of two types of microarray, BAC array and oligonucleotide array. The BAC array was applied for 298 patients to detect 58 CNVs in 47 patients, and among them 26 CNVs (8.7%) were determined to be causal (pathogenic). Conversely, the oligonucleotide arrays were applied for 703 patients to detect 1538 CNVs in 603 patients, and among them 74 CNVs (10.5%) were determined to be pathogenic. These results may lead to the following idea: a lower-resolution microarray detects a limited number of CNVs likely to be pathogenic, because such CNVs tend to be large, and a higher-resolution microarray detects an increasing number of bCNVs or VOUS.[38] Indeed, in studies using a high-resolution microarray, most of the CNVs detected were smaller than 500 kb but almost all pCNVs were relatively large.[54,81,83] Most of the small CNVs were judged not to be pathogenic, and the percentage of pCNVs stabilized at around 10%. This percentage may suggest a frequency of patients with MCA/MR caused by CNV affecting one or more genes, other than known syndromes and subtelomeric aberrations. The other patients may be affected by another cause undetectable by genomic microarray; for example a point mutation or microdeletion/duplication of a single gene, aberration of microRNA, aberration of methylation states, epigenetic aberration or partial uniparental disomy.

As recently hypothesized secondary insult, which is potentially another CNV, a mutation in a phenotypically related gene or an environmental event influencing the phenotype, may result in clinical manifestation.[84] Especially, in two-hit CNVs, two models have been hypothesized: (1) the additive model of two co-occurring CNVs affecting independent functional modules and (2) the epistatic model of two CNVs affecting the same functional module.[85] It also suggests difficulty in selecting an optimal platform in the clinical screening. Nevertheless, information on both pCNVs and bCNVs detected through studies using several types of microarrays is unambiguously significant because an accumulation of the CNVs will create a map of genotype–phenotype correlation that would determine the clinical significance of each CNV, illuminate gene function or establish a new syndrome.

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ORIGINAL COMMUNICATION

Genetic analysis of two Japanese families with progressive external ophthalmoplegia and parkinsonism

Kazunori Sato · Ichiro Yabe · Hiroaki Yaguchi · Fumihito Nakano · Yasuyuki Kunieda · Shinji Saitoh · Hidenao Sasaki

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Abstract Mutations in the progressive external ophthalmoplegia 1 (*PEO1*), adenine nucleotide translocator 1 (*ANT1*) and DNA polymerase gamma (*POLG*) genes were reported in patients with progressive external ophthalmoplegia and parkinsonism. However, the genotype–phenotype correlation and pathophysiology of these syndromes are still unknown. In order to define the molecular basis of progressive external ophthalmoplegia and parkinsonism, we screened for mutations in *PEO1*, *ANT1*, *POLG* genes and the whole mitochondrial genome in two families. In results, we identified a compound heterozygous *POLG* substitutions, c.830A>T (p.H277L) and c.2827C>T (p.R943C) in one of the families. These two mutations in the coding region of *POLG* alter conserved amino acids in the exonuclease and polymerase domains, respectively, of the POLG protein. Neither of these substitutions was found in the 100 chromosomes of ethnically matched control subjects. In the other family, no mutations were detected in any of the three genes and the whole mitochondrial genome in the blood sample, although mitochondrial DNA deletions were observed in the muscle biopsy sample. Progressive external ophthalmoplegia and parkinsonism are genetically heterogenous disorders, and part of this syndrome may be caused by mutations in other, unknown genes.

Keywords Progressive external ophthalmoplegia · DNA polymerase gamma gene · Parkinsonism · Mitochondria

K. Sato · I. Yabe (✉) · H. Yaguchi · F. Nakano · H. Sasaki
Department of Neurology,
Hokkaido University Graduate School of Medicine,
N15W7, Kita-ku, Sapporo 060-8638, Japan
e-mail: yabe@med.hokudai.ac.jp

Y. Kunieda
Department of Internal Medicine,
Wakkanai City Hospital, Wakkanai, Japan

S. Saitoh
Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Introduction

Mutations in genomic genes that alter mitochondrial DNA (mtDNA) are being increasingly reported, and can affect a variety of organs with variable ages of onset [1]. The hereditary forms are either autosomal dominant, or recessive, and rarely sporadic. DNA polymerase gamma (*POLG*, MIM ID #174763) encodes the catalytic subunit of DNA polymerase gamma, the only polymerase involved in replication of the mitochondrial genome [2]. A mutation in *POLG* associated with dominant progressive external ophthalmoplegia (PEO) was first described in 2001 [3]. In 2004, mutations in *POLG* in two individuals with a co-occurrence of dominant PEO and parkinsonism were reported [4]. Subsequently, mutations in the progressive external ophthalmoplegia 1 (*PEO1*, MIM ID #606075) and adenine nucleotide translocator 1 (*ANT1*, MIM ID #103220) genes were reported in patients with similar clinical phenotypes [5–11]. However, the genotype–phenotype correlation and pathophysiology of these syndromes are still unknown. We performed genetic analyses in two unrelated Japanese patients with PEO and parkinsonism and their families who had no maternal inheritance and found a compound heterozygotic missense mutation in *POLG* in one of the families.

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Subjects and methods

Subjects

Information from both families was not suggestive of maternal inheritance.

Family A (Fig. 1a): patient 1 (AII:2) was a 78-year-old man, who was healthy until the onset of slowly progressive bilateral ptosis with diplopia in his early 50s. At the age of 60 years, he developed left dominant hemi-parkinsonian features, such as rigidity with cogwheel phenomenon, bradykinesia, gait disturbance, resting tremor and postural instability. He was receiving no drugs that cause parkinsonism and there were no obvious infarctions on his brain MRI scan. Laboratory data and electrophysiological studies, including hyperammonemia, serum lactate and pyruvate values, nerve conduction studies, electrocardiogram, and electroencephalogram, all showed no abnormalities. He could not perform the exercise test because of his bradykinesia. Cardiac ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy showed slightly reduced heart-to-mediastinum (*H/M*) ratios at both the early and delayed phases. The mini mental state examination (MMSE) revealed no dementia (28/30). He showed a good response to L-DOPA (300 mg/day) treatment, so we diagnosed him with Parkinson's disease (PD). His father (AI:1) had a past history of blepharoplasty for bilateral ptosis but no parkinsonism and died of unknown cause at the age of 94 years. His mother (AI:2) was healthy until she died of stroke at the age of 96 years. His nine siblings are healthy and alive except for two brothers (AII:7 and AII:9) who died of intussusception at the age of 3 years. Neurological examinations confirmed that two siblings, AII:5 (68-year-old female) and AII:10 (60-year-old male), did not show any abnormalities including parkinsonism; however, AII:10 shows signs of slight ptosis without external ophthalmoplegia.

Family B (Fig. 1c): patient 2 (BII:3) was a 64-year-old man, who developed slowly progressive external ophthalmoplegie and ptosis at age 40 years and resting tremor of the left hand and stooped posture at age 60 years. Neurological examination at age 62 revealed other right dominant parkinsonian features, such as rigidity with cogwheel phenomenon of the bilateral arms, bradykinesia and postural instability, and mild proximal dominant muscle weakness. Pramipexole (1.5 mg/day) was started and thought to be effective. Laboratory findings showed increases in lactate and pyruvate in an exercise test but no other remarkable abnormalities; electrophysiological tests, including electroencephalogram, were also negative. MIBG cardiac scintigraphy showed markedly reduced *H/M* ratios at both the early and delayed phases. His

