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ORIGINAL ARTICLE

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A Recurrent De Novo *FAM111A* Mutation Causes Kenny–Caffey Syndrome Type 2

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ABSTRACT

Kenny–Caffey syndrome (KCS) is a rare dysmorphologic syndrome characterized by proportionate short stature, cortical thickening and medullary stenosis of tubular bones, delayed closure of anterior fontanelle, eye abnormalities, and hypoparathyroidism. The autosomal dominant form of KCS (KCS type 2 [KCS2]) is distinguished from the autosomal recessive form of KCS (KCS type 1 [KCS1]), which is caused by mutations of the tubulin-folding cofactor E (*TBCE*) gene, by the absence of mental retardation. In this study, we recruited four unrelated Japanese patients with typical sporadic KCS2, and performed exome sequencing in three patients and their parents to elucidate the molecular basis of KCS2. The possible candidate genes were explored by a de novo mutation detection method. A single gene, *FAM111A* (NM_001142519.1), was shared among three families. An identical missense mutation, R569H, was heterozygously detected in all three patients but not in the unaffected family members. This mutation was also found in an additional unrelated patient. These findings are in accordance with those of a recent independent report by a Swiss group that KCS2 is caused by a de novo mutation of *FAM111A*, and R569H is a hot spot mutation for KCS2. Although the function of FAM111A is not known, this study would provide evidence that FAM111A is a key molecule for normal bone development, height gain, and parathyroid hormone development and/or regulation. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: KENNY–CAFFEY SYNDROME; *FAM111A*; PARATHYROID-RELATED DISORDERS; HYPOMAGNESEMIA

Introduction

Kenny–Caffey syndrome (KCS) (OMIM #244460, %127000) is a rare dysmorphologic syndrome characterized by severe proportionate short stature with adult heights of 121 to 149 cm, cortical thickening and medullary stenosis of tubular bones, delayed closure of the anterior fontanelle, eye abnormalities, and hypocalcemia owing to hypoparathyroidism.[1–4] KCS is classified into two types according to its clinical features and inheritance pattern. Classical cases have normal intelligence and are transmitted as an autosomal dominant trait or sporadically and are called KCS type 2 (KCS2) (OMIM %127000).[5] Cases having mental and prenatal growth retardation and transmitted as an autosomal recessive trait are called KCS type 1 (KCS1) (OMIM #244460).[4,6,7]

In 2002, a study of 65 individuals from 34 pedigrees of Middle Eastern origin resulted in the identification of mutations of the tubulin-folding cofactor E (*TBCE*) gene as the cause of KCS1. *TBCE* encodes a molecular chaperone required for heterodimerization of α-tubulin with β-tubulin.[8] KCS2 is extremely rare, with only 5 sporadic cases reported in Japan.[9–12] Because of this rarity, the cause of KCS2 has been unknown until it was recently reported to involve the "family with sequence similarity 111, member A" (*FAM111A*) gene (NM_001142519.1) by a Swiss group in 2013.[13]

In this study, we recruited 4 Japanese patients with typical sporadic KCS2 having normal intelligence and performed whole exome sequencing in 3 unrelated trios to elucidate the molecular basis of KCS2. We hypothesized that KCS2 is caused by de novo mutations and built a de novo mutation detection pipeline to

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process the raw data from exome sequencing. Using this method, we found an identical de novo mutation in *FAM111A* in all 4 patients. This and the reported independent studies provide evidence that *FAM111A* is the cause of KCS2, and R569H is a hot spot mutation for KCS2.

Materials and Methods

Subjects

Case 1

This 10-year-old girl (Fig. 1, I-1)[9] was born at 40 weeks of gestation to nonconsanguineous, healthy Japanese parents. Polysyndactyly was noticed at birth. At 3 months of age, she was referred to a pediatric endocrinologist because of growth retardation. Her body length, body weight, and head circumferences were 55 cm (−2.5 SD), 5092 g (−1.8 SD), and 37.3 cm (0.2 SD), respectively. She was found to have liver dysfunction with a serum aspartate aminotransferase (AST) level of 227 U/L (reference range 21 to 75) and serum alanine aminotransferase (ALT) level of 227 U/l (reference range 11 to 69). Basal serum insulin-like growth factor (IGF-I), calcium (Ca), and phosphorus (P) levels were within normal limits. At the age of 1 year, hypocalcemia was revealed. Her serum Ca, P, and intact parathyroid hormone (PTH) levels were 1.6 mmol/L (reference range 2.1 to 2.4), 2.6 mmol/L (reference range 0.88 to 1.4), and 11 ng/L (reference range 15 to 50), respectively, with a normal magnesium (Mg) level of 0.86 mmol/L (reference range 0.74 to 0.90). Her serum $1,25(OH)_2D$ level, serum alkaline phosphatase level, and urine Ca/creatinine ratio were within normal ranges. Brain computed tomography (CT) revealed calcification in the basal ganglia (Fig. 2*A*). She was diagnosed with primary hypoparathyroidism and was treated with alfacalcidol [$1\alpha(OH)D_3$]. At 2 years of age, she was diagnosed with KCS2 based on clinical manifestations of proportionate short stature, cortical thickening and medullary stenosis confirmed by radioscopic study (Fig. 2*B*), macrocephaly with delayed closure of the anterior fontanelle, eye abnormalities (hypermetropia and pseudopapilledema), and normal intelligence. Magnesium oxide was administered because of a low serum Mg level (below 0.62 mmol/L) at 3 years of age.

