Figure 3 Immunoperoxidase staining of the organs, showing age dependent changes in AP2 immunoreactivity. Pancreas at (A) 11 years and (D) 32 years; seminiferous tubules in the testis at (B) 11 years and (E) 32 years; cortex of the adrenal gland at (C) 11 years and (F) 32 years. Arrows indicate AP2 positive cells. F, zona fasciculata; R, zona reticularis. Scale bar, l μm.

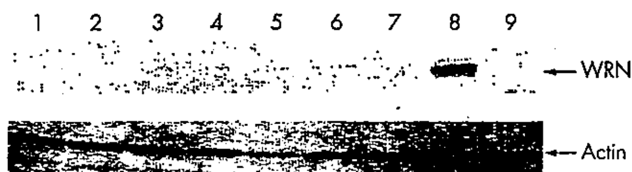

Figure 4 Immunoblot of cytosol extracts probed with the anti-WRNp antibody, showing age dependent changes in Werner's protein (WRNp) immunoreactivity. Lanes 1–3, tissues from the control subject at 27 gestational weeks: frontal lobe (lane 1), pancreas (lane 2), kidney (lane 3); lanes 4–6, tissues from the 11 year old control subject: frontal lobe (lane 4), pancreas (lane 5), kidney (lane 6); lanes 7–9, tissues from the 64 year old control subject: frontal lobe (lane 7), pancreas (lane 8), kidney (lane 9). Antiserum demonstrated the presence of the 170 kDa WRNp in the pancreas from the 64 year old control subject. An aliquot of 50 μg of extracted protein was applied to each lane. The lower figure shows immunoblot analysis of actin as a control for loading.

We demonstrated that the SP1 and AP2 transcription factors, which regulate WRN gene expression, also appeared in an age dependent manner in the regions where WRNp was expressed. In the controls after puberty, SP1 was expressed in the seminiferous epithelial cells and Leydig cells of the testis, and in the zona fasciculata and zona reticularis of the adrenal cortex, whereas AP2 was expressed in the glandular acini of the pancreas. These findings suggest that the copy number of these transcription factors is also increased after puberty, resulting in the upregulation of WRN gene expression.

It is noticeable that those organs that express high amounts of WRNp are also responsible for the secretion of sex hormones (androgen and testosterone). The sex hormonal disturbances seen in aging occur together with a decrease in the secretion of androgen and oestrogen in the testicles. An important decrease in adrenal androgen secretion has been noted in both sexes. These hormonal disturbances are thought to promote aging, especially in bone, muscle, skin, and mucous membranes.[16] Testosterone, which is not only responsible for the development of male secondary sexual characteristics at puberty, but is also essential for the continued function of the seminiferous epithelium, is the principal hormone secreted by Leydig cells. The zona fasciculata and the zona reticularis are thought to be responsible for the secretion of androgens. In the patient with WS, the lack of WRN gene expression in the Leydig cells, zona fasciculata, and zona reticularis may cause an accelerated cellular senescence as a result of a reduction in androgen and testosterone secretion. In fact, in the patient with WS, microscopic examination revealed atrophy of the zona fasciculata and zona reticularis, which was more pronounced than that seen in the older control patients.

To determine whether there was a disturbance of sex hormonal secretions in the patient with WS, we analysed the concentration of dehydroepiandrosterone (DHEA) in blood from this patient when she was admitted to our hospital.

Take home messages

- The WRN gene was expressed in selected tissues after puberty together with the transcription factors SP1 and AP2
- In the patient with WS, microscopic examination revealed atrophy of the zona fasciculata and zona reticularis, which was more pronounced than that seen in the older control patients
- Those organs that express high amounts of Werner's syndrome protein (WRNp) are also responsible for the secretion of sex hormones (androgen and testosterone)
- The lack of WRNp functions in selected organs closely correlate with the Werner's syndrome phenotype

DHEA, which is a precursor of androgen and acts as androgen itself, is produced principally in the zona fasciculata and the zona reticularis of the adrenal cortex, and after sulphate conjugation (to produce DHEA-S), is secreted into blood. The concentration of DHEA-S (230 ng/ml) in our patient with WS was lower than that seen in age matched female controls (normal range, 400–3500 ng/ml).

Endocrine and metabolic abnormalities have been reported in patients with WS when compared with normal, aged subjects.[17] The serum testosterone concentrations of patients with WS were lower than those of age matched controls, and testicular biopsy revealed more pronounced atrophy than that seen in aged subjects.[17]

In conclusion, the WRN gene was expressed in selected tissues after puberty, and the transcription factors were coexpressed. The lack of WRNp functions in selected organs closely correlate with the WS phenotype.

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Authors' affiliations

K Motonaga, M Itoh, Y Goto, Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
S Takashima, National Institute of Neuroscience, National Center of Neurology and Psychiatry
Y Hachiya, A Endo, Department of Clinical Laboratory, National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry
K Kato, H Ishikura, Department of Pathology, Chiba University School of Medicine, 1-8-1 Inohana, Chiba 260-0856, Japan
Y Saito, S Mori, The Second Department of Internal Medicine, Chiba University School of Medicine

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PERGAMON

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A point mutation of mitochondrial ATPase 6 gene in Leigh syndrome

Motohiro Akagi[a], Koji Inui[a,*], Hiroko Tsukamoto[a], Norio Sakai[a], Takashi Muramatsu[a], Minoru Yamada[a], Kouzi Matsuzaki[b], Yu-ichi Goto[c], Ikuya Nonaka[d], Shintaro Okada[a]

[a]*Department of Developmental Medicine (Pediatrics), Osaka University, Graduate School of Medical Science, 2-2 Yamadaoka, Suita, Osaka, Japan*
[b]*Suita Municipal Hospital, Suita, Osaka, Japan*
[c]*Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, NCNP, Kodaira, Tokyo, Japan*
[d]*Department of Ultrastructural Research, National Institute of Neuroscience, NCNP, Kodaira, Tokyo, Japan*

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Abstract

A T-to-G transition at nucleotide 9176 (T9176G) in the mitochondrial adenosine triphosphate 6 gene (MTATP6) was detected in two siblings with Leigh syndrome. Heteroplasmy was observed in the mother's leukocytes. The T9176G mutation changes a highly conserved leucine residue to an arginine in subunit 6 of ATPase and is maternally inherited like mutations in the other mitochondrial genes. Another mutation in the same codon (T9176C) has been previously reported in Leigh syndrome. This gives strong support to the relevance of MTATP6 dysfunction in Leigh syndrome and the importance of leucine at that position. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mitochondrial DNA; 9176 mutation; Leigh syndrome

1. Introduction

Leigh syndrome (subacute necrotizing encephalopathy) is a progressive neurodegenerative disorder in early infancy or childhood leading to death within months or years [1]. The disease is characterized with degeneration of the bilateral basal ganglia and brain-stem and consists of a group of heterogeneous metabolic disorders. In cases of Leigh or Leigh-like syndrome, the reported frequency of a mitochondrial DNA (mtDNA) defect is 18%, and most mtDNA defects have been associated with mutations at nucleotide (nt) 8993 or 9176 of the mtATPase 6 gene [1–7]. We surveyed reported mtDNA mutations [7] in fibroblasts, muscles and peripheral blood leukocytes of eight patients clinically diagnosed with Leigh syndrome and found a new T9176G mutation in the MTATP6 in a familial case of Leigh syndrome.

2. Patients and methods

2.1. Patient 1

The proband was delivered by cesarean section because of a breech presentation at 38 weeks of gestational age. Her birth weight was 2150 g and she developed normally until 6 months of age. Her development thereafter was gradually delayed: she crawled at 9 months, sat up at 14 months, and walked with support at 15 months of age. At 19 months of age, she had an episode of generalized convulsions, and the administration of phenobarbital was initiated. At 22 months of age, acetazolamide was added to control her convulsions. After 2 days, she showed tachycardia, tachypnea, a generalized convulsion, confusion, and became markedly spastic. Her brain computed tomographic (CT) scan showed symmetric hypodensities in the basal ganglia. The arterial plasma lactate and pyruvate levels were normal, but her cerebrospinal fluid (CSF) lactate and pyruvate levels were elevated to 55.0 and 2.65 mg/dl, respectively. She was diagnosed with Leigh syndrome and was unable to walk at 24 months of age. She had repeated episodes of pneumonia and died at 6 years and 1 month of age.

2.2. Patient 2

Her younger brother was delivered by cesarean section at 34 weeks of gestational age. His birth weight was 2190 g. Acute renal failure due to right renal dysplasia was found at birth. His developmental delay was first noticed at 5 months of age. He was suspected of having Leigh syndrome, but magnetic resonance imaging of his brain showed normal findings. At 2 years and 1 month of age, he could not

* Corresponding author. Tel.: +81-6-6879-3932; fax: +81-6-6879-3939.
E-mail address: koji@ped.med.osaka-u.ac.jp (K. Inui).