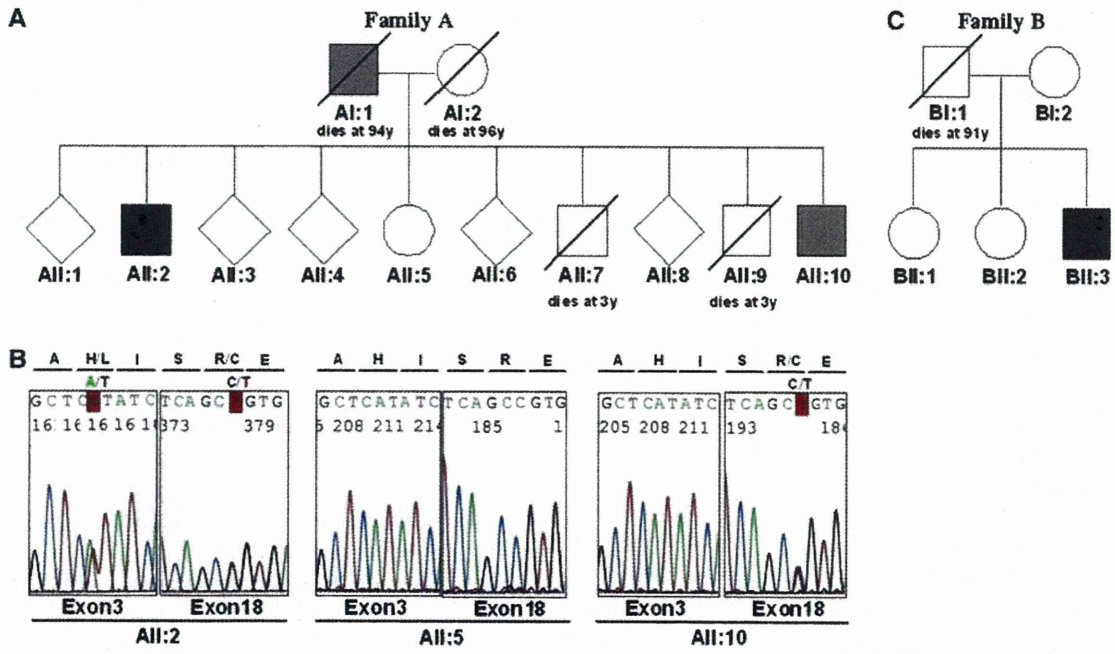

Fig. 1 Modified family pedigrees and electropherogram. **a** Family A (*open square*) man; (*open circle*) woman; (*slash*) deceased. *Open diamond,* family members not tested. *Solid symbols* show affected individuals with progressive external ophthalmoplegia and parkinsonism. *Gray symbols* show affected individuals with ptosis. **b** Electropherograms from members of family A. The proband (AII:2) of family A has two substitutions, c.830A > T (p.H277L) and c.2827C > T (p.R943C) in exons 3 and 18, respectively, of *POLG*. AII:5 exhibited neither of these substitutions and AII:10 has a single change of c.2827C > T (p.R943C). **c** None of the members of family B have substitutions in *POLG*, *PEO1*, or *ANT1*

MMSE score was 30. His father died of senile decay at the age of 91 years. We examined his mother (BI:2) and two sisters (BII:1 and BII:2) and found them to be healthy and with no neurological abnormalities.

Blood sampling and DNA extraction

All procedures used in this study were approved by the Hokkaido University Ethics Committee, and written informed consent was obtained from each individual (AII:2, AII:5, AII:10, BI:2, BII:1, BII:2 and BII:3) examined as well as from 50 ethnically matched control subjects. Blood samples were collected and genomic DNA and mtDNA were extracted from leukocytes using standard protocol.

Analysis of mitochondrial DNA deletion

The presence of mtDNA deletions was examined in muscle biopsy samples (see below) from patients 1 (AII; 2) and 2 (BII; 3) using Southern blot DNA hybridization (Mitsubishi Chemical Medience Corporation, Tokyo) according to the manufacturer's instructions. Whole cell DNA was prepared by phenol–chloroform extraction after incubation with proteinase K at 37°C overnight, and then purified by ethanol precipitation.

For Southern blotting and hybridization, 0.1 μg of genomic DNA or mtDNA were digested with 10 U of *Bam*HI (Roche) and *Pvu*II (Roche), respectively, at 37°C overnight. Digested DNA was separated by agarose electrophoresis (1% agarose gel, 55 V(CV)), hybridized with the probe recognizing mtDNA3307-4520 and exposed to X-ray film (XR, Fujifilm) at −70°C overnight.

DNA sequencing

Primers for PCR amplification of the 22 exons of the *POLG* gene, the 5 exons of the *PEO1* gene, and the 4 exons of the *ANT1* gene were as previously reported [12–14]. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems). Sequencing products were purified by BigDye X Terminator (Applied Biosystems) and analyzed on an ABI3130 genetic analyzer with sequencing analyzer software (Applied Biosystems). In addition, whole mtDNA genome analyses of blood from the two probands (AII:2 and BII:3) were conducted (mitoSEQr resequencing system, for resequencing the entire mitochondrial genome with 46 RSAs; Applied Biosystems, USA).

Muscle pathology

Open muscle biopsy was performed on the *rectus femoris* of both patients. Transverse frozen sections were prepared and stained with hematoxylin-eosin (HE), modified Gomori trichrome (m-GT), nicotinamide adenine dinucleotide-tetrazolium (NADH-tr), non-specific enolase (NSE), and alkaline phosphatase (ALP). Histochemical stainings for the mitochondrial enzymes succinate dehydrogenase (SDH) and cytochrome c oxidase (CCO) were also performed.

Results

Sequencing analyses revealed compound heterozygotic missense mutations in *POLG* in patient1: c.830A>T in exon 3, resulting in p.H277L and c.2827C>T in exon 18, resulting in p.R943C (Fig. 1b). The former was reported previously associated with Alpers syndrome [15] and the latter with autosomal dominant PEO [16]; however, neither of the substitutions have been reported in a phenotype with parkinsonism.

One of the brothers of patient 1 (AII:10) also exhibited the c.2827C>T substitution in exon 18, but did not have the c.830A>T substitution in exon 3 (Fig. 1b). The sister of patient 1 (AII:5) had no *POLG* mutations (Fig. 1b). Patient 1 had no mutations in either *ANT1* or *PEO1*. Neither of the substitutions was found in the 100 chromosomes of 50 ethnically matched control subjects. Patient 2 had no mutations in any of the three genes examined. No mutations were detected in the whole mtDNA of either blood sample of the patients. In the analysis of mtDNA, deletions were observed only in patient 2 (Fig. 2).

We found similar muscle pathologies in both patients (Fig. 3). There were a few atrophic fibers and basophilic fibers in HE staining and many ragged-red fibers in the m-GT staining. Absence of CCO activity was found in some fibers. Some fibers showed intense SDH activity but no strongly stained small vessels. The histological findings in both patients were compatible with chronic progressive external ophthalomoplegia among mitochondrial myopathies.

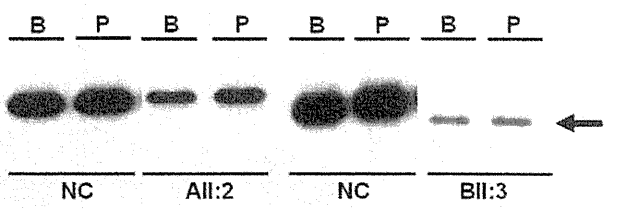

Fig. 2 Mitochondrial DNA deletion analysis. Southern blots of mitochondrial DNA isolated from muscle tissue. The muscle mitochondrial DNA was restricted with *Bam*HI (B) and *Pvu*II (P). The sample from patient 2 (BII:3) exhibited smaller restriction bands (*arrow*) than those from the normal control (NC), indicating the existence of mitochondrial DNA deletions. The size of normal band is 16.6 kb

