References

[1] Ganesan V, Kirkham FJ. Noonan syndrome and moyamoya. Pediatr Neurol 1997;16:256-8.

[2] Tang KT, Yang W, Wong J, Lee KY. Noonan syndrome associated with moyamoya disease: Report of one case. Acta Peditr Tw 1999;40:274-6.

[3] Booth F, Yanofsky R, Ross IB, Lawrence P. Primary antiphospholipid syndrome with moyamoya-like vascular changes. Pediatr Neurosurg 1999;31:45-8.

[4] Takahashi J, Sugita K, Miyazato S, Sakao E, Miyamoto H, Niimi H. Antiphospholipid antibody syndrome in childhood strokes. Pediatr Neurol 1995;13:323-6.

[5] Bonduel M, Hepner M, Sciuccati G, Torres AF, Tenembaum S. Prothrombotic disorders in children with moyamoya syndrome. Stroke 2001;32:1786-92.

[6] Shöning M, Klein R, Krägeloh-Mann I, et al. Antiphospholipid antibodies in cerebrovascular ischemia and stroke in childhood. Neuropediatrics 1994;25:8-14.

HIGH LIGHT

L-Arginine improves the symptoms of strokelike episodes in MELAS

Abstract—Based on the hypothesis that mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS) are caused by impaired vasodilation in an intracerebral artery, the authors evaluated the effects of administering L-arginine, a nitric oxide precursor. Patients were administered L-arginine intravenously at the acute phase or orally at the interictal phase. L-Arginine infusions significantly improved all strokelike symptoms, suggesting that oral administration within 30 minutes of a stroke significantly decreased frequency and severity of strokelike episodes.

NEUROLOGY 2005;64:710–712

Y. Koga, MD, PhD; Y. Akita, MD, PhD; J. Nishioka, MD; S. Yatsuga, MD; N. Povalko, MD; Y. Tanabe, MD, PhD; S. Fujimoto, MD, PhD; and Toyojiro Matsuishi, MD, PhD

Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes (MELAS) is a maternally inherited, multisystem mitochondrial disorder.[1] The primary cause of strokelike episodes in young patients with MELAS, whether mitochondrial cytopathy, angiopathy, or both, remains controversial. Based on the hypothesis that strokelike episodes in MELAS are caused by segmental impairment of vasodilation in intracerebral arteries, we administered L-arginine by IV administration during the acute phase of strokelike episodes and by oral administration during the interictal phase.

Methods. *Patients.* We studied 24 patients referred to the hospital with MELAS diagnosed according to clinical, muscle pathologic, and genetic studies and 72 healthy control subjects (table 1). Patients with congenital anomalies, sepsis, IV hyperalimentation, diabetes mellitus, cardiac failure, or a bedridden state were excluded from this study.

Study design. All patients or patients' parents gave written informed consent, and the L-arginine study protocol was approved (Kurume University IRB no. 9715). The study design was chosen because of patients' availability and finances did not permit a balanced, randomized design involving multiple centers. Our strokelike episodes fulfilled the criteria that patients have migraine headache, vomiting, convulsion, and transient blindness with brain image suggesting focal brain abnormality. Twenty-four patients with a total of 34 strokelike episodes took part in this study of L-arginine versus placebo, following a previously described protocol.[2] The severity of a strokelike attack (convulsion, cortical blindness, hemiparesis, or abnormality in brain images associated with headache and vomiting) was similar when either L-arginine or placebo was administered.

Six patients were treated by oral administration of L-arginine to prevent strokelike episodes. Four to 24 g of L-arginine (Arugi U, Ajinomoto Pharma; 0.15 to 0.3 g/kg/d) were given orally for 18 months. Patients were monitored clinically and biochemically as described previously once every 2 weeks. When patients were admitted to the hospital with a strokelike episode, the following symptoms were scored: headache (present: 1, none: 0), vomiting (present: 1, none: 0), teichopsia (present: 1, none: 0), convulsion (present: 1, none: 0) and hemiparesis (present: 1, none; 0).[3] For each admission during the study period, these scores were summed as the severity score for the stroke. Frequency of admission was taken to be the frequency of strokelike episodes. Severity and frequency were related to time as number and month and were compared between periods 18 months before and after oral administration of L-arginine in the same patient.

Analysis of amino acids, asymmetric dimethylarginine (ADMA),[4] nitric oxide (NOx),[5] cyclic guanosine monophosphate (cGMP)[6] were measured using described methods.