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walk by himself and his brain CT scan showed symmetric hypodensities in the basal ganglia and brain-stem. He was diagnosed with Leigh syndrome. At 2 years and 3 months of age, a muscle biopsy was performed and showed mild myopathic changes, but without ragged-red fibers. A biochemical analysis of his muscles showed normal enzymatic activities of NADH cytochrome *c* reductase, succinate *c* reductase, and cytochrome *c* oxidase. His neurological deterioration rapidly progressed, and he was intubated at 4 years and 2 months of age because of respiratory failure with periodic apnea. He died at 10 years and 7 months of age. Their mother was clinically normal.

2.3. Mutation analysis

DNA was isolated from fibroblasts of patient 1, biopsied muscle of patient 2, and peripheral blood leukocytes of their mother by the standard method. The MTATP6 was amplified from isolated DNA by polymerase chain reaction (PCR) methods using the following primer sets: (A), nt 8817–8836 (sense) 5′-ACT ATC TAT AAA CCT AGCCA-3′; (B), nt 9971–9990 (antisense) 5′-AAG ACC CTC ATC AAT AGATG-3′; and (C), nt 9207–9226 (sense) 5′-ATG ACC CAC CAA TCA CAT GC-3′. We amplified a 1174 bp fragment by PCR using primer sets of A and B under the follow-

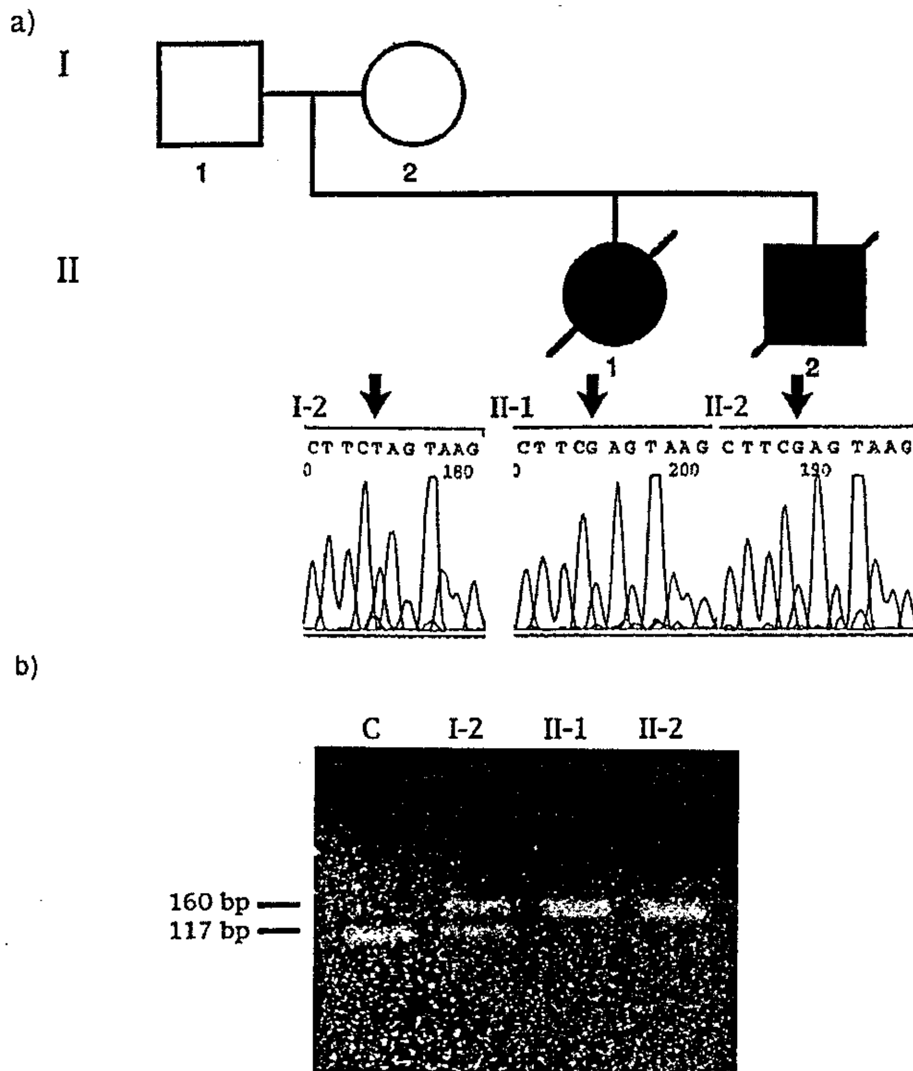

Fig. 1. (a) Direct sequence analysis of MTATP6. The patients have the homoplasmic T9176G mutation, whereas the mother has the 9176G and T sequence. (b) Restriction enzyme analysis encompassing the nt 9176 site. In a normal sequence, the amplified 160 bp PCR product containing one site of *Mae* I was digested to 117 and 43 bp products with *Mae* I, and only the 117 bp was visible in 1.5% NuSieve agarose gel electrophoresis (lower band). When there is a T-to-G transition at nt 9176, one site of *Mae* I is lost, resulting in 160 bp produced after *Mae* I digestion (upper band). In the patients, only 160 bp products were detected, which showed the homoplasmic state at the point (II-1,-2). While the mother showed a heteroplasmic pattern (I-2) and the mutant amount was estimated to be about 50% by densitometry, the controls showed only a lower band (c). These results are in agreement with direct sequence analysis.

ing conditions: 30 cycles of denaturation for 45 s at 94°C, annealing for 75 s at 53°C, and extension for 90 s at 72°C. The amplified products were purified and directly sequenced with primer sets of A and C by dye terminator cycle sequencing ready reaction (ABI 310 Genetic Analyzer, Perkin–Elmer, Foster City, CA). To confirm the mutation identified in the patient, the 160 bp PCR products encompassing the mutation site using the primer sets: (D), nt 9132–9151 (sense) 5′-AGA AAT CGC TGT CGC CTT AA-3′; and (E), nt 9273–9292 (antisense) 5′-GAG GTC ATT AGG AGG GCT GA-3′, were digested with 10 U of *Mae* I (*Mae* I site is lost due to T1976G transition) for 2 h at 45°C in a final volume of 20 μl and subjected to 1.5% NuSieve (FMC Bio Products, Rockland, ME) agarose and 1.5% Agarose S (Wako) gel electrophoresis.

3. Results

DNA sequence analysis revealed that the patients had a T-to-G transition at mtDNA nt 9176, resulting in an amino acid change of from Leu to Arg of ATPase 6. Their mother was found to be heteroplasmic (T and G) for the 9176 mutation (Fig. 1a). To confirm this evidence, restriction site analysis of mtDNA from blood samples was performed. The T9176G mutation obliterated a *Mae* I site, and fragments of 117 and 43 bp obtained with the digestion. In the patients, only a 160 bp fragment was detected. On the other hand, 160 and 117 bp fragments were obtained from the mother and the 160 bp fragment was estimated to be about 50% by densitometry, and only a 117 bp fragment was found in 30 normal controls (Fig. 1b). These data show that their mother has a heteroplasmic condition for the T9176G mutation. The T8993G and T8993C mutations were not detected (data not shown). The reported polymorphisms of G9055A [8] were found in patients and the mother (data not shown).

4. Discussion

We have described familial cases of Leigh syndrome with slow progressive symptoms and identified a new point mutation of T9176G in the MTATP with a G9055A polymorphism. Using DNA sequence analysis of amplified PCR products of MTATP6 and subsequent restriction enzyme analysis, homoplasmy for the T9176G mutation was detected in the patients. mtDNA analysis of their mother at that position showed that she was heteroplasmic for the mutation. Concerning the mutation, Carrozzo et al. recently demonstrated inactive ATP synthetase in *Escherichia coli* [9]. From this evidence, the mutation is expected to be pathological and important part of the amino acid of leucine at the position of ATPase 6. Along with an mtDNA mutation at nt 8993 having a T-to-G or T-to-C mutation, it provides strong evidence for the relevance of ATP synthetase dysfunction in maternally inherited Leigh syndrome.

A number of mutations causing Leigh syndrome have been identified in mtDNA [1–7]. The clinical presentations of our cases are similar to the reported cases of T9176C mutation [10], and the sister and brother had comparable clinical courses. However, there is a report of fulminant Leigh syndrome with a T9176C mutation [11]. Analysis of mtDNA mutations illustrates the complex genotype–phenotype correlation in mtDNA disorders, probably because there is variation in the mutant loads among tissues, and because the mutant load in a given tissue may change over time.