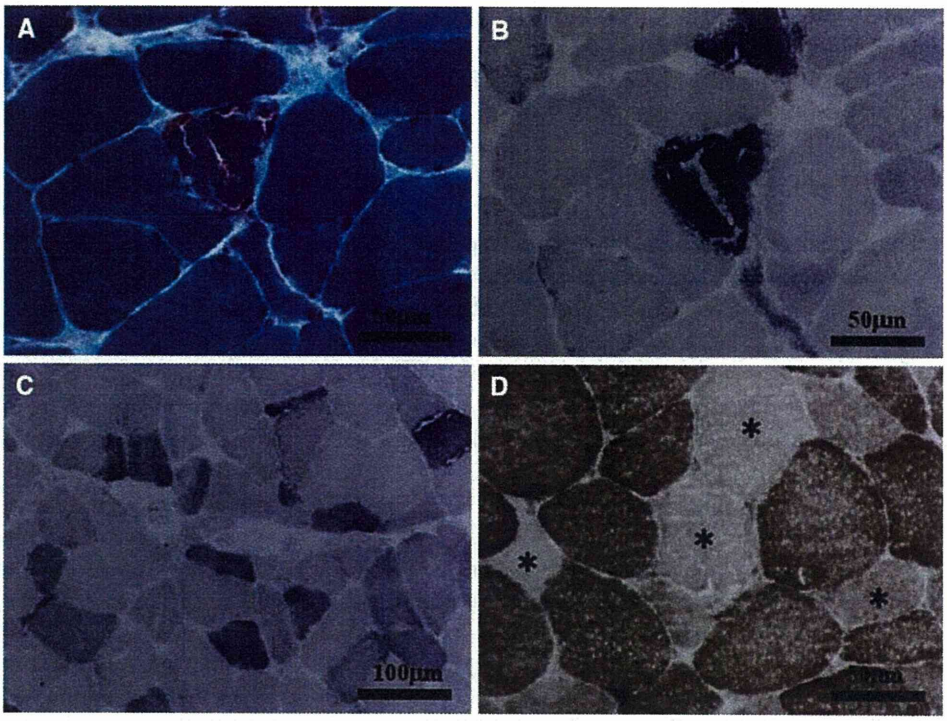

Fig. 3 Muscle pathology. **a–c** From BII:3. **a** Modified Gomori trichrome stained section showing ragged-red fiber in the center. **b** The same ragged-red fiber is darkly stained by succinate dehydrogenase (SDH) stain. **c** Darkly stained SDH fibers are scattered throughout the section. **d** In a section from patient AII:2, there are some cytochrome c oxidase-negative fibers (*asterisks*)

Discussion

We revealed a compound heterozygotic missense mutations in *POLG* in a patient with PEO and parkinsonism. To our knowledge, this is the first such compound mutation in a patient with PEO and parkinsonism and neither of these substitutions were previously reported in association with parkinsonism.

According to the genotypes of the siblings of patient 1, his mutations may be the result of transposition and each of his parents may have been heterozygotic for each of the mutations, because his brother (AII:10) has only the p.R943C substitution; however, a potential recombination can not be ruled out. In the POLG protein, p.H277L is involved in the exonuclease domain and p.R943C. in the polymerase domain. pR943C was previously reported in autosomal dominant PEO patients [16]. Most mutations in autosomal dominant PEO are in the polymerase domain [1], and, therefore, may be related to the onset of PEO in this case. In fact, the healthy sibling of patient 1 (AII:10) has slight ptosis without external ophthalmoplegia. However, it is unclear whether the difference between siblings can be explained only from the perspective of penetrance.

POLG is known as the causative gene of Alpers syndrome, which is a rare but severe autosomal recessive disorder that affects young children and causes mental retardation, seizures, deafness, liver failure, and eventual death [1]. Childhood myocerebrohepatopathy spectrum disorders (MCHS) are also known as POLG related disorders, and are defined by the clinical triad of myopathy or hypotonia, developmental delay or dementia, and liver dysfunction [17]. The p.H277L and p.R943C substitutions reported here are also known to occur in Alpers syndrome and MCHS, respectively [15, 17]. Although our patient had compound heterozygotic changes, he had no symptoms and signs suggesting either Alpers syndrome or MCHS. However, two siblings of patient 1 died of intussusception in their childhood. This may suggest that they had been affected with Alpers syndrome, although this could not be confirmed because their medical records were not available. It is reported that many *POLG* mutations are responsible for PEO and Alpers syndrome, and that the same substitutions cause PEO or Alpers syndrome [18]; however, the genotype–phenotype relationships are still unknown. In patients with PEO and parkinsonism, mutations are reported not only in the exonuclease domain [4] and the polymerase domain [4–6, 9] but also in the linker region [4, 7–9], therefore the correlation between mutation sites and development of parkinsonism is not clear. It could not be determined from our limited data whether both allele changes are required for the development of PEO and parkinsonism.

Although mtDNA deletions were not observed in our patient with the *POLG* mutation, other patients were also reported with *PEO1* or *POLG* mutations but with no apparent mtDNA deletions in muscle specimens observed

with Southern blotting [19, 20]. Real time PCR may be required to demonstrate the defect.

In spite of the presence of a mtDNA deletion and typical findings of muscle pathology indicative of mitochondrial disorders, patient 2 shows neither mtDNA mutations nor *POLG*, *PEO1*, or *ANT1* mutations. These results suggest wide heterogeneity in this phenotype and possibly the presence of mutations in other genes involved in the maintenance of mtDNA, particularly those involved in replicating and repairing mtDNA as does *POLG*.

PD is one of the common neurodegenerative diseases and its prevalence generally increases with age [21, 22]. The slowly progressive course, hemi-parkinsonism, and good response to anti-parkinsonian drugs observed in the parkinsonism in our patients is compatible with PD. Therefore, it seems possible that our elderly PEO patients may have developed Parkinson's disease by chance. However, detection of not only 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) [23, 24] but also mutations in phosphatase and tensin homolog (PTEN)-induced kinase (*PINK1*) in familial parkinsonism [25], support the relationship of mitochondrial dysfunction and the pathogenesis of PD. Therefore, it seems possible that mitochondrial dysfunction due to the *POLG* mutation in our patient participated in the pathogenesis of PEO and parkinsonism. In addition, although parkinsonism caused by *POLG* mutations is a rare situation, such cases may also be included among clinically diagnosed progressive supranuclear palsy (PSP) patients, as these patients often have oculomotor abnormalities as well.

There was no apparent association between *POLG* variants and sporadic idiopathic PD in two previous studies [26, 27]; however, these studies examined only some common variants of *POLG* and over 100 substitutions in all regions of *POLG* have been reported to date [28]. Although, to our knowledge, the percentage of PEO patients with PD is not reported, it seems to be rare; however, PD with PEO may have a high rate of genetic mutations of nuclear genes functioning in the maintenance of mtDNA. Not only in PD patients with PEO, but also in PD patients who have family histories of PEO, nuclear genes functioning in the maintenance of mtDNA, including *POLG*, should be considered as etiologies.

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Conflict of interest The authors report no conflicts of interest.

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RESEARCH ARTICLE

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A Loss-of-Function Mutation in the *SLC9A6* Gene Causes X-Linked Mental Retardation Resembling Angelman Syndrome

Yumi Takahashi,[1] Kana Hosoki,[1] Masafumi Matsushita,[2] Makoto Funatsuka,[3] Kayoko Saito,[4] Hiroshi Kanazawa,[2] Yu-ichi Goto,[5] and Shinji Saitoh[1]*

[1]Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[2]Deparrtment of Biological Sciences, Graduate School of Science, Osaka University, Osaka, Japan

[3]Department of Pediatrics, Tokyo Womens' Medical University, Tokyo, Japan

[4]Institute of Medical Genetics, Tokyo Womens' Medical University, Tokyo, Japan

[5]Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan

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SLC9A6 mutations have been reported in families in whom X-linked mental retardation (XMR) mimics Angelman syndrome (AS). However, the relative importance of *SLC9A6* mutations in patients with an AS-like phenotype or XMR has not been fully investigated. Here, the involvement of *SLC9A6* mutations in 22 males initially suspected to have AS but found on genetic testing not to have AS (AS-like cohort), and 104 male patients with XMR (XMR cohort), was investigated. A novel *SLC9A6* mutation (c.441delG, p.S147fs) was identified in one patient in the AS-like cohort, but no mutation was identified in XMR cohort, suggesting mutations in *SLC9A6* are not a major cause of the AS-like phenotype or XMR. The patient with the *SLC9A6* mutation showed the typical AS phenotype, further demonstrating the similarity between patients with AS and those with *SLC9A6* mutations. To clarify the effect of the *SLC9A6* mutation, we performed RT-PCR and Western blot analysis on lymphoblastoid cells from the patient. Expression of the mutated transcript was significantly reduced, but was restored by cycloheximide treatment, indicating the presence of nonsense mediated mRNA decay. Western blot analysis demonstrated absence of the normal NHE6 protein encoded for by *SLC9A6*. Taken together, these findings indicate a loss-of-function mutation in *SLC9A6* caused the phenotype in our patient.