Analysis. Plasma concentrations of amino acids, NOx, and ADMA in patients in the acute or interictal phase of MELAS were compared with those in controls using unpaired *t* tests, with Bonferroni corrections for outlying values. Concentrations of L-arginine, L-citrulline, NOx, ADMA, and cGMP in plasma obtained before, 30 minutes after, and 24 hours after L-arginine infusion were compared with those in controls using paired *t* tests. Statistical analysis of clinical improvement was performed using Fisher's exact test. Frequency and severity of strokelike episodes in six patients with MELAS after long-term oral L-arginine supplementation were compared with those in the same patients without supplementation using a nonparametric Mann-Whitney *U* test. All data are presented as means ± SD. p Values of 0.05 or less were considered to indicate significance.

Results. Baseline characteristics of the 24 patients and 72 controls are shown in table 1. Mean plasma concentrations of L-arginine and L-citrulline were lower in both acute (L-arginine: 47 ± 13 μmol/L; L-citrulline: 23 ± 10 μmol/L) ($p < 0.01$) and interictal phases (L-arginine: 84 ± 26 μmol/L; L-citrulline: 26 ± 10 μmol/L) ($p < 0.01$) of MELAS than in controls (L-arginine: 108 ± 28 μmol/L; L-citrulline: 35 ± 9 μmol/L). Concentrations of L-arginine in the acute phase were also significantly lower than in the interictal phase, whereas those of L-citrulline did not show a significant phase-related change. NOx concentrations were lower in the acute phase (24 ± 10 μmol/L) ($p < 0.01$) of MELAS than in controls (45 ± 30 μmol/L), whereas in the interictal phase (91 ± 44 μmol/L) ($p < 0.01$), they were higher than in controls. Conversely, concentrations of ADMA did not significantly differ between controls and acute phase, although the ADMA/L-arginine ratio was higher in the acute phase (0.011 ± 0.004) ($p < 0.01$) than in the controls (0.005 ± 0.001) or in the interictal phase (0.005 ± 0.001).

From the Department of Pediatrics and Child Health (Drs. Koga, Akita, Nishioka, Yatsuga, Povalko, and Matsuishi), Kurume University School of Medicine, Fukuoka, Japan; Department of Pediatric Neurology (Dr. Tanabe), Chiba Children's Hospital, Chiba, Japan; Department of Pediatrics (Dr. Fujimoto), Nagoya City University School of Medicine, Nagoya, Japan; and Research Centre for Medical Genetics RAMNS (Dr. Povalko), Moscow, Russia

Supported in part by grants 13670853 and 16390308 from the Ministry of Culture and Education in Japan; H14-006, and H14-team(syouni)-005 from Evidence-based Medicine, Ministry of Health, Labor and Welfare in Japan; Uehara Memorial Foundation; and Morinaga Hoshikai.

Received June 18, 2004. Accepted in final form October 4, 2004.

Address correspondence and reprint requests to Dr. Yasutoshi Koga, Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-Machi, Kurume City, Fukuoka 830-0011, Japan; e-mail: yasukoga@med.kurume-u.ac.jp

***Table 1** Baseline characteristics of the patients with MELAS and controls*

Variable	Patients with MELAS (n = 24)	Controls (n = 72)
Age, y (range)	19.6 ± 12.5 (8.2–30.3)	21.5 ± 10.4 (4.3–35.4)
Gender, M/F	8/16	27/45
BMI	17.8 ± 3.6*	20.4 ± 2.3
Height	−2.2 ± 0.8*	0.2 ± 0.9
Alanine, μmol/L plasma	514 ± 164*	406 ± 121
Pyruvate, μmol/L	0.22 ± 0.06*	0.08 ± 0.05
Lactate, μmol/L	4.5 ± 1.8*	0.8 ± 0.2
L/P ratio	19.8 ± 2.9*	10.5 ± 1.8
Total cholesterol	139 ± 27	135 ± 38
LDL cholesterol	13.8 ± 3.7	14.6 ± 5.9
A3243G in muscle, %	68 ± 16	ND
Ragged-red fibers in muscle, %	3.6 ± 1.9	ND

Plus-minus values are means ± SD.
* $p < 0.05$ compared with controls.

MELAS = mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes; BMI = body mass index; L/P = lactate pyruvate; LDL = low density lipoprotein; ND = not detectable.

Symptoms and biochemical measurements after L-arginine therapy in the acute phase of strokelike episodes in MELAS are shown in table 2 and the figure. After administration of L-arginine, all symptoms suggesting stroke dramatically improved. No adverse effects occurred, except headache, when L-arginine was infused too rapidly in two patients. With treatment, concentrations of lactate and pyruvate, L-arginine, L-citrulline, NOx, cGMP, and ADMA returned to interictal-phase concentrations within 24 hours.