Note: After submission of this paper, Carrozzo et al. reported the same mtDNA T9176G point mutation in a child with Leigh syndrome [12].

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SHORT COMMUNICATION

Masakazu Mimaki · Akemi Ikota · Aya Sato
Hirofumi Komaki · Jun Akanuma · Ikuya Nonaka
Yu-ichi Goto

A double mutation (G11778A and G12192A) in mitochondrial DNA associated with Leber's hereditary optic neuropathy and cardiomyopathy

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Abstract We report a male patient with Leber's hereditary optic neuropathy (LHON) and hypertrophic cardiomyopathy. Besides a G11778A mutation in the *ND4* gene of the mitochondrial DNA (mtDNA), one of the most common mutations in LHON patients, sequencing of total mtDNA revealed a G12192A mutation in the tRNA (His) gene that was recently noted to be a risk factor for cardiomyopathy. Because no case of LHON presenting with cardiomyopathy has been reported, the present finding suggests that the G12192A mutation caused cardiomyopathy as an additional symptom. In the present case, the double pathogenic mtDNA mutations may be associated either synergistically or concomitantly with two different clinical manifestations.

Key words Leber's hereditary optic neuropathy (LHON) · Cardiomyopathy · Mitochondria · DNA · Mutation

Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by acute or subacute visual impairment in both eyes. The association between LHON and mitochondrial DNA (mtDNA) was first identified in 1988 when a G11778A mutation in the *ND4* gene of mtDNA was found in nine unrelated families (Wallace et al. 1988). Since then, it has come to be understood that this is one of the most common mutations causing LHON (Riordan-Eva et al. 1995). It is also recognized that some idiopathic cardiomyopathy is associated with mtDNA mutations (Chan and Allen 1995), and a G12192A mutation in tRNA (His) was recently reported to be a risk factor for cardiomyopathy (Shin et al. 2000).

Here, we report a unique patient with LHON presenting with hypertrophic cardiomyopathy, who carried both the G11778A and G12192A mutations.

M. Mimaki · H. Komaki · J. Akanuma · Y. Goto (✉)
Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
Tel. +81-42-346-1713; Fax +81-42-346-1743
e-mail: goto@ncnp.go.jp

A. Ikota
Department of Internal Medicine, Kin-ikyo Sapporo Hospital, Sapporo, Japan

A. Sato
Department of Medical Chemistry, Kagawa Nutrition University, Saitama, Japan

I. Nonaka
National Center Hospital for Mental, Nervous and Muscular Disorders, NCNP, Tokyo, Japan

Patient and methods

A 51-year-old Japanese man was admitted to hospital because of subacute body weight loss and general malaise. His past history was unremarkable except for mild diabetes mellitus of 5 years' duration. He smoked 20–40 cigarettes and drank 1000ml of beer and a third of a bottle of whisky almost every day. On admission, he was suffering from chronic renal failure accompanied by hypertension. A son of his sister's daughter had suffered visual loss since the age of 12 years, but there was no other family history related to the patient's condition.

Six months after he presented at the hospital, he began to experience rapid progressive visual loss. On examination using the Landolt ring visual chart, his visual acuity in the right eye was found to have decreased from 1.2 to 0.01 in only 3 months, and successively it decreased rapidly in the left eye. Finally, he became blind about 6 months later. Examination of the ocular fundus showed peripapillary telangiectasia without findings of diabetic retinopathy. His renal failure was also becoming worse simultaneously with the decrease of visual acuity, and hemodialysis became necessary 2 years after his hospital stay. A renal biopsy revealed pathological findings mimicking those of the end stage of arteriosclerotic kidney without the characteristic findings of diabetic nephropathy. The diabetes mellitus was

Table 1. MtDNA sequence variants in the patient

Gene product	Nucleotide number	Base change	Amino acid change	MitoMap database
D-loop	514	C to deletion		Reported polymorphism
D-loop	515	A to deletion		Reported polymorphism
12S rRNA	709	G to A		Reported polymorphism
12S rRNA	750	A to G		Reported polymorphism
12S rRNA	1598	G to A		Reported polymorphism
16S rRNA	2706	A to G		Reported polymorphism
NADH dehydrogenase 2	4769	A to G	Synonymous	Reported polymorphism
Cytochrome c oxydase 1	7028	C to T	Synonymous	Reported polymorphism
ATP synthase 6	8784	G to A	Synonymous	Reported polymorphism
ATP synthase 6	8829	C to T	Synonymous	Unreported
ATP synthase 6	8860	A to G	T to A	Reported polymorphism
Cytochrome c oxydase 3	9950	T to C	Synonymous	Reported polymorphism
NADH dehydrogenase 4	11719	G to A	Synonymous	Reported polymorphism
NADH dehydrogenase 4	11778	G to A	R to H	
NADH dehydrogenase 4	11914	G to A	Synonymous	Reported polymorphism
tRNA histidine	12192	G to A		
NADH dehydrogenase 5	12361	A to G	T to A	Reported polymorphism
NADH dehydrogenase 6	14470	T to C	Synonymous	Reported polymorphism
Cytochrome b	14766	C to T	T to I	Reported polymorphism
Cytochrome b	15326	A to G	T to A	Reported polymorphism
Cytochrome b	15508	C to T	Synonymous	Unreported
Cytochrome b	15662	A to G	Synonymous	Unreported
Cytochrome b	15851	A to G	I to V	Reported polymorphism
tRNA threonine	15927	G to A		Reported polymorphism
tRNA threonine	15951	A to G		Reported polymorphism
D-loop	16243	T to C		Reported polymorphism
D-loop	16318	A to T		Reported polymorphism
D-loop	16319	G to A		Reported polymorphism
D-loop	16519	T to C		Reported polymorphism

Total mtDNA sequencing identified 29 base changes. Twenty-four changes were previously reported polymorphisms and three were unreported but synonymous polymorphisms. The other two changes are G11778A and G12192A

well controlled without serious complications. Echocardiography showed hypertrophic cardiomyopathy with severe loss of compliance and marked thickening of the entire wall of the left ventricle, which continued even after the start of hemodyalysis.

Because of the acute visual loss, LHON was suspected and diagnosed on detection of a G11778A mutation in his mtDNA based on an analysis of restriction fragment length polymorphism. However, because the causes of his clinical manifestations other than visual loss remained unclear, we investigated further.

Genomic DNA was isolated from peripheral leukocytes of the patient using conventional methods. As in a previous study (Akanuma et al. 2001), to avoid nuclear pseudogene amplification, we applied the long polymerase chain reaction (PCR)-based sequencing method. With 96 primer sets designed for sequencing, we sequenced the PCR products using a BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA). The reaction product was then analyzed with an ABI 3700 automated sequencer according to the manufacturer's protocol. The sequence data was compared with MITOMAP (http://www.mitomap.org/mitomap/mitoseq.html).

To identify the G11778A mutation, we amplified the 261-bp PCR fragment and performed an endonuclease *Sfa*NI digestion. In the absence of the mutation, cleaved fragments of 133 and 128 bp were detected. To detect the G12192A mutation, the 174-bp fragment with the mismatched and reverse primers was digested by *Bp1*407I. If the fragment had the G12192A mutation, cleaved fragments of 147 and 27 bp could be obtained. Each fragment was detected in a 4% agarose gel (Nusieve 3:1 agarose; Bio Whittaker Molecular Applications, Rockland, ME, USA) stained with ethidium bromide.

Results

Total mtDNA sequencing of the patient revealed 29 base changes (Table 1). Twenty-four changes were previously reported polymorphisms and three were unreported but synonymous polymorphisms. The other two changes are thought to be associated with the patient's diseases. One was a G11778A mutation, which is associated with LHON (Fig. 1A), and the other was a G12192A mutation (Fig. 1B). Both mutations were confirmed by restriction fragment length polymorphism, and revealed to be homoplasmic (Fig. 1C, D).