Key words: *SLC9A6*; sodium/hydrogen exchanger 6; Angelman syndrome; X-linked mental retardation; nonsense mediated mRNA decay

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INTRODUCTION

SLC9A6 mutations were first reported by Gilfillan et al. [2008] in families exhibiting an X-linked mental retardation (XMR) syndrome mimicking Angelman syndrome (AS). Angelman syndrome is characterized by severe developmental delay with absent or minimal speech, ataxia, easily provoked laughter, epilepsy, and microcephaly. The syndrome is caused by loss-of-function of the *UBE3A* gene which is subject to genomic imprinting. Patients with *SLC9A6* mutations resemble patients with AS, but also demonstrate distinctive clinical features including cerebellar atrophy, slow progression of symptoms, increased glutamate/glutamic acid peak on magnetic resonance spectroscopy (MRS), and lack of characteristic abnormalities seen AS patients examined using electroencephalography (EEG). Following the first report in 2008, in 2010 Schroer et al. reported two other families with AS due to *SLC9A6* mutations, and confirmed the findings of Gilfillan et al.

The *SLC9A6* gene is located on Xq26.3, and encodes the ubiquitously expressed Na^+/H^+ exchanger protein member 6, NHE6. The NHE protein family consists of nine members and includes

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*Correspondence to:
Shinji Saitoh, Department of Pediatrics, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan.
E-mail: ss11@med.hokudai.ac.jp
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NHE1-5 which is found in the plasma membrane, and NHE6-9 which is found in the membranes of intracellular organelles such as mitochondria and endosomes. NHE6 is predominantly present in the early recycling endosome membranes, and is believed to have a role in regulating luminal pH and monovalent cation concentration in intracellular organelles [Brett et al., 2002; Nakamura et al., 2005]. Moreover, Roxrud et al. demonstrated that NHE6 in combination with NHE9 participated in regulation of endosomal pH in HeLa cells by means of the procedure of co-depletion of NHE6 and NHE9 [Roxrud et al. 2009], indicating the significant role of NHE6 in fine-tuning of endosomal pH in human cells. In the brain, exocytosis from recycling endosomes is essential for the growth of dendritic spines which grow during long-term potentiation (LTP). In the absence of recycling endosomal transport, spines are rapidly lost, and LTP stimuli fail to elicit spine growth [Park et al., 2006]. Thus, NHE6 has an important role in the growth of dendritic spines, and also in the development of normal brain wiring. Thus far, five *SLC9A6* mutations have been reported in six AS families; two nonsense mutations, one inframe deletion, one frameshift deletion, and one splicing mutation [Gilfillan et al., 2008; Schroer et al., 2010]. The precise pathogenesis by which these mutations produce disease remains to be clarified.

The aim of this study was to clarify the incidence and importance of *SLC9A6* mutations in AS-like patients and patients with XMR, and to shed light on the molecular pathogenesis of disease due to *SLC9A6* mutations.

MATERIALS AND METHODS

Enrolled Patients

We examined 22 affected Japanese males clinically suspected of having AS but who lacked the genetic abnormalities reported in AS (AS-like cohort). These patients had AS excluded by having negative results for the *SNURF-SNRPN* DNA methylation test (which identifies a deletion, uniparental disomy, or imprinting defect) and *UBE3A* mutation screening (performed as described previously) [Saitoh et al., 2005]. We also examined DNA samples from 104 Japanese patients suspected of having XMR (XMR cohort). The XMR samples were collected as a part of a project for the Japanese Mental Retardation Consortium [Takano et al., 2008]. This study was approved by the Institutional Review Board of Hokkaido University Graduate School of Medicine, and written informed consent was obtained from the parents of the enrolled patients.

Mutation Analysis of the *SLC9A6* Gene

We amplified each exon, including exon–intron boundaries, of the *SLC9A6* gene using polymerase chain reaction (PCR), and all amplicons were directly sequenced on an ABI 3130 DNA analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator V.1.1 Cycle Sequencing Kit (Applied Biosystems). *SLC9A6* encodes two alternatively spliced transcripts produced from alternative splicing donor sites in exon 2 which give rise to a long form designated as variant 1, and a short form called variant 2. Variant 1 and variant 2 code for NHE6.1 (isoform a) and NHE6.0 (isoform b), respectively (Fig. 1). The primers were designed to amplify each transcript variant. The primers sequence used for amplification and sequencing are available on request. Genomic DNA (10 ng) extracted from peripheral blood was amplified in a total PCR volume of 20 μl containing 1× buffer, 0.4 μM of each primer (forward/reverse), 0.18 mM dNTPs, 0.5 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems). The PCRs for all exons except exon one were performed at 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then one cycle at 72°C for 7 min. The high CpG content of exon 1 required it to be amplified in a total reaction volume of 20 μl containing 1× buffer, 0.4 μM of each primer, 0.2 mM dNTPs, 0.4 U Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland), and 3% DMSO. The thermocycling conditions for exon 1 were 98°C for 3 min followed by 35 cycles of 98°C for 10 sec, 65°C for 30 sec and 72°C for 30 sec and then one cycle of 72°C for 5 min. The PCR products were purified with Wizard® PCR Preps DNA Purification System (Promega, Madison, WI) prior to sequencing. All mutations are referred to in relation to reference sequence NM_001042537.

Cell Culture and Cycloheximide Treatment

Epstein–Barr virus (EBV)-transformed lymphoblastoid cells lines were established from peripheral blood cells using standard methods. To prevent potential degradation of transcripts containing premature translation termination codons (PTCs) by nonsense mediated mRNA decay (NMD), lymphoblastoid cells from the patient with the *SLC9A6* mutation and normal controls were treated with 100 μg/ml cycloheximide (CHX) (Sigma, St. Louis, MO). This compound interferes with NMD through inhibition of protein synthesis [Aznarez et al., 2007]. CHX or a 0.1% DMSO control vehicle was used 4 hr prior to RNA extraction from the cell lines [Carter et al., 1995].

RT-PCR

Total RNA from cultured lymphoblastoid cells from the patient and four normal controls, was extracted using the RNAqueous® Kit (Applied Biosystems). Reverse transcription was performed using 100 ng of total RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a total reaction volume of 20 μl containing 1× Random primers, 4 mM dNTP mix, 2.5 U of Multiscribe™ Reverse Transcriptase, and 1 μl of RNase Inhibitor. The reactions were incubated at 25°C for 10 min, then at 37°C for 120 min and then followed by 85°C for 5 min to inactivate the reverse transcriptase. Complementary DNA was then amplified using a primer set designed to amplify exon 2–5; forward 5′-GTCTTTTGGTGGGCCTTGT-3′, reverse 5′-GTCCCGTTACC-TTCATCAG-3′. PCR products for NHE6.1 (transcript variant 1) and NHE6.0 (transcript variant 2) were 399 and 303 bp, respectively.