After oral L-arginine supplementation, the frequency and severity of symptoms caused by the stroke had decreased dramatically. Frequency of strokelike episodes after treatment (0.09 ± 0.09) ($p < 0.05$) decreased compared with before supplementation (0.78 ± 0.42). The severity score after treatment (0.17 ± 0.18) ($p < 0.05$) was also lower than before supplementation (2.04 ± 0.34). After L-arginine supplementation, no patient with MELAS had a major strokelike attack, including hemiconvulsion or hemiparesis, but only headache or teichopsia. Plasma concentrations of L-arginine in patients with MELAS ranged from 82 to 120 μmol/L (mean ± SD 92 ± 17 μmol/L) after initiation of L-arginine supplementation.

Discussion. L-Arginine, which plays an important role in endothelium-dependent vascular relaxation, was significantly lower in both the acute and interictal phases of MELAS than in control subjects. Why plasma L-arginine is decreased in the acute phase of MELAS remains to be elucidated. We analyzed the

***Table 2** Effects of L-arginine on the clinical symptoms in acute phase of MELAS*

	Time after administration			
	Before	15 min	30 min	24 h
Headache (improvement of score from 3/2 to 1/0)				
L-Arginine	0/22	2/22	18/22*	21/22*
Placebo†	0/12	0/12	1/12	1/12
Clinical disability (improvement of score from 3/2 to 1/0)				
L-Arginine	0/22	3/22	16/22*	20/22*
Placebo†	0/12	0/12	1/12	1/12
Nausea				
L-Arginine	0/22	2/22	15/22*	22/22*
Placebo†	0/12	0/12	0/12	1/12
Vomiting				
L-Arginine	0/22	3/22	18/22*	22/22*
Placebo†	0/12	0/12	0/12	1/12
Hemi-blindness (transient)				
L-Arginine	0/7	2/7	4/7*	7/7*
Placebo†	0/4	0/4	1/4	1/4
Teichopsia				
L-Arginine	0/22	0/22	8/22*	19/22*
Placebo†	0/12	0/12	0/12	0/12

Numbers indicate the number of occasions when improvement was seen relative to the total number of episodes.
* $p < 0.05$ by Fisher's exact test.
† 5% dextrose (0.5 g/kg/dose) in eight episodes, and D-arginine in four episodes.

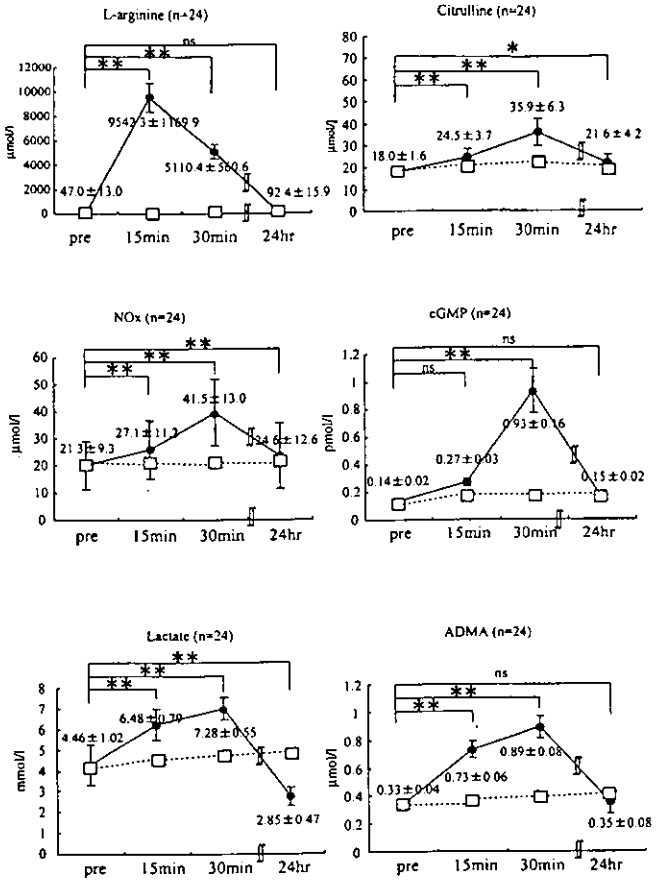

*Figure. Plasma concentrations of L-arginine, L-citrulline, nitric oxide (NOx), cyclic guanosine monophosphate (cGMP), lactate, and asymmetric dimethylarginine (ADMA) before and after L-arginine therapy in the acute phase of strokelike episodes in MELAS. Data represent mean ± SD (μmol/L) (n = 24). *p < 0.05; **p < 0.01 vs values before L-arginine therapy. ns = not significant. Filled circles show biochemical analysis after L-arginine therapy. Open squares show biochemical analysis after administration of placebo.*