Discussion

LHON patients have been reported to manifest a variety of ancillary symptoms such as altered reflexes, ataxia,

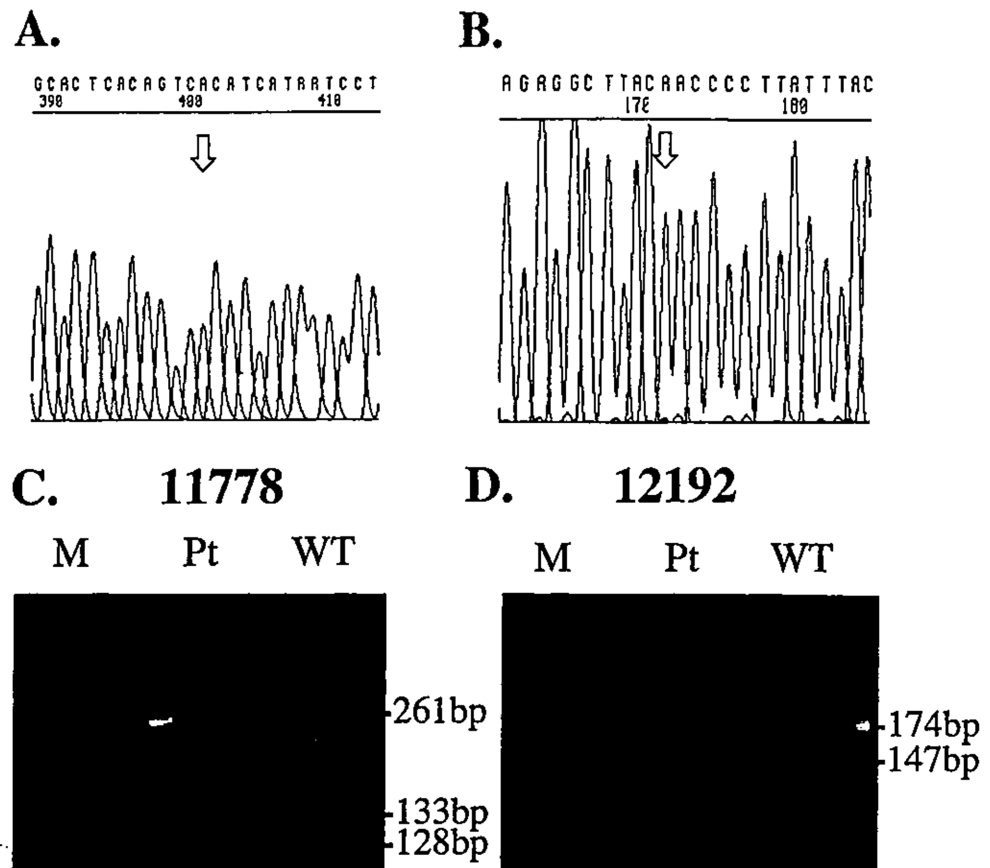

Fig. 1.A,B Identification of mutations in the mtDNA. Sequences of polymerase chain reaction-amplified products from the mtDNA of the patient, showing **A** a G-to-A substitution at nucleotide position 11778 (*arrow*) and **B** a G-to-A substitution at nucleotide position 12192 (*arrow*). **C, D** Detection of G11778A and G12192A mutations. **C** For the G11778A *Sfa*NI digestion, mutant mtDNA is indicated by a 261-bp band. Wild-type mtDNA is indicated by the presence of a 133- and 128-bp band. **D** For the G12192A *Bp1*407I digestion, mutant mtDNA is indicated by a 147-bp band. Wild-type mtDNA is indicated by the presence of a 174-bp band. *M*, Molecular weight marker; *Pt*, patient; *WT*, wild-type control

sensory neuropathy, and skeletal abnormalities. Cardiac diseases such as preexcitation syndromes including Wolff-Parkinson-White and Lown-Ganong-Levine (Nikoskelainen et al. 1985) have also been noted in these patients, but there is no report of a patient with cardiomyopathy. Recently, it was shown that a G→A substitution at position 12192 in tRNA (His) strongly predisposes carriers to cardiomyopathy (Shin et al. 2000). The second mutation, G12192A, may be related to the additional symptom of hypertrophic cardiomyopathy in the present patient.

This patient also had an A→T substitution at position 16318 in the D-loop, which was also detected in all five reported patients with cardiomyopathy carrying the G12192A mutation. The fact that the five patients shared this rare single-nucleotide polymorphism suggests a close evolutionary relationship among the mtDNAs of these patients (Shin et al. 2000). On the other hand, phylogenetic analysis of LHON mtDNA has indicated multiple independent occurrences of the G11778A mutation in people with this condition (Brown et al. 1995). According to these findings, one may consider the possibility that the G11778A mutation occurred in a member of a family carrying the G12192A mutation.

Multiple tissue involvement is a key characteristic of mitochondrial diseases with heteroplasmic mtDNA mutations. One patient was reported who carried a heteroplasmic 2532-bp deletion of the type seen in Kearns-Sayre syndrome, as well as a heteroplasmic A3243G mutation in the tRNA-Leu (UUR) gene of the type seen in cases of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (Ohno et al. 1996). This patient showed progressive ptosis, ophthalmoparesis, mitochondrial myopathy, pigmentary retinopathy, and autoimmune polyglandular syndrome type II. The two heteroplasmic mutations can be related to these multisystem disorders, but their effects on individual clinical symptoms cannot be separated clearly. Our patient, however, carried two homoplasmic mutations, and the G11778A mutation is in a gene that codes for a protein. To our knowledge, only one male patient with LHON accompanied by chronic renal failure has been reported (Souied et al. 1997) and no cases of LHON accompanied by diabetes mellitus or hypertension have been reported. Thus, it is likely that the effect of the G11778A mutation in this patient is confined to visual impairment typical of LHON, apart from the other symptoms, and it is nearly inconceivable that all of these conditions could be due to smoking and drinking. The G12192A mutations in the tRNA gene may be related to the additional multisystem symptoms, including cardiomyopathy and renal failure accompanied by hypertension and diabetes mellitus.

In several reports, "secondary point mutations" of mtDNA were simultaneously found in LHON patients with "primary mutations," such as the G11778A mutation

(Matsumoto et al. 1999), which may alone cause LHON. The exact pathogenic role of these mutations however remains unclear and there has been no reported case that includes additional symptoms with which they may be associated. Only two patients carrying the two primary mutations of LHON, G11778A and T14484C, have been reported (Riordan-Eva et al. 1995; Brown et al. 2001). The authors of these reports noted that these mutations might have some influence on the symptoms of LHON, but that they did not cause additional symptoms other than those of LHON.

The present patient suggests the possibility of multiple tissue involvement based on individual polymorphic mutations in mtDNA. To confirm this, further investigations on the separate or synergistic effects of mtDNA sequence on cellular function will be required.

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亜急性硬化性全脳炎 (SSPE) に対する薬物療法の有用性

本田真美* 二瓶健次*

要旨

亜急性硬化性全脳炎（subacute sclerosing panencephalitis：SSPE）は麻疹ウイルスが関連する遅発性ウイルス感染症で，小児期に発症し，知能障害，運動障害が徐々に進行する予後不良の疾患である．その想定される発生機序から，免疫抑制剤，免疫賦活剤や抗ウイルス剤が用いられてきたが，絶対的な治療法はいまだ確立されていない．しかし，そのなかでinosinpranobex（INP；イノシンプラノベクス）あるいはINPとinterferon（IFN）の髄腔内投与が延命率，臨床症状の改善率を向上させる治療として有用とされている．また，最近ではリバビリン併用療法も試みられているが，今後の研究が期待される．いずれの薬物も有用性と副作用発現の両者を念頭に置き，薬物の種類，量あるいは投与方法を慎重に検討していく必要がある．

はじめに

亜急性硬化性全脳炎 (SSPE) は1967年Bouteille[1)]により脳組織から麻疹ウイルスに類似したヌクレオカプシドが発見され，さらにConnoly[2)]により患者血清中の麻疹抗体が高値を示すことが示され，麻疹感染後長い潜伏期間の後に発症する遅発性ウイルス感染症であると考えられるようになった．しかしその発症機序については現在もなお不明で，確実な治療法も見出されていないのが現状である．

本症には，麻疹ウイルスの感染の後，長い潜伏期間を伴って発症すること，麻疹抗体の高値を示すにもかかわらず感染が継続することより，何らかの免疫異常が関連している可能性があることが想定され，これまで抗ウイルス剤，免疫賦活剤，免疫抑制剤などが治療として試みられてきた．すなわち，ステロイド剤，インターフェロン誘導剤，トランスファーファクター(TF)，インターロイキン(IL)，TRH，γグロブリン，イノシンプラノベクス (inosinpranobex：INP)，インターフェロン(interferon：IFN)，リバビリン，麻疹抗原賦活リンパ球などがあげられる．その臨床効果は報告によりさまざまである．しかし，これらの治療により，従来2～4年以内に死亡する例が圧倒的に多かったが，最近では10年以上の延命の例が多くなっていることは事実である[3)]．

これまでのなかで試みられた治療と現在もっとも有用とされているINPとIFN髄注（脳室内投与）の併用療法を中心に述べる．

* Manami HONDA, Kenji NIHEI 国立成育医療センター神経科
[連絡先] 〒157-8535 東京都世田谷区大蔵2-10-1 国立成育医療センター神経科

I．試みられた治療

1．ステロイド剤

本症にみられる異常麻疹抗体価の高値，自己免疫的な機序の関与の可能性から免疫抑制としてステロイド剤が用いられたことがあるが，髄液のIgGが減少したとする報告があるものも臨床的には効果はみられていない．しかし，臨床的悪化を示したとも報告されていない．現在用いられているという報告はない．