Real-Time Quantification of *SLC9A6* mRNA

To measure *SLC9A6* transcript variant 1 and variant 2, both of which are alternative splicing products, primers and TaqMan® MGB probes were designed with Primer® Express Software (Applied Biosystems; Fig. 1). The Primer and MGB probe sequence

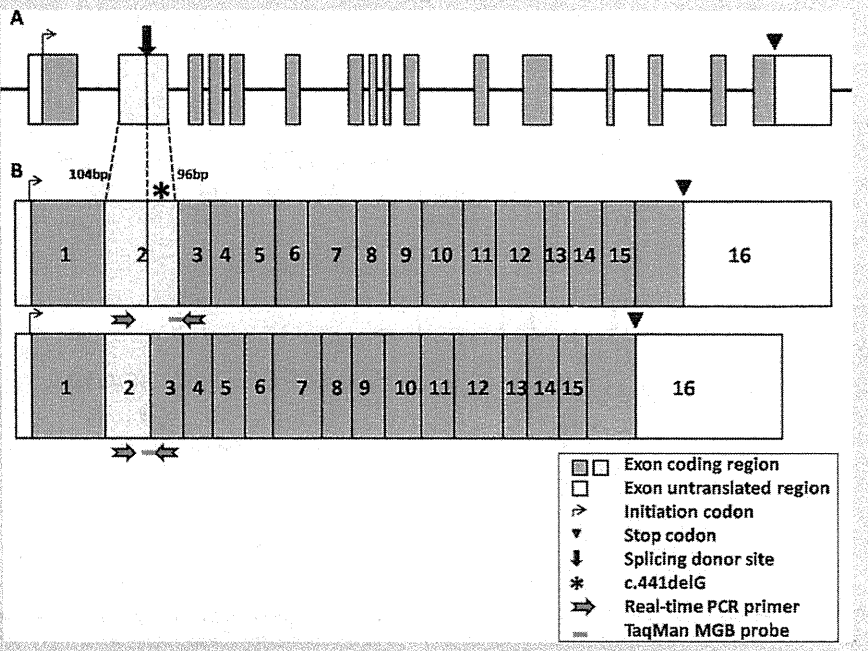

FIG. 1. A: Genomic structure of the *SLC9A6* gene. B: Two alternatively spliced transcripts of the *SLC9A6* gene. Above: *SLC9A6* transcript variant 1 (encodes NHE6.1 or isoform a). Below: *SLC9A6* transcript variant 2 (encodes NHE6.0 or isoform b). The location of the *SLC9A6* mutation in our patient is shown with *. Primers and probes used in real-time quantitative PCR are shown (horizontal arrows).

for variant 1 were forward primer 5′-TGAGTATATGCTGAAAGGAGAGATTAGTTC-3′, reverse primer 5′-GATAGGAGGAAGTAATATGTTGAAAAATACTTC-3′, TaqMan MGB probe 5′-CTTAGAAAGGTTACTTTTGATCC-3′; and for variant 2 forward primer 5′-CTGTGAAGTGCAGTCAAGTCCAA-3′, reverse primer 5′-GATAGGAGGAAGTAATATGTTGAAAAATACTT-3′, TaqMan MGB probe 5′-CTACCTTACTGGTTACTTTTGA-3′. Human *GAPDH* MGB probe and primers purchased from Applied Biosystems were used as the internal control. Patient cDNA was transcribed from 10 ng of total RNA in a total volume of 25 μl containing 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.9 μM of each primer (sense/antisense) and 0.25 μM of probe. Thermocycling was 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Real-time quantitative PCR was performed using the ABI PRISM 7700 (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used for relative quantification.

Western Blot Analysis

HeLa cells and cultured lymphoblastoid cells from the patient, mother and normal controls were washed with phosphate buffered saline and suspended in lysis buffer (phosphate buffered saline containing 1% Triton-X, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). HeLa cells expressing the NHE6.1 were used as a control. The cells were disrupted by sonication and centrifuged at 20,000*g* for 10 min at 4°C. The supernatants were then resolved by SDS-polyacrylamide electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). NHE6 was detected with rabbit polyclonal anti-NHE6 antibody [Ohgaki et al., 2008], anti-rabbit IgG antibody conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA) and chemiluminescence reagent (ECL Western Blotting Detection System; GE Healthcare, Waukesha, WI).

RESULTS

Identification of a *SLC9A6* Mutation

We identified only one male patient with a frameshift mutation (c.441delG, p.S147fs) in exon 2, out of 22 male patients in the AS-like cohort (Fig. 2). This frameshift mutation causes a PTC. His healthy mother was heterozygous for the mutation.

No mutation in the *SLC9A6* gene was identified in the XMR cohort. However, two common polymorphisms (rs2291639, rs2307131), and one putative novel polymorphism in intron 12 (c.1692 +10 A>G) were detected.

Clinical Features of the Patient With the *SLC9A6* Mutation

The affected male patient at birth suffered from mild neonatal asphyxia, however he had no other perinatal problems. His parents

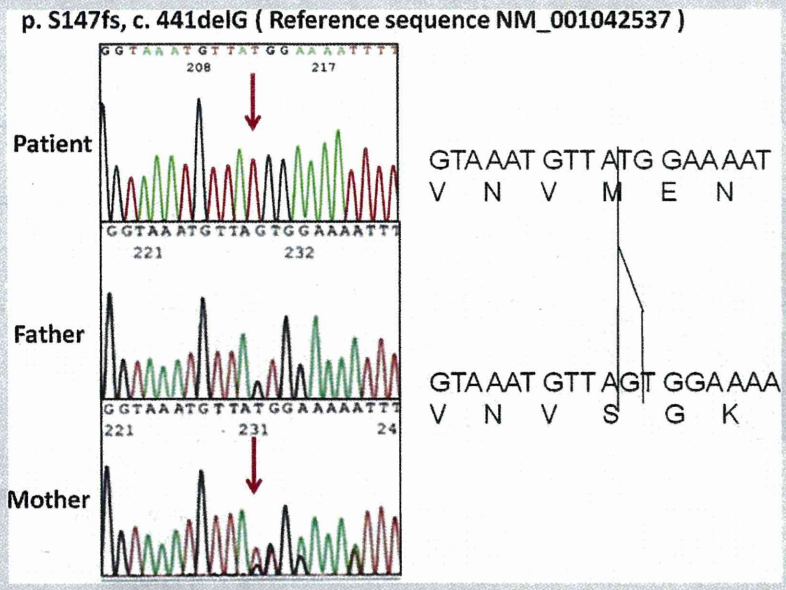

FIG. 2. Chromatographs showing the *SLC9A6* mutation in our patient, and the equivalent genomic region in both his parents. The mutation c.441delG is located in exon 2 and is only present in transcript variant 1. His mother was heterozygous for this mutation, while his father did not have the mutation. This mutant transcript leads to premature protein truncation. The mutation is described relative to reference sequence NM_001043537. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-485X]

were non-consanguineous and he did not have any family history of neurological diseases. Although formal clinical assessment was not conducted to the mother, she is healthy and does not have intellectual disability. His clinical features are summarized in Table I. He showed typical findings of AS; severe developmental delay with absence of verbal language, generalized hypotonia, easily provoked laughter, epilepsy, ataxia, strabismus, and microcephaly. His occipitofrontal head circumference at birth was 33.8 cm (+0.4 SD), but his head growth has decelerated into 51.5 cm (−3.0 SD) at 18 years of age. He acquired head control at three months of age, sat and crawled at 6 months of age, and walked unassisted at 18 months of age. His first epileptic attack occurred at 4 years of age. After this first attack, he lost his ability to walk until he was 5 years old. His epileptic attacks consisted of multiple types of seizures, and they were difficult to control with ACTH or several anti-epileptic drugs. TRH treatment improved his awakening and activity levels, and he transiently acquired the ability to walk. However, subsequently his ability to walk was lost, probably due to exacerbation of ataxia. His deep tendon reflex was not increased and no other features of spasticity or peripheral neuropathy were identified. His EEG findings included a background frequency of 5–6 Hz theta waves and spontaneous appearance of 3 Hz diffuse high voltage slow waves. TRH did not change the frequency of his seizures or his EEG findings. He showed no cerebellar atrophy on magnetic resonance imaging (MRI) at 5 years of age. MRS was not performed. He had a normal G-banding karyotype.

Downregulation of the *SLC9A6* Variant 1 in the Patient With the Mutation

The identified mutation c.441delG is located in exon 2 and is only present in variant 1 (Fig. 1). Therefore, the mutation only affects NHE6.1, leaving NHE6.0 intact. Reverse transcriptase PCR demonstrated that *SLC9A6* variant 1 mRNA expression decreased in our patient (Fig. 3A) compared to that in four normal controls. On the other hand, variant 2 expression was increased in the patient compared to the controls. To further investigate mutant *SLC9A6* gene expression, real-time quantitative PCR (qPCR) was performed using cDNA from the patient and normal controls. Quantitative PCR confirmed that *SLC9A6* variant 1 was significantly downregulated in the patient, while it was not downregulated in normal controls (Fig. 4A). Furthermore, the *SLC9A6* variant 2 mRNA in the patient was significantly increased compared to normal controls (Fig. 4B).