correlation in all amino acids and found that the decrease of L-arginine in the acute phase is not influenced by urea cycle activities but may be caused by endothelial dysfunction (data not shown). A low L-arginine concentration and a relatively high ADMA concentration may predispose to strokelike episodes in MELAS. Impairment of endothelial function associated with relatively increased ADMA concentrations is reversed by IV L-arginine.[7] Consistent with these data, L-arginine infusion improved the ischemic process during the acute phase of MELAS.

Focal cerebral hyperemia has been reported in MELAS.[8] Although the underlying mechanisms are incompletely understood, hyperemia is thought to reflect vasodilation caused by local metabolic acidosis in the area of the infarct or by the foci of periodic epileptiform discharge.[9] Because the above studies were performed several days or several weeks after the onset of a strokelike episode, secondarily induced NOx production generated by inducible NOx synthase in the injured region may alter evidence of the primary pathophysiologic abnormality. In an analysis of SPECT findings in young patients with MELAS at a very early stage of strokelike episodes (within 3 hours after onset), we found hypoperfusion in the region affected by the strokelike episode. We cannot explain conclusively why our findings differ from those reported by neurologists treating adults. If the sites of angiopathy in MELAS most likely include small cerebral arteries, arterioles, and capillaries, small infarcts would be expected rather than the large confluent region of infarction described in many reports of MELAS. L-Arginine is an important precursor of NOx, which may reduce ischemic damage in the acute phase of focal brain ischemia by increasing microcirculation in the cerebral blood flow. The symptoms improved earliest, and magnetic resonance spectroscopy abnormality was minimal when L-arginine was given during the acute phase of strokelike episodes in MELAS.[10]

We evaluated the effects of oral L-arginine supplementation on long-term occurrence of strokelike episodes. The frequency and severity of clinical symptoms of strokelike episodes decreased without serious adverse effects. Prophylactically treated patients with MELAS have not had major strokelike attacks such as hemiconvulsion and hemiparesis. Headache and teichopsia have occurred.

References

1. Pavlakis SG, Phillips PC, DiMauro S, et al. Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes: a distinctive clinical syndrome. Ann Neurol 1984;16:481–488.
2. Koga Y, Ishibashi M, Ueki I, et al. Effects of L-arginine on the acute phase of strokes in three patients with MELAS. Neurology 2002;58:827–828.
3. Lassen LH, Ashina M, Christiansen I, et al. Nitric oxide synthase inhibition in migraine. Lancet 1997;349:401–402.
4. Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive diseases. Circulation 1997;95:2068–2074.
5. Nims RW, Darbyshire JF, Saavedra JE, et al. Colorimetric methods for the determination of nitric oxide concentration in neutral aqueous solutions. Methods Enzymol 1996;268:93–105.
6. Honma M, Satoh T, Takezawa J. An ultrasensitive method for the simultaneous determination of cyclic GMP in small-volume samples from blood and tissue. Biochem Med 1977;18:257–273.
7. Piatti PM, Fragasso G, Monti LD, et al. Acute intravenous L-arginine infusion decreases endothelin-1 levels and improves endothelial function in patients with angina pectoris and normal coronary arteriograms. Circulation 2003;107:429–436.
8. Gropen TI, Prohovnik I, Tatemichi TK, Hirano M. Cerebral hyperemia in MELAS. Stroke 1994;25:1873–1876.
9. Iizuka T, Sakai F, Suzuki N, et al. Neuronal hyperexcitability in stroke-like episodes of MELAS syndrome. Neurology 2002;59:816–824.
10. Kubota M, Sakakihara Y, Mori M, et al. Beneficial effect of L-arginine for stroke-like episode in MELAS. Brain Dev 2004;26:481–483

Title: A new sequence variant in mitochondrial DNA associated with high penetrance of Russian Leber hereditary optic neuropathy

Authors: Nataliya Povalko, MD[1,2], Ekaterina Zakharova, MD, PhD[2], Galina Rudenskaia, MD, PhD[2], Yukihiro Akita, MD, PhD[1], Koji Hirata, MD, PhD[1], Matsuishi Toyojiro, MD, PhD[1], Yasutoshi Koga, MD, PhD[1]*