2．トランスファーファクター（TF）

胸腺内に存在する細胞性免疫を賦活する因子で，細胞性免疫の障害を有する免疫不全症に用いられて有用とされてきた．本症も麻疹抗原に対する細胞性免疫の障害があるとする仮説に基づき，TFの投与の試みがなされた．有効，無効の報告がなされたが，安定した供給がなされないこともあり，現在ではほとんど用いられていない．また，歴史的には胸腺摘出術も用いられたが効果はみられていない．

3．γグロブリン

自己免疫学的な機序が関与していると考えられている神経疾患（重症筋無力症，ギランバレー症候群，慢性脱髄性ニューロパチー，など）にγグロブリンが有用とされているが，SSPEでも自己免疫的な機序の関与も考えられることから試みられた報告がある．しかし明らかな有用性は認められない．

4．TRH（thyrotropin releasing hormone）

中枢神経系の細胞に広範囲にTRHに感受性をもつ細胞が存在し，意識障害，呼吸障害，脊髄小脳変性症の治療に用いられて効果があるとされている．SSPEに試みられている例もあるが，遅発性ウイルス感染として有用であるというよりは，2次的な神経症状改善に役立っているものと考えられる．

5．イノシンプラノベクス（INP）

INP（イソプリノシン，持田製薬）は宿主感染防御に対する作用と抗ウイルス作用を有し，本来インフルエンザなどに対する抗ウイルス剤として1978年に米国で開発されたものである．リンパ球幼若化現象の増強や主としてTリンパ球に作用し，active-T細胞の増加，killer細胞の増強，IFN産生細胞の増加が報告され，1974年Mattsonら[4]がSSPE患者への投与が有効であったと報告した．本邦では1980年代から使用されるようになり[5][6]，1980年SSPE 4症例中1例に有効であったとする二瓶らの報告が最初で，1986年154例のSSPE患者を対象にINP投与群と非投与群で比較検討され[7]有効性が認められ，1987年に使用が認可された．

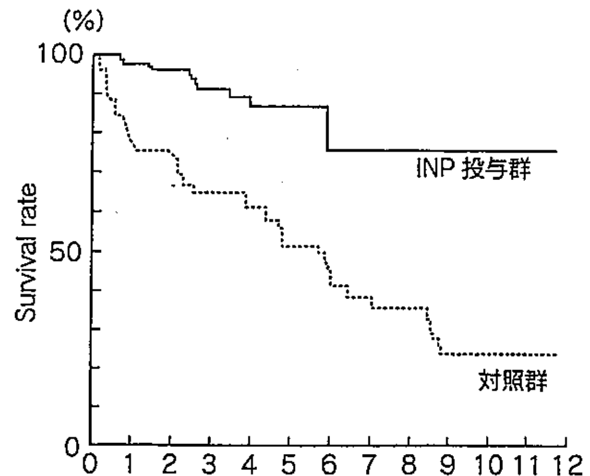

図　イノシンプラノベクス（INP）の効果
（福山幸夫ほか，1986[7]）

投与量は通常1日50〜100 mg/kgを分3〜4で経口的に投与する．とくに大きな副作用は報告されていないが，高尿酸血症およびそれに基づく尿路結石やトランスアミナーゼの上昇が報告されている．定期的に血液検査を行い，高値を示す場合，抗尿酸剤の投与やINPの減量，中止を行う．その効果については，図に示すごとくで，自然経過に比べ明らかに延命率が上昇している．

6．INP＋インターフェロン（IFN）髄腔内投与

IFNは抗ウイルス作用を有し，変異麻疹ウイ

表1 IFNの有用性

	著効	有効	不変	悪化	合計
α-IFN	25.0%	8.3%	29.2%	37.5%	100%(26例)
β-IFN	11.1%	5.5%	44.4%	39.0%	100%(18例)

(鴨下重彦ほか，1997[13)14)])

ルスに対して *in vitro* で増殖抑制作用を示すことが報告され，SSPEへの有用性が示唆されていた．それまでも本症に対してのIFNの有用性についての報告がみられていたが[8)~12)]，1997年，αIFNに対して26例，βIFNに対して20例について後に示すような方法で検討された[13)14)]．その結果，表1に示すような結果が報告され，SSPEの治療として有効であるとされ，1999年にINPとの併用で認可された．Yalazらも INPとIFNとの併用を推奨している[15)]．

IFNは脳血流関門を通過しないことから，筋注，静注では効果がなく，髄腔内投与が行われている．実際には，脊髄穿刺による髄腔内投与あるいはOmmaya reservoirを留置し脳室内への直接投与の両者が試みられている．前者に比し後者では，脳外科的にreservoir留置術を施行しなければならないために，脳外科的処置ができる環境にあることが要求されることや，病初期で脳室が小さい時期では脳室内に設置することが困難であることがあるなどの欠点はあるが，頻回投与される場合はその都度脊髄穿刺しなくてよいので，患者の負担を減らし，外来での投与も可能である．また，操作による髄膜炎などの感染の危険性を減らし，IFN投与後のIFN脳室内濃度が初期から上昇し，高濃度を維持できるなどの利点がある[16)]．

一般的な脳室内投与の手順として，①reservoir部の剃毛，②イソジンによる消毒，③27Gの翼状針で穿刺し，はじめの1 m*l* 程度はreservoir内の残留液が混入し，見かけ上の細胞数増多が起こりうる可能性があるため使用せず，以降の髄液を後述する検査検体として提出する．④髄液を一定量採取後，IFNを注入し生理食塩水で流す．⑤刺入部を1分ほど圧迫し，消毒圧迫固定する．

IFNはαIFN（スミフェロン，住友製薬），βIFN（持田製薬）が用いられている．投与量は通常1回100～300万単位を週1～3回，前記の方法で投与する．副作用としては[13)14)]，投与後12時間前後で発熱がほとんどの症例で認められるが一過性である．髄液中の細胞数の増多（通常100/3以内で単球優位である）が一過性に認められることがある．IFNによるchemical meningitisと考えられているが，医原性の髄膜炎を常に鑑別に入れておかなければならない．反応性の発熱に対しては，解熱剤の投与，体を冷やすなどの対症療法で対応するが，IFN投与に同時にハイドロコートン20～30 mgを髄腔内に投与することにより発熱が抑制される[17)]．いずれにしても回数を経るにつれて発熱を起こすことが少なくなる傾向がある．

このほかにけいれん，筋硬直，排尿障害，意識障害，精神症状，間質性肺炎やニューロパチー，心筋症などがまれに認められるが，副作用なのか病勢の進行なのかを見極めて，投与の継続か中止を考えなくてはならない．副作用の場合は多くの場合薬剤の中止や減量で改善される．

効果については，効果判定の項に後述する．

7．リバビリン

リバビリンは抗ウイルス剤として開発されたもので，わが国では1994年にハムスターでSSPEウイルスの増殖を抑制することが報告され，SSPE患者に対し臨床的に投与され始め有用とする報告がなされている[18)19)]．C型肝炎でIFNとリバビリンとの併用療法が有用と認められているが，SSPEに対しては症例数も少ないことから，有効性と安全性の検討が今後期待される．C型肝炎に使用する場合のリバビリンは経口剤であり，SSPEに使用される静脈，髄注用の製剤は入手が困難である．

投与量は1～3 mg/kg/日を分2～3で静脈内

表2　SSPEの臨床スコア

項　目	ス　コ　ア	
意識状態	0．正常 1．やや低下 2．呼びかけに反応する 3．物理的な刺激に対して反応する 4．昏睡	
行動・知能	言語表出	言語の理解
	0．正常 1．やや障害（文章が簡単になる，構文が乱れる） 2．軽度障害（2語文程度になる） 3．中等度障害（わずかの単語で意思表示） 4．重度障害（ほとんど発語なし）	0．正常 1．やや障害（複雑な話ができない） 2．軽度障害（簡単な話ができない） 3．中等度障害（簡単な命令，指示しかわからない） 4．重度障害（ほとんど発語なし）
	認知（見当識，物品，家族などの認知）	記　憶
	0．正常 1．やや障害 2．軽度障害 3．中等度障害 4．重度障害	0．正常 1．やや障害（ときどき記憶が悪いことがある） 2．軽度障害（よく覚えていた言葉や歌を忘れる） 3．中等度障害（家族をやっと覚えている程度） 4．重度障害（記憶がない）
運動・感覚・反射	感情表出	操　作
	0．正常 1．やや障害（通常より少し感情表出が悪い） 2．軽度障害（好きなものを食べたり，呼びかけに笑う） 3．中等度障害（強い呼びかけにかすかに笑う） 4．重度障害（ほとんどしなくなる）	0．正常 1．やや障害（手操作が鈍くなる，字が下手になる） 2．軽度障害（服の着脱に時間がかかる） 3．中等度障害（食事の操作が難しくなる） 4．重度障害（ほとんどなにもできない）
	歩　行	協調運動
	0．正常 1．やや障害（拙劣な歩行） 2．軽度障害（少し介助があると歩ける） 3．中等度障害（全面介助でやっと歩ける） 4．重度障害（歩行不能）	0．正常 1．やや障害 2．軽度障害 3．中等度障害 4．重度障害
	四肢の動き	筋緊張
	0．正常 1．やや障害 2．軽度障害 3．中等度障害 4．重度障害	0．正常 1．やや亢進 2．軽度亢進 3．中等度亢進 4．除脳－除皮質硬直
	腱反射	ミオクロニーの頻度
	0．正常 1．やや亢進 2．軽度亢進 3．中等度亢進 4．高度亢進	0．みられない 1．ときにみられる 2．律動的になる 3．頻回になる 4．消失（Ⅳ期）
	ミオクロニーの程度	感覚（触覚）