Nonsense Mediated Decay Was Involved in the Downregulation of Mutant *SLC9A6* in the Patient

To investigate the possible involvement of NMD in the downregulation of mutant *SLC9A6* in the patient's lymphoblastoid cells, we treated the cells with CHX. After CHX treatment, the expression level of *SLC9A6* variant 1 increased compared to normal control samples on RT-PCR (Fig. 3B). It was also proved that the expression level of variant 1 was significantly increased by performing qPCR, while the expression level in normal control samples

TABLE I. Clinical Findings in Affected Males Previously Reported and Our Patient

Family number: report affected males number (examined number)	1: Gilfillan et al. [2008] 3 (3)	2: Gilfillan et al. [2008] 2 (1)	3: Gilfillan et al. [2008] 3 (3)	4: Gilfillan et al. [2008], Christianson et al. [1999] 16 (4)	5: Schroer et al. [2010] 6 (6)	6: Schroer et al. [2010] 1 (1)	Our patient
Development and behavior							
Profound delay	+	+	+	+	+	+	+
Verbal language absent	+	+	+	+	+	+	+
Easily provoked laughter	+	+	+	+	3/6	−	+
CNS findings							
Epilepsy	+	+	+	+	+	+	+
Ataxia	+	+	+	+	NR	NR	+
Hyperkinetic movements	2/3	−	+	−	2/6	NR	−
Strabismus	+	+	+	+	5/6	+	+
Physical findings							
Microcephaly	+	+	+	3/4	5/6	+	+
Open mouth + drooling	2/3	+	+	NR	4/6	+	+
Swallowing difficulty	2/3	+	1/3	1/4	NR	+	−
Flexed arms	+	NR	1/3	+	3/6	−	−
Electroencephalography							
Epileptiform activity	+	+	+	+	+	+	+
Background activity	10–11 Hz	1.5–3 Hz	4–7 Hz	3–6 Hz to 11–14 Hz	NR	α rhythm	5–6 Hz
Brain MRI/autopsy							
Cerebellar atrophy	1/3	NR	NR	2/4	2/6	+	−
Mutation	p.E287_S288del c.936_941delAAAGTG	p.R500X c.1574C → T	p.V176_201del c.679 +1 delGTAA	p.H203fs c.684_685delAT	p.R500X c.1574C → T	p.Q437X c.1391C → T	p.S147fs c.441delG

+, present with all the patients; −, not present; NR, not recorded.

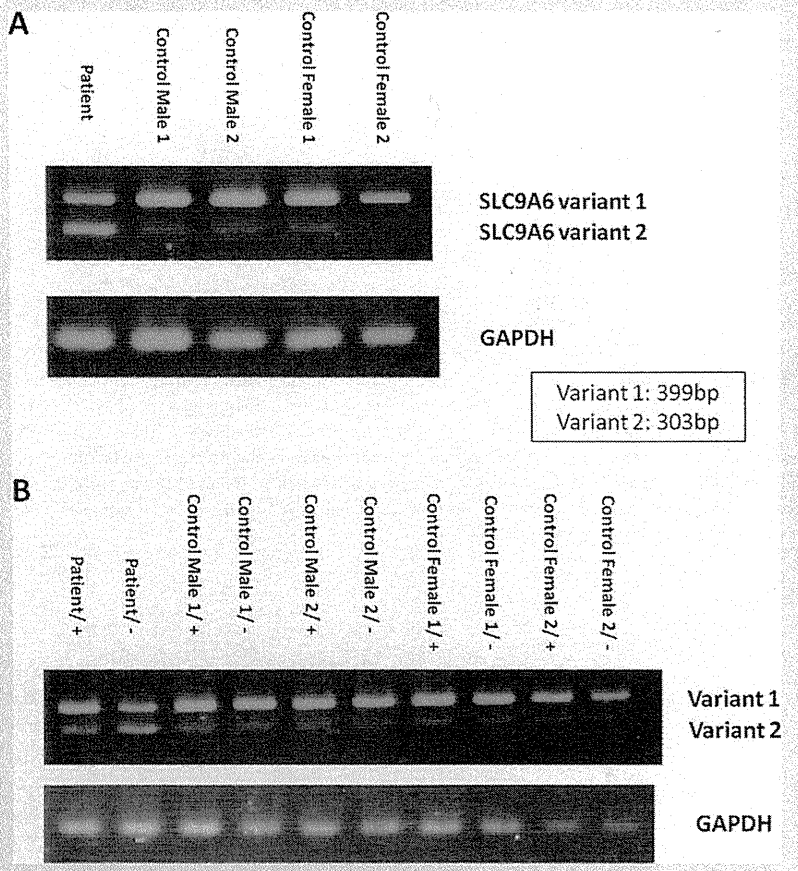

FIG. 3. RT-PCR amplification of the *SLC9A6* gene. A: *SLC9A6* variant 1 mRNA expression was decreased in the patient compared to that in four normal controls. On the other hand, variant 2 expression was increased in the patient compared to that in the controls. B: CHX treatment increases the mutant *SLC9A6* variant 1 mRNA expression, leading to similar expression levels in the patient and four normal controls samples. (+) After CHX treatment, (−) no CHX treatment.

was unchanged (Fig. 4A). The expression level of *SLC9A6* variant 2 increased in all samples after CHX treatment, however the increase was significant only in control samples (Fig. 4B).

Decreased Expression of the NHE6 Protein From Mutant *SLC9A6*

Western blotting was performed to investigate expression of the NHE6 protein in the homogenate of lymphoblastoid cell lines from the patient and his mother. As a result, protein expression of NHE6.1 was not detected in the patient (Fig. 5A,B). The same NHE6.1 was detected in HeLa cells and cells from the patient's mother as well as in the controls. NHE6.0, which was expected to be 10–20 kDa smaller than NHE6.1on SDS–PAGE [Ohgaki et al., 2008], was not detected in any sample (Fig. 5B).

DISCUSSION

In this study we investigated 22 male AS-like patients and 104 male patients with XMR, and identified only one AS-like patient with a *SLC9A6* frameshift mutation. This result further confirms *SLC9A6* is not a major cause of AS-like cases, as reported by Fichou et al. [2009]. Although the number of patients with XMR in this study was small, *SLC9A6* is likely to account for only small proportion of XMR cases.

Patients with *SLC9A6* mutations reported by Gilfillan et al., exhibit cardinal features similar to those of AS including severe developmental delay, mental retardation with absent or minimal use of words, easily provoked laughter, ataxia, epilepsy, hyperkinetic movement, nystagmus, and microcephaly.

Gilfillan et al. also identified possible features of difference between these patients and AS patients, including slow progression of symptoms, thin body, cerebellar atrophy, increased glutamate/

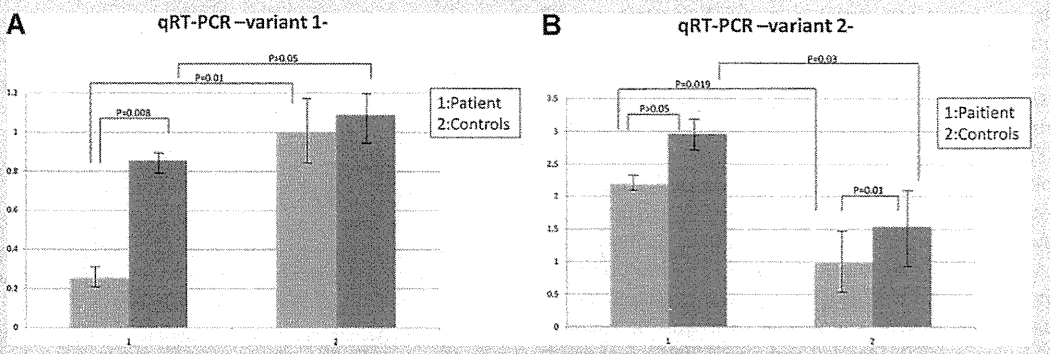

FIG. 4. Real-time quantitative PCR in samples from cell lines from the patient and four normal controls containing two males and two females. The light gray bars indicate the expression levels of *SLC9A6* before CHX treatment, while deep gray bars after CHX treatment. We performed statistical analysis using paired and unpaired Student's *t*-test. Error bars show standard deviation. A: The *SLC9A6* variant 1 was significantly downregulated in samples from the patient while it was not downregulated in samples from four normal controls. After CHX treatment, expression level of the *SLC9A6* variant 1 mRNA in the patient's sample was significantly increased. B: The *SLC9A6* variant 2 in the patient's sample was significantly increased compared to normal controls. Expression level of *SLC9A6* variant 2 increased in all samples after CHX treatment, but a significant increase was only seen in samples from controls.