Affiliations:[1]Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi Machi, Kurume, Fukuoka 830-0011, Japan, [2] Research Centre for Medical Genetics RAMNS, Moskvorechie St. 1 Moscow 115478, Russia

***Correspondence to:** Yasutoshi Koga, Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-machi, Kurume city, Fukuoka 830-0011, Japan

Tel.: (81) 942-31-7565. Fax: (81) 942-38-1792. E-mail: yasukoga@med.kurume-u.ac.jp

Acknowledgements and Fundings: Ministry of Culture and Education in Japan:#13670853, #16390308, Evidence-based Medicine, Ministry of Health, Labor and Welfare in Japan: #H14-006, and #H14-team(syouni)-005, and Uehara Memorial Foundation.

Abstract

We have analyzed mitochondrial DNA sequence in 15 Russian LHON patients and found the new mtDNA sequence variant in one family (2 patients) who showed 100% penetrance of the disease in men. This family has a T14484C primary mutation, and 4 secondary mutations (T4216C, G 13708A, G 15812A, G15257A), which belong to the European haplogroup J. The new sequence variant of A9016G in the ATPase 6 gene changed highly conserved amino acid of isoleucine to valine, has not been found in the rest of 13 LHON patients and controls. This novel sequence variant may contribute to the 100% penetration of LHON disorder in men of this family.

Key words: LHON, point mutation, penetrance, mitochondrial DNA

Introduction

Leber Hereditary Optic Neuropathy (LHON [MIM 535000]) is a maternally inherited genetic disorder associated with point mutations in the mitochondrial DNA (mtDNA) that cause blindness in young adults (Wallace et al., 1988; Howell. et al., 2003). It is characterized by acute or subacute, progressive, and bilateral loss of central vision due to focal degeneration of the retinal ganglion cell layer and optic nerve. Common primary LHON mutations are G3460A, G11778A, and T14484C not in association with each other, heteroplasmic and are absent among control are necessary for expression of the disease but not sufficient (Man et al., 2002). Secondary/intermediate LHON mutations showed different frequency pattern among the three classes of above common primary mutations in positive patients and controls, usually homoplasmic, and may contribute to the LHON by increasing the probability of expressing the phenotype (Torroni et al., 1997).

Patients and methods.

Patients.

Fifteen patients from 13 unrelated Russian families were fulfilled the clinical criteria of LHON (Wallace et al., 2001), however, patients 8, 9, 11, 12, 13, 14 and 15 have no family history. Three women and twelve men from 10 to 56 years old with mean age of onset about 22.3 years. The research followed the tenets of the Declaration of Helsinki. Informed consent

was obtained from the patients after explanation of the nature and possible consequences of the study. DNA was taken and studied for the various mtDNA mutations.

One family which has the highest penetrance among our 13 unrelated families is shown in Figure 1. The proband is a 35-year-old Caucasian man, who suffered from a rapid bilateral painless loss of central vision. The loss of vision began at the age of 18 years, and at the same period, the patient was admitted to the hospital with diabetic coma due to insufficient insuline production. He had a 6-month interval between the blindness in both eyes which was not caused by diabetic retinopathy. The visual acuity is 0.02-0.03 with the central scotomas. Bilateral loss of central vision was also detected in patient's mother, a 56-year-old woman, when she was 47 years old. She had two brothers and two uncles who were completely blind from their youth, showing 100% penetrance of LHON disease in men of maternal lineage. There is no family history of diabetes mellitus, deafness or neuromuscular disorders except blindness.

Sequencing analysis.

Total DNA was extracted from white blood cells using the standard method (King et al., 1992). The complete mitochondrial genome was amplified by long-PCR method in 7 overlapping fragments using 14 primers (Table1). The PCR conditions were: first, one cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 3 min, and finally, one cycle of 72 °C for 5 min followed by cooling to 4 °C. The quality and quantity of

template DNA were determined by 1% agarose gel electrophoresis. ExoSap-IT (USB Corporation, Ohio USA) utilizes two hydrolytic enzymes Exonuclease I and Shrimp Alkaline Phosphatase together in a specially formulated buffer, to remove unincorporated dNTPs and primers. The ExoSap-IT was added directly to the PCR product. After treatment, ExoSap-IT was inactivated simply by heating to 80 °C for 15 minutes. Using 40 forward primers (Table 2) and CEQ Dye Terminating Cycle Sequencing Kit (Beckman Coulter, Inc., Fullerton, CA), sequence reaction was performed as following: first, one cycle of 96 °C for 5 min, followed by 30 cycles of 96 °C for 20 sec, 50 °C for 20 sec, 60 °C for 3 min, and finally, one cycle of 60 °C for 5 min followed by cooling to 4 °C. The sequences were assembled in a contig using the program DNASIS Pro (Hitachi Software Engineering Co, Ltd, Tokyo, Japan) and the resulting contig was aligned to the Cambridge sequence (Anderson et al., 1981; Andrews et al., 1999).

