | 0.00 (11.25) |
| MTHFR 677 TT | 5 (23.8) | 8 (5.3) |        |
| MTHFR 1298 AA | 8 (38.1) | 67 (44.7) |        |
| MTHFR 1298 AC | 9 (42.9) | 64 (42.7) | 0.69 (0.73) |
| MTHFR 1298 CC | 4 (19) | 19 (12.7) |        |
| MTRR 66 AA | 1 (4.8) | 47 (31.3) |        |
| MTRR 66 AG | 14 (66.7) | 93 (62) | 0.00 (14.14) |
| MTRR 66 GG | 6 (28.6) | 10 (6.7) |        |
**Fig 1:** PCR-RFLP of MTHFR C677T polymorphism via *Hinf*I, lane 1: PCR product, lane 2: size marker, lane 3: heterozygote, lane 4: mutant homozygote, lane 5: normal

**Fig 2:** PCR-RFLP of MTHFR A1298C polymorphism via *MboII*, lane 1: PCR product, lane 2: size marker, lane 3: heterozygote, lane 4: normal, lane 5: mutant homozygote
Discussion
This study has illustrated the high prevalence and significant association of the C677T MTHFR and A66G MTRR gene polymorphisms with the LHON syndrome as has been supported by a P-value of 0.00 and emphasizing their significance in LHON etiology. Folate gene alteration has been seen as an important factor in influencing basic cellular, mitochondrial and respiratory dysfunctions. Folate-dependent enzymes are compartmentalized between the cytoplasm and mitochondrion and their pathways contribute to the entire cellular metabolism in mammalian cells (13, 27). Hence, in the regards to LHON the disruption of mitochondrial metabolism due to mtDNA mutations and in conjunction with folate pathway gene alteration may affect electron transport leading to mitochondrial dysfunction and reduced ATP levels which may contribute to the severity of LHON neuropathology.

The MTRR protein is a member of the Ferredoxin-NADP⁺ Reductase (FNR) family of electron transferases and shares 38% identity with the human cytochrome P450 reductase (22). The function of MTRR and its homology with the NADPH genes may suggest a possible role for the A66G MTRR mutation in the defective electron transport system as observed in LHON.

In rats suffering from folate deficiency mitochondrial degeneration and degenerative appearance in the cerebrocortical microvascular wall has been observed. However, it has been suggested that oxidative stress may be responsible for the damage to mitochondrial DNA and function in LHON (6). Altered folate metabolism also induces sensitivity to oxidative stress. The conjunction of these two gene mutations (C677T and A66G) may enhance mitochondrial degeneration and energy deficit in LHON, thus triggering a wave of optic nerve dysfunction.

A significant difference in the frequency distribution of the C677T MTHFR and MTRR A66G genotypes has been observed between affected patients and the control group. This observation adds to the knowledge regarding the etiology of LHON and is thus of great value in the better
understanding of biochemical targets for the future treatment of LHON.

Ethical considerations
Ethical issues (including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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