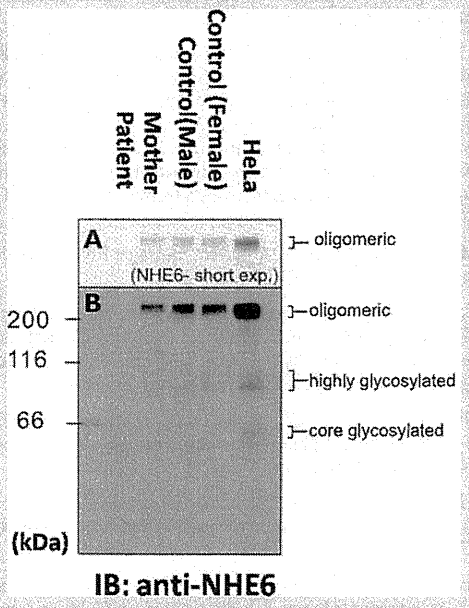

FIG. 5. Protein expression of NHE6 in cultured lymphoblastoid cells and HeLa cells. In the patient, no protein expression of NHE6 isoforms was detected with Western blotting using anti-NHE6 antibody. A: A cropped image taken using a short exposure time demonstrating the oligomeric form of NHE6. Protein size in kDa is shown by numbers on the left of the image. B: A chemiluminescence image of Western blotting taken with a longer exposure time.

glutamic acid peak on MRS, and rapid frequency of 10–14 Hz waves on EEG (Table I). Our patient lost his ability to walk although he did not demonstrate spasticity, demonstrating a slowly progressive clinical course consistent with findings in Gilfillan's report. Indeed, slow progression may be a distinctive clinical feature for patients with *SLC9A6* mutations. One of the families which Gilfillan et al. investigated was previously reported by Christianson et al. [1999], and designated as Christianson syndrome. Schroer et al. reported patients with Christianson syndrome, and they showed that the patients demonstrated an AS-like phenotype. However, while the clinical features of our patient were consistent with those of most patients previously reported by Gilfillan, there were differences including the EEG findings and lack of cerebellar atrophy. Despite this, our patient did meet the diagnostic criteria for AS [Williams et al., 2006]. Therefore, this study further demonstrated that a patient with a *SLC9A6* mutation may resemble patients with AS. Further, this striking similarity between patients with AS and those with *SLC9A6* mutations suggests a possible relationship between the gene function of *UBE3A* and *SLC9A6* in the developing brain.

Our patient's mutation created a frameshift resulting in 7 missense amino acids followed by a stop codon. This mutation was present only in *SLC9A6* transcript variant 1. *SLC9A6* mRNA has two transcript variants caused by alternative splicing in exon 2 (Fig. 1), but the role of each variant has not been clarified. The mutation detected in our patient only affects variant 1 sequence, but the phenotype of the patient was as severe as those in previously reported patients. Therefore, our finding suggests that the NHE6.1 plays an important role in brain function.

Nonsense mediated decay is involved in regulating the expression of alternatively spliced forms containing PTCs [Lareau et al., 2007; Ni et al., 2007]. Since the identified mutation was predicted to result in a PTC, we speculated that NMD could be involved in disease pathogenesis. The result of qRT-PCR showed a significant

decrease in *SLC9A6* variant 1 mRNA expression in the patient sample. This reduction was restored by CHX treatment, while *SLC9A6* variant 1 expression was unaltered by CHX treatment in normal control samples. Expression of *SLC9A6* variant 2 in the patient on the other hand, was significantly increased compared to that in control samples, however it was not influenced by CHX treatment. Therefore, the c.441delG mutation in the patient seems to have modified the alternative splicing pattern, leading to an increase in variant 2 expression. Alternatively, low variant 1 could trigger a regulatory feed back on transcription causing the apparent increase in variant 2 expression. A mutation causing premature protein truncation could alter the splicing pattern and lead to exon skipping, use of alternative splice sites, and intron retention [Hentze and Kulozik, 1999; Mendell and Dietz, 2001]. Our results indicated that the c.441delG mutation caused a PTC altered the splicing pattern, and activated NMD machinery then downregulated *SLC9A6* variant 1 expression.

As protein NHE6.1 was not detected, this indicates an absence of intact NHE6.1. NHE6.0 was also not detected. These findings conclusively indicated that the identified mutation should cause total loss-of-function. Recently, Garbern et al. identified cases with an in-frame deletion of three amino acids, who showed milder dysmorphic features and higher gross motor abilities than those in cases previously reported [Garbern et al., 2010]. Their in-frame deletion should not cause total loss-of-function but create a mildly dysfunctional protein. Therefore, severe phenotypes including severe developmental delay and progressive neurological deterioration may be caused by truncated mutations and less severe phenotypes may be caused by missense or in-frame mutations, and such mild phenotypes are likely missed in patients with mild developmental delay.

Given that the *SLC9A6* variant 2 was upregulated, we speculated that upregulated variant 2 might partially compensate for the absence of NHE6.1. However, we could not establish the upregulation of the NHE6.0 protein, rather it was not detected in the patient's lymphoblastoid cells. NHE6.0 may be unstable compared to NHE6.1. Alternately, NHE6.0 translation may be inhibited. Further investigation is required to definitively answer this question.

NHE6 is found in the membranes of early recycling endosomes and transiently in plasma membranes. Its distribution is regulated by RACK1 [Ohgaki et al., 2008]. Recycling endosomal trafficking is essential for the growth of dendritic spines during LTP in the brain [Park et al., 2006]. The function of the protein product of *UBE3A*, E3 ubiquitin ligase, is also associated with dendritic spine morphology. Mice with a maternal null mutation in *Ube3a* are also reported to have defects in LTP, and manifest motor and behavioral abnormalities [Jiang et al., 1998]. In a recent study, *Ube3a* deficient mice demonstrated dendritic spine dysmorphology [Dindot et al., 2008]. Thus, *UBE3A* and *SLC9A6* could interact in a common pathway involved in dendritic spine development, with a mutation in either leading to an AS-like phenotype.

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CLINICAL RECORD

Successful cochlear implantation in a patient with mitochondrial hearing loss and m.625G>A transition

A SUDO[1], N TAKEICHI[2], K HOSOKI[3], S SAITOH[3]

[1]*Department of Pediatrics, Sapporo City General Hospital, and the Departments of* [2]*Otolaryngology and* [3]*Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan*

Abstract

Objective: We present a patient with mitochondrial hearing loss and a novel mitochondrial DNA transition, who underwent successful cochlear implantation.

Case report: An 11-year-old girl showed epilepsy and progressive hearing loss. Despite the use of hearing aids, she gradually lost her remaining hearing ability. Laboratory data revealed elevated lactate levels, indicating mitochondrial dysfunction. Magnetic resonance imaging showed diffuse, mild brain atrophy. Cochlear implantation was performed, and the patient's hearing ability was markedly improved. Whole mitochondrial DNA genome analysis revealed a novel heteroplasmic mitochondrial 625G>A transition in the transfer RNA gene for phenylalanine. This transition was not detected in blood DNA from the patient's mother and healthy controls. Mitochondrial respiratory chain activities in muscle were predominantly decreased in complex III.

Conclusion: This case indicates that cochlear implantation can be a valuable therapeutic option for patients with mitochondrial syndromic hearing loss.

Key words: Sensorineural Hearing Loss; Cochlear Implantation; Mitochondrial DNA

Introduction

There have recently been many reported cases of sensorineural hearing loss of mitochondrial origin. In such patients, the effectiveness of cochlear implantation has been recognised in those with the m.1555A>G and m.3243A>G mutations.[1] However, the efficacy of such treatment for patients with other mitochondrial DNA mutations has not yet been defined.