PCR-RFLP analysis.

To confirm the heteroplasmic condition in primary and secondary mutation reported in LHON, or novel sequence variant, we performed the PCR-RFLP analysis using suitable sets of primers and restriction enzymes (Brown et al., 1995; Brown et al., 2001). Conditions for all PCR amplifications were as follows: first, one cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55.5 °C for 1 min, 72 °C for 45 sec, and finally, one cycle of 72 °C for 5 min followed by cooling to 4 °C. Diagnostic restriction endonuclease digestions were resolved by 12% polyacrylamide gel electrophoresis, and the DNA fragments were identified by ethidium

bromide.

For new A9016G sequence variant, we designed a set of primers as following: forward from np8833 to np8852 and reverse from np9140 to np9121 (5'-3').This fragment contains a single restriction site for HpyCH4IV in the presence of mutation. To eliminate partial enzymatic digestion, all restriction enzyme reactions were carried out overnight, followed by the addition of 10 units of enzyme and 2 additional hours of incubation. Percentage of heteroplasmy was quantitated by one-dimensional densitometry using Image Quant software version 4.1 (Molecular Dynamics, Sunnyvale, CA, USA).

Results.

All nucleotide changes found in our study are shown in Table 3. Seven patients from 5 unrelated families have one of the primary mutations. Two unrelated patients (patients 1 and 7) have a G11778A mutation. One of them (patient 7: haplogroup J) has two secondary mutations of T4216C and G13708A, however, the other (patient 1: haplogroup W) has no secondary mutation. Three patients (2 families) have a G3460A mutation, one family (patients 4 and 5: haplogroup X) has no secondary mutations, and the other (patient 6: haplogroup T1) has a T4216C and A4917G mutations. Patients 2 and 3 (mother and son) belong to haplogroup J and have a primary mutation of T14484C and secondary mutations of a T4216C, G13708A, G15257A, and G15812A. Eight patients from 8 families (patients from 8 to 15) have no known

primary mutation in their mtDNA, however 3 patients (patients 8, 12, and 13) from this group have one of the secondary mutations of G13708A or A4917G. Remaining 5 patients have none of pathogenic mutations reported before. Moreover, we have found a novel sequence variant of A9016G in the ATPase 6 gene in mother and son (patients 2 and 3) who have a primary mutation of T14484C (Figure 2).

Among 13 unrelated families from Russian LHON, distribution of mtDNA haplogroups were as follows: 23% for haplogroup H (n=3) ; 23% for haplogroup J (n=3) ; 15.4 % for haplogroup X (n=2) ;15.4 % for haplogroup T (n=2) ; 7.7 % for haplogroup U2 (n=1) ; and 7.7 % for haplogroup M (n=1).

Discussion.

LHON is characterized by incomplete penetrance and men are preferentially affected (~68%) when the primary mutation of T14484C exist (Wallace et al., 2001). Since our family (patients 2 and 3), who showed 100% penetrance of the disease in men in three generations, has the nucleotide changes at C5633T, A11251G, A12612G, and C15452A in addition to primary mutation of T14484C in the homoplasmic condition, (Herrnstadt et al., 2002) they are constituent of European mtDNA haplogroup J background which is present in European population at a frequency of 9 %. The other factors such as smoking, alcohol excess, diet, psychological stress, exposure to toxins, head trauma have been among the epidemiologic

factors suspected of increasing the penetrance of LHON (Tsao et al., 1999). However in our family, there are no obvious risk factors to increase the penetrance of this disorder. We found the novel A9016G sequence variant in both mother and son in this family. This sequence variant was heteroplasmic and results in a substitution of isoleucine for valine at a highly conserved residue in the ATPase 6 polypeptide (Figure 3). Hence, this is likely to be pathogenic mutation according to above information (Riordan-Eva and Harding ,1995; Brown et al., 2002). This sequence variant has not been reported in the literature, MITOMAP and mtSNP database, and has not been found in 13 Russian LHON patients and in 30 Japanese individuals. Though a number of studies have failed to make the important distinction between frank pathogenic mtDNA mutations and haplogroup-associated polymorphism (Chagnon et al., 1999; Lin et al., 1992), this A9016G sequence variant may increase the penetrance of the disease in LHON, when it is associated with the primary mutation of T14484C and secondary mutations of a T4216C, G13708A, G15257A, and G15812A. In addition, this family has the unique combination of the following polymorphisms of A9494G, G11718A, A15662G, and C16193T which are reported in the literature, however, they are not present in the rest of 12 families in this study.

