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Original Communication

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

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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.

Key words: progressive external ophthalmoplegia, deoxyribonucleic acid polymerase gamma gene, parkinsonism, mitochondria

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.

Subjects and Methods

Subjects

Information from both families was not suggestive of maternal inheritance.

Family A (Figure 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, 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 (300mg/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. His mother (AI:2) was healthy until she died of stroke at the age of 96. 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. 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 (Figure 1C): Patient 2 (BII:3) was a 64-year-old man, who developed slowly progressive external ophthalmoplegie and ptosis at age 40 and resting tremor of the left hand and stooped posture at age 60. 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 MMSE score was 30. His father died of senile decay at the age of 91. 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 (Figure 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 (Figure 1B). The sister of patient 1 (AII:5) had no *POLG* mutations (Figure 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 (Figure 2).

We found similar muscle pathologies in both patients (Figure 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.

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 might have 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, hemiparkinsonism, 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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LEGENDS

Figure 1. Modified family pedigrees and electropherogram

A; Family A. □, man; ○, woman; ⁄ deceased. ◇, 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*.

Figure 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.

Figure 3. Muscle pathology

A, B, and C are 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).

Figure 1

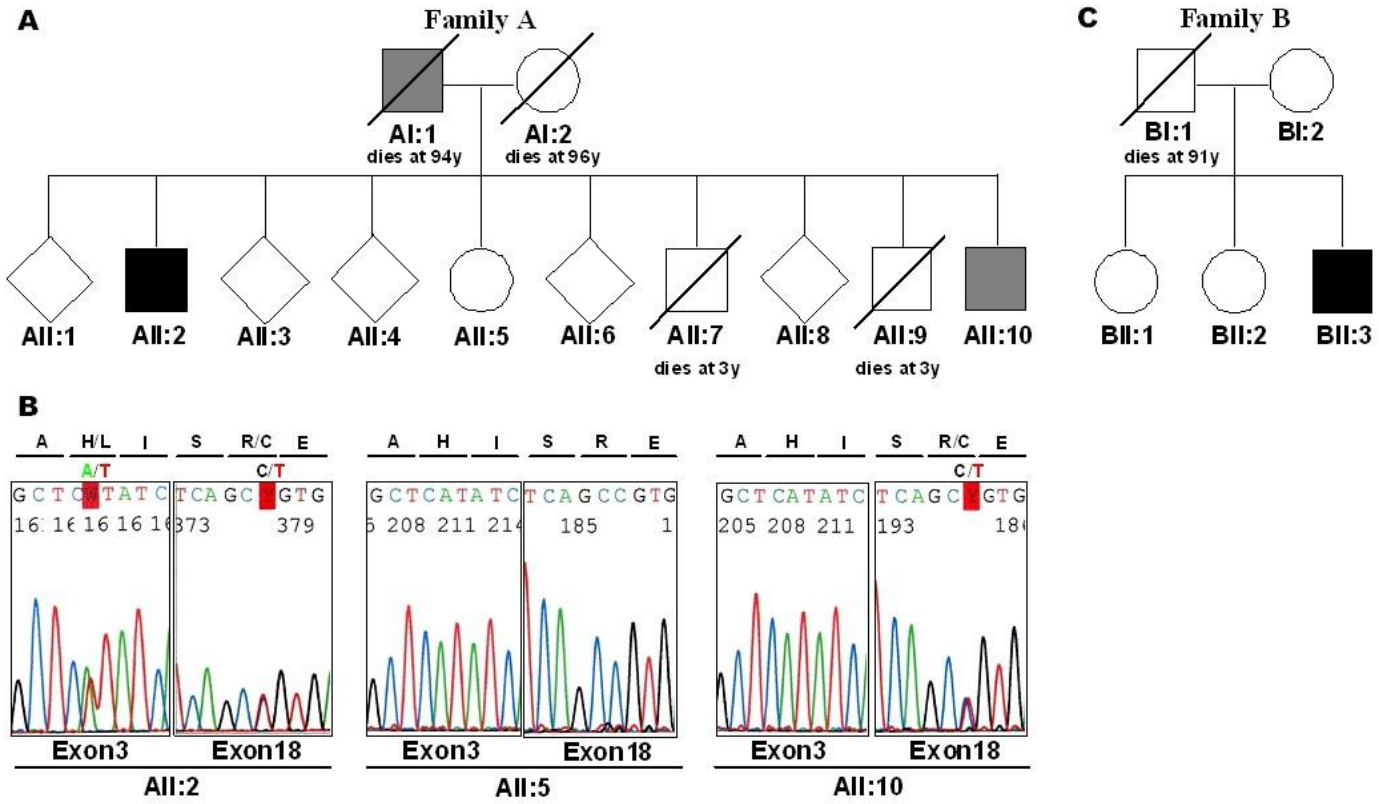

A
Family A
AI:1
dies at 94y
AI:2
dies at 96y
AII:1
AII:2
AII:3
AII:4
AII:5
AII:6
AII:7
dies at 3y
AII:8
AII:9
dies at 3y
AII:10
C
Family B
BI:1
dies at 91y
BI:2
BII:1
BII:2
BII:3
B
A H/L I S R/C E
A/T
C/T
Exon3
Exon18
AII:2
A H I S R E
Exon3
Exon18
AII:5
A H I S R/C E
C/T
Exon3
Exon18
AII:10

Figure 2

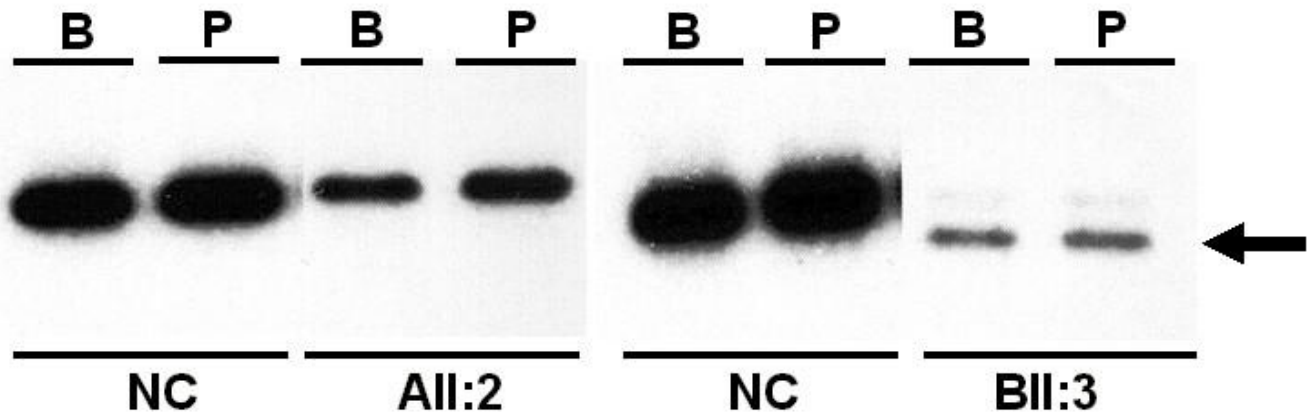


Figure 3