Here, we present a patient with syndromic hearing loss, probably caused by a novel mitochondrial DNA mutation (m.625G>A), who gained excellent benefit from cochlear implantation.

Case report

The patient, an 11-year-old girl, was the first child of healthy and nonconsanguineous Japanese parents. There was no family history of hearing loss or epilepsy, and the patient had had no perinatal problems. Her motor and cognitive development was normal, but she displayed an abnormally short stature for her age.

The patient's hearing difficulty had first been noticed by her mother at the age of six years. Two years later, the patient had been examined by an otolaryngologist for the first time, and bilateral hearing aids had been prescribed. However, her hearing ability continued to deteriorate. There had been no previous exposure to aminoglycoside antibiotics. In addition to hearing loss, at the age of eight years the patient had begun to suffer generalised tonic seizures, uncontrolled by valproic acid. At the age of 10 years, she had been referred to our institution, as her family had moved to the locality near our hospital.

On physical examination, the patient had a height of 119.0 cm (−3.0 standard deviations (SD)), a weight of 21.9 kg (−1.7 SD) and a head circumference of 53.6 cm (+0.9 SD). Cranial nerve and cerebellar functions were normal. Hypertrichosis was observed. Although her muscle force did not decrease, she was unable to exercise for extended periods of time. Deep tendon reflexes were normal, without spasticity. She was unable to communicate verbally, although her intelligence appeared normal as she could communicate in writing and could solve age-appropriate arithmetic problems. Otitis media was not found.

Laboratory data revealed mildly elevated blood lactate levels (24.0 mg/dl (normal range, <17 mg/dl), with a pyruvate level of 1.0 mg/dl (normal range, <0.9 mg/dl)), and noticeably elevated cerebrospinal fluid lactate levels (55.8 mg/dl, with a pyruvate level of 2.0 mg/dl).

Electroencephalography revealed no distinct epileptic discharge during waking and sleeping states.

Computed tomography showed no internal ear malformations. Magnetic resonance imaging (MRI) revealed mild brain atrophy without focal lesions (Figure 1a).

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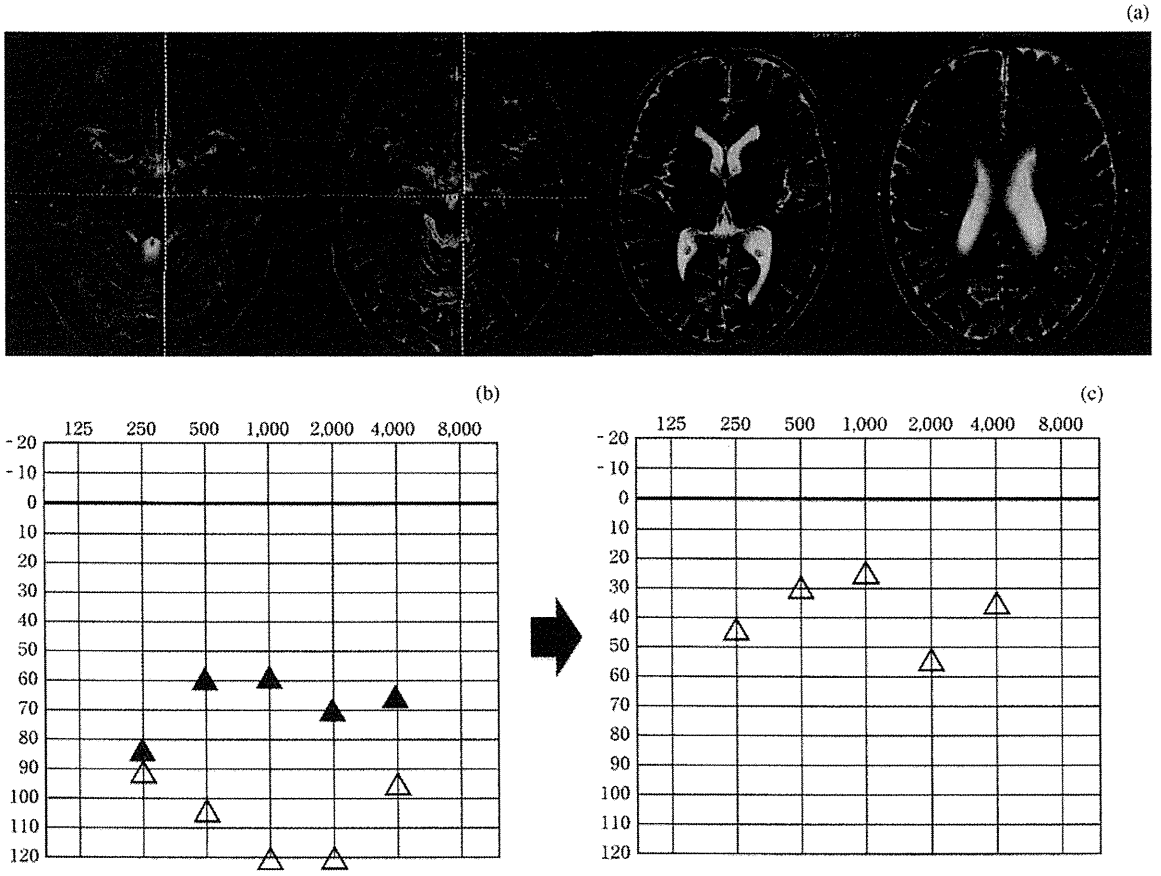

FIG. 1

(a) Axial magnetic resonance imaging brain scans, showing mild brain atrophy without focal lesions. (b) Left ear audiogram taken at 11 years, before cochlear implantation, following progression of hearing loss (hearing aids were no longer useful). (c) Left ear audiogram taken one month after implantation, showing significant improvement, with hearing thresholds of almost 25 – 45 dB. Δ = sound source 1 m away, without hearing aids; ▲ = with hearing aids in both ears

Formal pure tone audiography revealed hearing thresholds of between 90 and 120 dB at 250 through to 4 kHz. The patient's hearing aids only minimally improved her hearing thresholds (Figure 1b).

Auditory evoked potential testing showed a barely detectable auditory reaction at maximum intensity stimulation of 105 dB.

Therefore, the patient was considered to be a candidate for cochlear implantation.

Informed consent for participation in academic research was obtained from the patient and her parents.

During cochlear implantation, temporalis muscle and skin specimens were obtained.

Genomic DNA was extracted from blood, skin and muscle specimens. Sequencing of the whole mitochondrial genome was performed using the mitoSEQ resequencing system (Applied Biosystems, Foster City, California, USA). Polymerase chain reaction amplification was conducted, using forward mismatch primer (nucleotides 601–624, 5′-GCAATACACTGAAAATGTTTAGC-3′; where G = guanine, C = cytosine, A = adenine and T = thymine) and reverse primer (nucleotides 768–786, 5′-CGTTTTGAG CTGCATTGCT-3′). This enabled the m.625G>A sequence to be specifically recognised, and cut using the restriction enzyme BstOI (Promega, Madison, WI, USA). The proportion of heteroplasmy was approximately measured by using a mixture-template standard curve of wild type and mutant clones.

The activities of the mitochondrial respiratory chain complexes I, II, III and IV were assayed, using methods previously described.[2] We used the diagnostic criteria for respiratory chain disorders previously published by Bernier *et al.*[3]

Cochlear implantation and clinical course

The patient underwent left-sided cochlear implantation (using a CI24RCS device; cochlear LTD, Lane Cove, Australia) at the age of 11 years.

One month after implantation, she was able to use the telephone, clearly indicating improvement in her hearing function. Audiological data indicated a good response (Figure 1c). Her speech perception score increased to almost 100 per cent, from 0 per cent before surgery.

Twenty months after surgery, the patient and her parents were satisfied with her improved communication, and she continued to attend regular school classes. Her epileptic seizures were well controlled by carbamazepine and clonazepam. Her neurological signs and symptoms remained nonprogressive, possibly due to vitamin B1 supplementation.