	0．なし 1．わずかにみられる 2．中等度（大きくなる） 3．激しい（ベットがきしむほど） 4．消失（Ⅳ期）	0．正常 1．やや障害 2．軽度障害（痛みに対する反応が弱くなる） 3．中等度障害（強い刺激にも反応が悪い） 4．高度障害（ほとんど反応しない）
	視 覚	聴 覚
	0．正常 1．やや障害 2．軽度障害（近くのものがわかる） 3．中等度障害（明るい方を見る） 4．重度障害（ほとんど光に反応しない）	0．正常 1．やや障害 2．軽度障害（小さい音には反応しない） 3．中等度障害（大きな音でないと反応しない） 4．重度障害（ほとんど反応しない）
自律神経症状	0．なし 1．軽度発汗，分泌，発熱など 2．中等度発汗，分泌，発熱など 3．激しい発汗，口内分泌，不定期発熱など 4．消失（Ⅳ期）	
全身症状	基本状態	食 事
	0．正常 1．立位までは可能 2．座位までは可能 3．ベッド上の生活だが寝返り可能 4．ベッド上寝たきり	0．正常 1．食事に時間がかかるようになる 2．一部介助が必要 3．全面的に介助が必要，むせることが多い 4．チューブ栄養
	排 泄	呼 吸
	0．完全自立 1．時に知らないうちに失敗している 2．一部介助 3．ほとんど介助 4．終日おむつ	0．正常 1．呼吸が荒くなることがある 2．夜間，感染時にしばしば呼吸障害を認める 3．気管切開が必要になる 4．補助呼吸が必要

あるいは脳室内投与する．10日間投与し20日間休薬する．半減期は2～6時間とされているが，治療濃度域が50～200 μg/ml と推定され非常に狭いため慎重に投与する必要がある．ヒトでの脳内移行性は明らかでない．

副作用としては本剤は濃度依存的に赤血球内に移行し，リン酸化を受け赤血球内に蓄積され，赤血球の性状異常を起こし網内系での除去が促進されることから，静脈内投与ではほぼ全例で貧血が認められる．また発生機序は不明であるが口唇腫脹や口唇炎も高頻度に認められる．その他に毛髪変化，歯肉炎，湿疹の出現が報告されているが，多くの場合薬剤の中止や減量で改善される．

Ⅱ．効果判定

以上に述べた治療の効果判定は定期的な診察による臨床症状，麻疹抗体価，脳波，MRIなどの検査からなされる．

1．臨床スコア

SSPEの病期による分類として4つのステージに分類するJabbourの分類がしばしば用いられている．しかしこのステージ分類の変動はかなり大きなもので，微妙な変動を把握するためには臨床症状を細かく分類してスコア化する必要がある．Dykenのスコア評価が有名であるが，IFNの効果判定に用いられたスコア評価を

表3 脳波による評価

I. 基礎波 a) 覚醒時：下記の6段階の分類に従い，いずれかを選択する. 0. 正常 1. 軽度徐波化（後頭部α律動あり*） 2. 中等度徐波化（α律動なし*） 3. 高度徐波化（高振幅大徐波主体*） 4. 低電位化 5. 局在性異常 * 一括判定による基準 b) 睡眠時：正常・異常のいずれかを選択する．また異常の内容を記入する. 0. 正常 1. 異常 II. てんかん波：無・有のいずれかを選択する. 0. 無 1. 有：有の場合，散発か頻発のいずれかを選択する. 1. 散発 2. 頻発 ・下記の3段階の分類に従い，いずれかを選択する. 1. Diffuse irregular spike-waves 2. Diffuse slow spike-waves 3. 焦点性棘波	III. 周期性群発：無・有のいずれかを選択する. 0. 無 1. 有：有の場合，下記4段階の分類に従い，いずれかを選択する．また，電位を記入する. 1. 周期（begin-to-begin）20秒以上 2. 周期（begin-to-begin）10〜20秒 3. 周期（begin-to-begin）5〜10秒 4. 周期（begin-to-begin）5秒以下 睡眠時については，自然睡眠か薬剤睡眠のいずれかを選択する．また薬剤睡眠の場合は使用薬剤を記入する. 1. 自然睡眠 2. 薬剤睡眠 〔脳波の一括判定による評価〕 改善：基礎波点数減少 不変：基礎波点数不変 悪化：基礎波点数増加 なお，着目すべきより微細な脳波学上の変化があった場合は，基礎波の点数に変化がなくとも，「改善」または「悪化」と評価した.

（鴨下重彦ほか，1997[13][14]）

表2に示す[13][14].

表に示すごとく，意識状態，行動・知能，運動・感覚・反射，自律神経症状，全身症状に分類し，これらの項目について5段階評価を行いそれぞれの得点を合計してスコア化する．①4点以上の減少を著効，②2点以上の減少を有効，③±1点の変動を不変，④2点以上の増加を悪化とした.

2. 血液検査

SSPE患者では血清および髄液中の麻疹抗体価が中和抗体(NT)，血液凝集素価(HI)，補体結合抗体（CF）のいずれも上昇する．一般的には初期には1,024倍以上の高値をとることが多いが，必ずしも高値を示さないこともあるので診断時には注意が必要である．麻疹抗体価の推移はJabbourのI期からII期にかけて抗体価が上昇し，III期では高値を維持，IV期ではむしろ低下傾向となるので，臨床病期と併せた抗体価の推移が治療効果の判定に役立つ．髄液の推移もほぼ一致している.

3. 髄液検査

血液検査同様に麻疹抗体価の推移は治療効果判定に役立つ．細胞数の増多は多くの場合ないか，あっても軽微である．糖も正常のことが多いが，末期では総蛋白は増加する．また髄液中のIgG，とくにIgGインデックスは症状の改善とともに低下する傾向がある.

4. 脳波所見

脳波は特徴的な所見を呈するため，診断的意義および病期決定に重要である．I期では特異的な所見はみられないが，徐波成分が多くみられたり，全般性の速波が周期的にみられることがある．II期では，周期性の100〜200 μV も高圧徐波群が出現しこれに続いて脳波が平坦化す

表4 MRIによる評価

評価部位
①前頭葉 ②頭頂葉 ③側頭葉 ④後頭葉
⑤尾状核 ⑥レンズ核 ⑦視床 ⑧小脳 ⑨脳幹

評価基準
下記基準に基づき，脳萎縮および萎縮以外の脳病変を各々の部位について評価し，各々のスコア合計値を（1）脳萎縮MRIスコア，（2）脳病変MRIスコアとして算出した．

(1) 脳萎縮MRIスコア
a．大脳：前頭葉，頭頂葉，側頭葉，後頭葉（5段階評価）
（0点）萎縮なし，（1点）軽度萎縮，（2点）中等度萎縮
（3点）重度萎縮，（4点）スケールアウト（著明な水頭症など）
b．尾状核（4段階評価）
（0点）萎縮なし，（1点）volume少し減，（2点）volume少し残
（3点）消失
c．レンズ核・視床・小脳・脳幹（2段階評価）
（0点）萎縮なし，（1点）萎縮あり

(2) 脳病変MRIスコア（4段階評価）
（0点）病変なし，（1点）PVH（+），T_1/T_2 low/high intensity
（2点）Leukoariosis，（3点）激烈あるいは形状消失のため判定不可能

MRIスコア改善度判定基準
HLBI投与前後の脳萎縮MRIスコアおよび脳病変MRIスコアの変化（「投与終了時MRIスコア」−「投与開始時MRIスコア」）より，脳萎縮および萎縮以外の脳病変の改善度を判定した．
改善：MRIスコア変化が「−1点」以下
不変：MRIスコア変化が「0点」
悪化：MRIスコア変化が「1点」以上