We could not perform further biochemical analysis in this index family, because specimens were not available. Thirty-seven to fifty percent of LHON having T14484C primary mutation are reported to be recovered within 16 months interval (Wallace DC et al., 2001, Web

site of Mitochondrial Research Society). However we have not observed such recovery of blindness in the members of this family. Though the pedigree is so small and additional biochemical date is not available in this study, we suggest that an A9016G sequence variant is specific for this family and may increase the penetration of LHON with the combination of secondary mutations (Mackey, 1994). Above information will continue to provide us with new insights into the pathophysiology of mitochondrial disease.

Acknowledgements

This work was supported in part by grants #13670853, #16390308 from the Ministry of Culture and Education in Japan, #H14-006, and #H14-team(syouni)-005 from Evidence-based Medicine, Ministry of Health, Labor and Welfare in Japan; and Uehara Memorial Foundation.

References

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. 1981. Sequence and organization of the human mitochondrial genome. *Nature.*290:457-465.

Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet.*23(2):147.

Brown MD, Starikovskaya E, Derbeneva O, Hosseini S, Allen JC, Mikhailovskaya IE, Sukernik RI, Wallace DC. 2002. The role of mtDNA background in disease expression: a new primary LHON mutation associated with Western Eurasian haplogroup *J. Hum Genet* 110:130-138.

Brown MD., Torroni A., Record CL., Wallace DC. 1995. Phylogenetic analysis of Leber's hereditary optic neuropathy mitochondrial DNA's indicates multiple independent occurrences of the common mutations. Hum. Mutat 6:311-325

Brown MD, Allen JC, Van Stavern GP, Newman NJ, Wallace DC. 2001. Clinical, genetic, and biochemical characterization of a Leber heredatiry optic neuropathy famyli containing both the 11778 and 14484 mutations. Am.J.Med.Genet. 104:331-338.

Chagnon P, Gee M, Filion M, Robitaille Y, Belouchi M, Gauvreau D. 1999. Phylogenic analysis of the mitochondrial genomes indicates significant differences between patients with

Alzheimer disease and controls in a French-Canadian founder population. *Am J Med Genet.* 85(1):20-30.

Herrnstadt C, Elson JL, Fahy E, Preston G, Turnbull DM, Anderson C, Ghosh SS, Olefsky JM, Beal MF, Davis RE, Howell N.2002. Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups. *Am J Hum Genet.* 70(5):1152-1171.

Howell N, Oostra RJ, Bolhuis PA, Spruijt L, Clarke LA, Mackey DA, Preston G, Herrnstadt C. 2003 Sequence analysis of the mitochondrial genomes from Dutch pedigrees with Leber hereditary optic neuropathy. *Am J Hum Genet.*; 72 (6):1460-1469.

King MP, Koga Y, Davidson M, Schon EA. 1992. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the tRNALeu (UUR) mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. *Mol Cell Biol.*12 : 480-490.

Lin FH, Lin R, Wisniewski HM, Hwang YW, Grundke-Iqbal I, Healy-Louie G, Iqbal K. 1992. Detection of point mutations in codon 331 of mitochondrial NADH dehydrogenase subunit 2 in Alzheimer's brain. *Bioch Biophys Res Commun.* 182:238-246.

Mackey DA. 1994. Three subgroups of patients from the United Kingdom with Leber hereditary optic neuropathy. *Eye.* 8(Pt 4) : 431-436.

Man PYW, Turnbull DM, Chinnery PF. 2002. Leber hereditary optic neuropathy. *J Med Genet* .

39:162-169.

Riordan-Eva P, Harding AE. 1995. Lever's hereditary optic neuropathy: the clinical relevance of different mitochondrial DNA mutations. *J Med Genet.* 32:81-87.

Tsao K, Aitken PA, Johns DR. 1999. Smoking as an aetiological factor in a pedigree with Leber's hereditary optic neuropathy. *Br J Ophthalmol.* 83:577-581.

Torroni A, Petrozzi M, D'Urbano L,Sellitto D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A, Scozzari R. 1997. Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber Hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. *Am J Hum Genet.* 60:1107-1121.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ2nd, Nikoskelainen EK. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science.* 242(4884):1427-1430.