（鴨下重彦ほか，1997[13)14)]）

る．いわゆるburst supressionが認められる．III期では周期が延長し，IV期では周期性徐波が消失し，δ波優位の脳波となる．表3に示すような評価[13)14)]を用いて参考にした．

5．MRI所見

表4に示すような評価[13)14)]を用いて参考にした．

III．まとめ

SSPEに対する治療として，INPあるいはINP+IFN髄腔内投与が有用とされている．これらの治療が用いられて以後，明らかに死亡までの年齢が延長し，これまで臨床症状が改善することがほとんどなかった疾患であったが，症状が改善する例が増えている．確実な治療法ではないが，現時点ではもっとも有用な治療法と考えられる．

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The Human Secretin Gene: Fine Structure in 11p15.5 and Sequence Variation in Patients with Autism

Takanori Yamagata,[1,2] Swaroop Aradhya,[1] Masato Mori,[2] Ken Inoue,[1] Mariko Y. Momoi,[2] and David L. Nelson[1,*]

[1]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA*
[2]*Department of Pediatrics, Jichi Medical School, Tochigi, Japan*

**To whom correspondence and reprint requests should be addressed. Fax: (713) 798-1116. E-mail: nelson@bcm.tmc.edu*

Secretin is a peptide hormone involved in digestion that has been studied as a potential therapeutic agent in patients with autism. We characterized the human secretin locus to determine whether mutations in this gene might play a role in a fraction of autism patients. While the secretin gene (*SCT*) was not found to be mutated in the majority of autistic patients, rare heterozygous sequence variants were identified in three patients. We also investigated length variation in a variable number of tandem repeats (VNTR) immediately upstream of *SCT* and found no significant differences in length between patients with autism and normal controls. *SCT* is located on 11p15.5, adjacent to *DRD4* and *HRAS*. This region has been reported to be associated with both autism and attention deficit hyperactivity disorder (ADHD). Although imprinting is a characteristic of some genes in the vicinity, we could find no evidence for methylation of *SCT* in lymphoblast cells from patients or control individuals.

Key Words: autism, secretin, *SCT*, gene mutation, VNTR, *DRD4*, *HRAS*, 11p15.5, ADHD

INTRODUCTION

Three autistic patients showed improvement of symptoms upon injection with a preparation of porcine secretin [1], a peptide hormone produced by the gut. This event has led to speculation that secretin treatment may serve to modify behavioral features of autistic patients. Clinical trials are in progress to test this hypothesis, and initial findings suggest that secretin administration is unlikely to be effective in the majority of patients [2]. However, should secretin deficiency prove to be involved in the generation of autism, even in a small number of patients, the finding would be important. These results provided impetus to characterize the human gene encoding secretin as a potential candidate for mutations in patients with a diagnosis of autism.

Autism is a relatively common condition (1 or 2 in 1000 children) marked by behavioral features that include impairment of reciprocal social interaction, impairment in verbal and nonverbal communication, and a markedly restricted repertoire of activities and interests [3]. The disorder is usually apparent in children by the age of 3 years. There is a significant genetic component to autism, with monozygotic twin concordances in the range of 36-100%, while dizygotic twins are concordant at a rate between 0 and 24% [4]. Numerous sib-pair and other linkage studies have failed to identify a single genomic region likely to harbor mutations that are responsible for the disease in the majority of patients. Many loci are expected to contain alleles that predispose for autism [5-9].

Secretin is a small (27 amino acids) peptide hormone that is secreted by the gut in response to food passage. It induces the pancreas to secrete pancreatic juice, a mixture of products that includes proteases and bicarbonate. Secretin was first described in 1902 [10] and was the first hormone to be isolated. Secretin is proteolytically cleaved from a prohormone that varies in length in the three species in which it has been described [11-16].

Secretin extracted from pig gut is routinely used in diagnostic procedures to determine pancreaticobiliary secretion on gastrointestinal endoscopy. It was in this context that Horvath and colleagues administered secretin to autistic patients complaining of diarrhea. The patients' behaviors (eye contact, alertness, and expression of language) were found to be markedly improved [1].

The secretin gene and mRNA have been characterized from several mammalian species [11-15,17], and recently the human gene was described [18]. We chose to characterize the human secretin locus to determine whether mutations in this gene might play a role in a subset of autism patients. We report here that the secretin gene (*SCT*) is not mutated in the majority of autistic patients. However, rare heterozygous

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A

```
        10        20        30        40        50 .      60        70        80        90       100
tgcgcgcagtccctggaagcgctgaatccacagacagaaagtggacggggcgccggggctgaggaggggagggggcgtttgatggggacagagcttcatt

       110       120       130|      140       150       160 |     170       180       190 |     200
ctgggaggacaggagagtctgagaacggatttgggaggacgggagcgtctgtgaacggatttgggaggacgggagcgtctgtgaacggatttgggag

       210       220  |    230       240       250  |    260       270       280  |  290       300
gacgggagcgtctgtgaacagatttgagaggacgggagagtctgagaacggatttgggaggacggaagagtctgagaatggattttggaggacggaa

       310      |320       330       340      |350       360       370      |380       390       400
gagtctgagaacggatttgggaggacgggagcgtctgtgaacgatttgggaggacgggagcgtctgtgaacgatttgggaggacgggagcgtctgtg

      |410       420       430      4|40       450       460      4|70       480       490      50|0
aacgatttgggaggacgggagcgtctgtgaacggatttgggaggacgggagcgtctgtgaacggatttgggaggacgggagcgtctgtgaacggatt

       510       520       530       540       550       560       570       580       590       600
ttgggaggacgggaaagtctgagaacggatggtggggatggatgcaccgaatgatcctgaatcgcgcacttcaaagcagataaaatggcaacaccggccc

       610       620       630       640       650       660       670       680       690       700
cccaacagtcacgccacgctgaagctggagacgaactcgccccagcaaggtgctccaggggcctccggaactccggccgcagccccttccccggggcccc

       710       720       730       740       750       760       770       780       790       800
ccagtccccccccaaaccctccccaagccctccggcccctccgcccccgcaggctccgaggcttcgcggtgcggttccctctgcgggtcccaggcccgc

       810       820       830       840       850       860       870       880       890       900
gctccgttcgcccccaggccgggacgtcccccctcccccagccggccgagggcgccagcgcggcggagggggcggaggccgggccgggggcgacagctg
                                                                                                   c/t
       910       920       930       940       950       960       970       980       990      1000
gggggcggccctgaccttcccgggatcgctgggcgcggccgggccgtcccctttccgggcgccgccataaaggggccttgcgggggcccggggccgtgg
                                                              a
      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
ccgcagcgctcagctcctgcgccccgaccccgccATGGCCCCCCGGCCCCTCCTGCTGCTGCTGCTGCTCCTCGGGGGCTCCGCCGCGCGCCCCGCGCCC
                                  M  A  P  R  P  L  L  L  L  L  L  L  L  G  G  S  A  A  R  P  A  P

      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CCCAGgtgagccgcccgccaggcccgcccgcccccgggccccacccgcccccagtgcgccctgaccccgacccccgaccgcccgcagGGCCCGGCGACA
 P  R                                      g (insertion)                                   A  R  R  N
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
CTCAGACGGGACGTTCACCAGCGAGCTCAGCCGCCTGCGGGAGGGCGCGCGGCTCCAgCGGCTGCTACAGGGCCTGGTGGGAAGCGCAGgtgaggccgg
  S  D  G  T  F  T  S  E  L  S  R  L  R  E  G  A  R  L  aR  L  L  Q  G  L  V  G  K  R  S
                                                         Q/Q
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
gtggggtggggggtcctggcgtggtgccccagcagcaacgcgcgacccccccagctccaggactaactctgttttgggcgggggcagCGAGCAGGACGC
                                                                                         E  Q  D  A

      1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
AGAGAACAGCATGGCCTGGACCAGGCTCAGCGCGGGTCTGCTCTGCCcGTCAGGGTCCAACATGCCCATCCTGCAGGCCTGgtgagcacccgccccaccg
  E  N  S  H  A  W  T  R  L  S  A  G  L  L  C  t  S  G  S  N  M  P  I  L  Q  A  W
                                              P/L
      1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
cagaccccactccagaccccaccccagacccctccttctgtcctggcctccccgggtcagcgctggtggccactggaggcccaattccagcagcggggcc

      1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
ggggtctccagccccagccctcagaccctcacgtgctgtctctgcagGATGCCCCTGGACGGGACCTGGTCTCCCTGGCTGCCCCCTGGCCCTATGGTTT
                                                  M  P  L  D  G  T  W  S  P  W  L  P  P  G  P  M  V

      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
CAGAACCAGCTGGCGCTGCTGCAGAAGGAACCTTGCGGCCCAgATGAggaaggaacccccctcaccacctgcccggcccaggagcgcagctgcatttgggg
S  E  P  A  G  A  A  A  E  G  T  L  R  P  c  X
                                        R/T
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
tgggggcaggatgggggagaggggggagggggtggtacttggcaccaataaaggaggaatcagacccagacgctgggagttgtctgtgtccactcagtgta

      1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
atcgaggcaacccctgtgtagcggggctgagggatggatggaggcccatccacccaggagggactgaccacacccagctgccctttacacctgcaggcac

      2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
ggcggcccctcatccttatctctggcatcagggccacctcctgtacctcgactaagtactgcagggccccgccccaccccttgtcatccattaggaaaca

      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
ttctggggctcgggggatcccggacaccctctcagctcctgcccggggcccatgtggtcagtgtttctgtaaggggtagtggggagggtgtgaaggggc

      2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
caggggggctcaggcgggcaggaagaggggagggtggaggtgtcacctggggtgccaggaatccactctggcgtgaggactccggaccctctgtctcgtg

      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
tggcctcggaccagtggcttgacccctcagagcctgtggagcacggagcgtgcagggcttgaaggcgaccatgagggcaccgtgcagggttaggacactg
```