Wallace DC, Lott MT, Brown MD, Kerstann K. 2001. Mitochondria and neuro-ophthalmologic diseases. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. *The metabolic & molecular bases of inherited disease.* 8th ed. New York. McGraw-Hill, Inc, pp. 2425-2509.

MITOMAP. The human mtDNA database: http://www.mitomap.org

GIFU International Institute of Biotechnology. GIIB-JST mitochondrial mtSNP database: http://www.giib.or.jp

Web site of Mitochondrial Research Society.

http//www.neuro.wustl.edu/neuromuscular/mitosyn.html

Table 1. PCR primers used for amplification of the entire mtDNA of Russian LHON patients.

Fragment	Forward primer	Reverse primer	Length(bp)
1	Gn549F:5'-caa cca aac ccc aaa gac ac-3'	Gn3424B:5'-aac gtt ggg gcc ttt gcg ta-3'	2876
2	Gn3205F:5'-cca cac cca ccc aag aac ag-3'	Gn5910B:5'-cga aca tca gtg ggg gtg ag-3'	2706
3	Gn5662F:5'-cca atg gga ctt aaa ccc ac-3'	Gn8028B:5'-gct tca atc ggg agt act ac-3'	2367
4	Gn7794F:5'-cca tca tcc tag tcc tca tc-3'	Gn10515B:5'-gtg aga tgg taa atg cta g-3'	2722
5	Gn10289F:5'-agc cct aca aac aac taa cc-3'	Gn12360B:5'-ggt tat agt agt gtg cat gg-3'	2072
6	Gn12144F:5'-aca tca tta ccg ggt ttt cc-3'	Gn14762B:5'-tgc gta ttg ggg tca ttg g-3'	2619
7	Gn14641F:5'-acc cac act caa cag aaa c-3'	Gn706B:5'-gga tgc ttg cat gtg taa tc-3'	2635

Table 2. Forward primers used for direct sequence.

Number	Primer	5'-position
1.	CQ549:5'-caa cca aac ccc aaa gac acc	549
2.	CQ611:5'-gaa aat gtt tag acg ggc tc	611
3.	CQ 1161:5'-aac tca aag gac ctg gcg gt	1161
4.	CQ1690:5'-cca ctc cac ctt act acc aga c	1690
5.	CQ2164:5'-ccc ata gta ggc cta aaa gca gcc	2164
6.	CQ2629:5'-atg aat ggc tcc acg agg gtt c	2629
7.	CQ3116:5'-cct ccc tgt acg aaa gga c	3116
8.	CQ3205:5'-cca cac cca ccc aag aac agg g	3205
9.	CQ3712:5'-gta gcc caa aca atc tca ta	3712
10.	CQ4240:5'-tcc agc att ccc cct caa ac	4240
11.	CQ4621:5'-gtt cca cag aag ctg cca tc	4621
12.	CQ5151:5'-cta cta cta tct cgc acc tg	5151
13.	CQ5662:5'-cca atg gga ctt aaa ccc ac	5662
14.	CQ6251:5'-tat agt gga ggc cgg agc ag	6251

15.	CQ6801:5'-gac aca cga gca tat ttc ac	6801
16.	CQ7321:5'-gag aag cct tcg ctt cga ag	7321
17.	CQ7794:5'-cca tca tcc tag tcc tca tc	7794
18.	CQ8239:5'-ctt tga aat agg gcc cgt att tac c	8239
19.	CQ8634:5'-tct cat caa caa ccg act aa	8634
20.	CQ9091:5'-aca ctt atc atc ttc aca at	9091
21.	CQ9621:5'-gca tca gga gta tca atc ac	9621
22.	CQ10133:5'-aca act caa cgg cta cat ag	10133
23.	CQ10289:5'-agc cct aca aac aac taa cc	10289
24.	CQ10616:5'-caa cac cca ctc cct ctt ag	10616
25.	CQ11183:5'-cgc ctg aac gca ggc aca ta	11183
26.	CQ11671:5'-aac ccc ctg aag ctt cac cg	11671
27.	CQ11900:5'-gtg cta gta acc acg ttc tcc	11900
28.	CQ12144:5'-aca tca tta ccg ggt ttt cc	12144
30.	CQ12602:5'-tca tcc ctg tag cat tgt tc	12602
31.	CQ13101:5'-agg aat ctt ctt act cat cc	13101
32.	CQ13490:5'-cct cac agg ttt cta ctc c	13491
33.	CQ13949:5'-cct atc tag gcc ttc tta cga gcc	13949
34.	CQ14511:5'-cta tta aac cca tat aac ct	14511