FIG. 1. Secretin gene sequence. (A) Nucleotide sequence of the secretin gene region. A region of 2400 nucleotides is shown, with the four coding exons and translated amino acid sequence of the secretin mRNA and polypeptide in capital letters. The processed hormone amino acid sequence is in bold type. A number of features are underlined, including intronic splice junction sequences and promoter elements. The poly(A) addition signal is in bold type. In addition, a variable number of tandem repeats (VNTR) region 5′ to the gene is in bold type, and individual repeats are indicated by vertical lines above the sequence. Finally, positions of the six base-pair alterations identified in normal and autistic individuals are indicated in bold type, with altered amino acid sequences indicated where appropriate. (B) (Opposite page) Nucleotide sequence alignment of the rat and human secretin promoter regions. A ClustalW alignment of the promoter regions of secretin genes from rat and human is provided, with boxes indicating blocks of identity. Arrowhead and underlined regions mark the base that was mutated in autism patients, and E-box and GC-boxes, respectively. (C) (Opposite page) Peptide sequence alignment of the secretin prohormone sequences from human, mouse, rat, and pig. A ClustalW alignment of the peptide sequences of the secretin prohormone from four species. The peptide hormone region is underlined, and the positions of the two human amino acid variants found in autistic individuals are indicated with asterisks.

sequence variants were identified in three patients. The role of these alterations in the phenotypes of these patients is not clear. The gene is located on 11p15.5, adjacent to *DRD4* and *HRAS*. This region has been reported to be associated with both autism and attention deficit hyperactivity disorder (ADHD) [19–21]. A VNTR sequence is located immediately upstream of the secretin coding sequence; however, allele lengths do not differ significantly between autism patients and normal control subjects. Although imprinting is a characteristic of some genes in the vicinity, no evidence was found that *SCT* is subject to parent-of-origin effects on expression.

Results

Isolation of the Human Secretin Gene

Comparison of the earlier described pig, mouse, and rat secretin sequences revealed that the highest levels of conservation were found in exons 2 and 4. We designed PCR primers in conserved sequences to amplify the region

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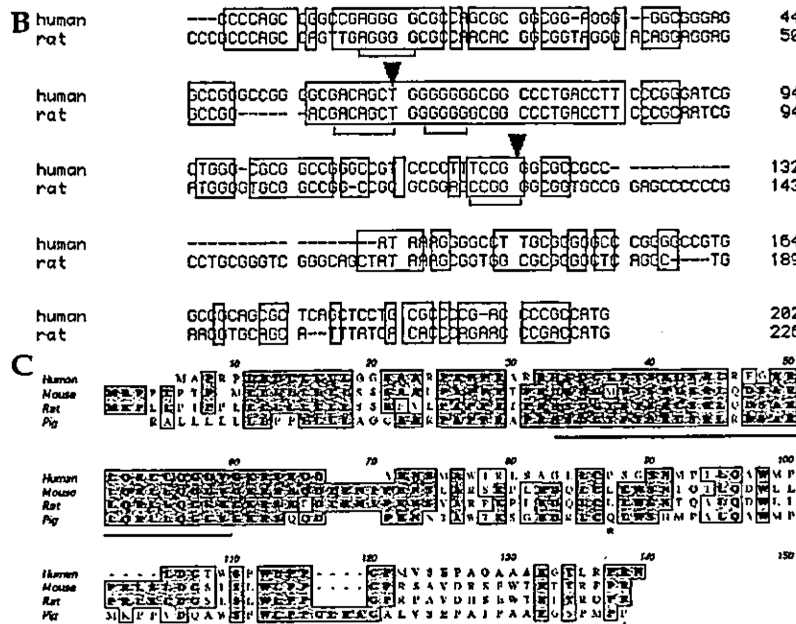

between exons 2 and 4. Amplification of human genomic DNA resulted in a 460-bp fragment. Sequencing this fragment demonstrated significant similarity with the pig sequence and suggested that a fragment of the human secretin gene had been isolated.

We screened a gridded bacterial artificial chromosome (BAC) library (RPCI-11 [22]) with the human fragment and identified four positive clones (41I8, 412M16, 199P23, and 496I9). Characterization of the clones by restriction digestion with *Eco*RI revealed that two clones were identical (41I8 and 199P23). Southern hybridization with the secretin genomic amplification product demonstrated fragments of identical size in each of the three unique clones. These were subcloned from the BACs and characterized by DNA sequencing.

The human secretin gene is small, composed of four exons that span 800 bp from the ATG to the poly(A) addition signal (Fig. 1A). The peptide sequence predicted for the human hormone was identical to the previously determined human protein sequence [23]. The hormone-encoding domain is contained in exon 2, and alignment of the predicted human amino acid sequence with those of the pig, mouse, and rat demonstrates high similarity in this region (Fig. 1B). The prohormone is 122 amino acids in length and is not nearly as well conserved with the other species in which it has been described. There are some regions of high conservation, such as the WLPP motif at position 99–102 (Fig. 1B). The gene is very GC-rich (76%), and there are six GC-box elements and one E-box found in the promoter region. Figure 1C illustrates rather high similarity between the human and rat promoters.

Variable Number of Tandem Repeats 5' to *SCT*

As noted by Whitmore *et al.* [18], a region carrying a VNTR consisting of repeats of 30 or 31 bases is located upstream of the promoter region, 500 bp from the ATG (Fig. 1A). PCR-based analysis of this VNTR in 50 Caucasian subjects showed alleles of 11, 13, and 14 repeats. Of 100 alleles analyzed, 75 carried 14 repeats, 24 alleles were 13 repeats in length, and one allele was 11 repeats. We also tested a population of 50 Japanese subjects and found the 14-repeat allele on 93 chromosomes, with four instances of 13 repeats, and three alleles at 15 repeats (Table 1). Both populations exhibited no significant differences from expected frequencies of hetero- or homozygotes. Sequence variation within repeat units allows definition of individual repeats. From sequencing, it was possible to determine that each of three alleles with 13 repeats lacked the 10th unit and one allele of 11 repeats was missing the 10th through 12th units.

Precise Position of *SCT*

Fluorescent *in situ* hybridization was used to determine the chromosomal origins of BAC clones 41I8 and 412M16. Signals were obtained consistently at the distal end of the short arm of chromosome 11 (data not shown). Mapping in the G3 Radiation Hybrid Panel suggested that *SCT* is closely linked to the markers *SHGC-31256* (ROD score 11.30), *SHGC-58456* (ROD score 10.27), and *SHGC-64262* (ROD score 9.69). Cross-reference of these markers in the GB4 Radiation Hybrid map (NCBI; http://www.ncbi.nlm.nih.gov/genemap/) indicated an interval between *D11S922* and *D11S1318* at 11p15.5, near *IGF2*.

We tested several genes and markers near *SHGC-31256*, including *IGF2, H19, TH, MUC2, ASCL2, CTSD, L23, CD81, D11S922*, and *D11S1318*, on each secretin-carrying BAC clone by PCR. None of these genes and markers was present in any of the three BAC clones. We tested genes farther distal (*RNH, DRD4, HRAS*, and *HRC1* [24,25]) and found these to be present (data not shown). *DRD4* is present on 41I8 and 496I9.

TABLE 1: Secretin VNTR alleles in autism and control samples*

VNTR repeat length	Caucasian control	Caucasian autistic	Japanese control	Japanese autistic
15	0/100	0/100	3/100	0/50
14	75	68	93	47
13	24	31	4	3
11	1	1	0	0

*Numbers are alleles observed for the indicated number of chromosomes studied.

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