Case 2

This 16-year-old boy (Fig. 1, II-4)[10] was born at 41 weeks of gestation to nonconsanguineous, healthy Japanese parents. When he was 23 days old, he had a generalized convulsion because of hypocalcemia. At this time, his serum Ca, P, Mg, and intact PTH levels were 1.5 mmol/L, 3.1 mmol/L, 0.74 mmol/L, and undetectable, respectively. T-cell subset was normal. He was treated with alfacalcidol on the basis of a diagnosis of primary hypoparathyroidism. Magnesium sulfate was added because of his low serum Mg level at the age of 1 year. He suffered repeated bouts of acute otitis media until the age of two years. His serum IgG level was within the normal range. At 3 years and 1 month, his height, weight, and head circumference were 77.9 cm (−4.4 SD), 9.9 kg (−2.7 SD), and 47.4 cm (−1.5 SD), respectively. He had normal intelligence for his age. He was diagnosed with KCS2 based on clinical findings of proportionate short stature, medullary stenosis revealed by radiography, a widely open anterior fontanelle (Fig. 2*C*, skull radiograph at 9 years), and hypermetropia. He also suffered severe atopic dermatitis after normalization of his serum Ca levels. His growth chart is shown in Fig. 2*D*.

Case 3

This 22-year-old woman (Fig. 1, III-9)[11] was born at 40 weeks of gestation to nonconsanguineous, healthy Japanese parents following an uneventful pregnancy. At 1 month, she had an episode of generalized convulsions because of hypocalcemia. At this episode, her serum Ca, P, Mg, and intact PTH levels were 1.3 mmol/L, 2.9 mmol/L, 0.49 mmol/L, and undetectable, respectively. Oral alfacalcidol administration was started on the basis of a diagnosis of primary hypoparathyroidism. At the age of 5 years 1 month, she was referred to another hospital. Her height was 84.2 cm (−5.3 SD), and her weight was 12.2 kg (−2.2 SD). She had normal intelligence. Brain CT revealed fine calcification in the basal ganglia. Based on clinical manifestations of proportionate short stature, medullary stenosis of the long bones typical of KCS, a 1 × 1-cm opening of her anterior fontanelle, normal intelligence, and hypermetropia, she was diagnosed with KCS2. The patient was started with a combination therapy of vitamin D and magnesium sulphate. Fig. 2*E* shows her radiograph at 14 years of age.

Case 4

This 38-year-old man (Fig. 1, IV-13)[12] was born at 40 weeks of gestation to nonconsanguineous, healthy Japanese parents following an uneventful pregnancy. At 8 days of age, he had a generalized convulsion, and hypocalcemia (0.75 mmol/L) and hypomagnesemia (0.18 mmol/L) were detected. The convulsion was controlled by intravenous administration of Ca gluconate and magnesium sulfate until he was 15 days old. At 4 years of age, he again had an episode of generalized convulsion because of hypocalcemia. At this episode, his serum Ca, P, and intact PTH levels were 1.2 mmol/L, 2.6 mmol/L, and undetectable, respectively. He was diagnosed with primary hypoparathyroidism, and oral alfacalcidol and Ca lactate administration were started. He suffered repeated acute otitis media during infancy and was affected with empyema and bacterial meningitis at 4 years of age. Hypogammaglobulinemia was found, and he was administered gamma globulin intermittently. At 12 years of age, he was referred to another hospital for further investigation. His height was 99 cm (−6.3 SD), and his weight was 16.2 kg (−3.3 SD). He had normal intelligence with an intelligence quotient score of 105. Brain CT revealed fine calcification in the basal ganglia. Based on clinical manifestations of proportionate short stature, medullary stenosis of the long bones, a 4.2 × 1.8-cm opening of his anterior fontanelle, and eye abnormalities (hypermetropia, amblyopia, and pseudopapilledema), he was diagnosed with KCS2. Mg loading and Ca restriction tests revealed that his hypoparathyroidism was secondary to hypomagnesemia. The patient was then changed from vitamin D and Ca lactate to magnesium sulfate treatment, which successfully corrected his serum Ca levels.

We recruited these 4 Japanese patients with clinically diagnosed typical sporadic KCS2 (Fig. 1). Supplemental Table S1{TBL S1} summarizes the clinical characteristics of the 4 patients. We obtained peripheral blood samples from all 4 patients, together with those of 9 unaffected parents or siblings, with informed consent for DNA analysis (Fig. 1). The study was performed with the approval of the Ethics Committee of The University of Tokyo and of each institution where the samples

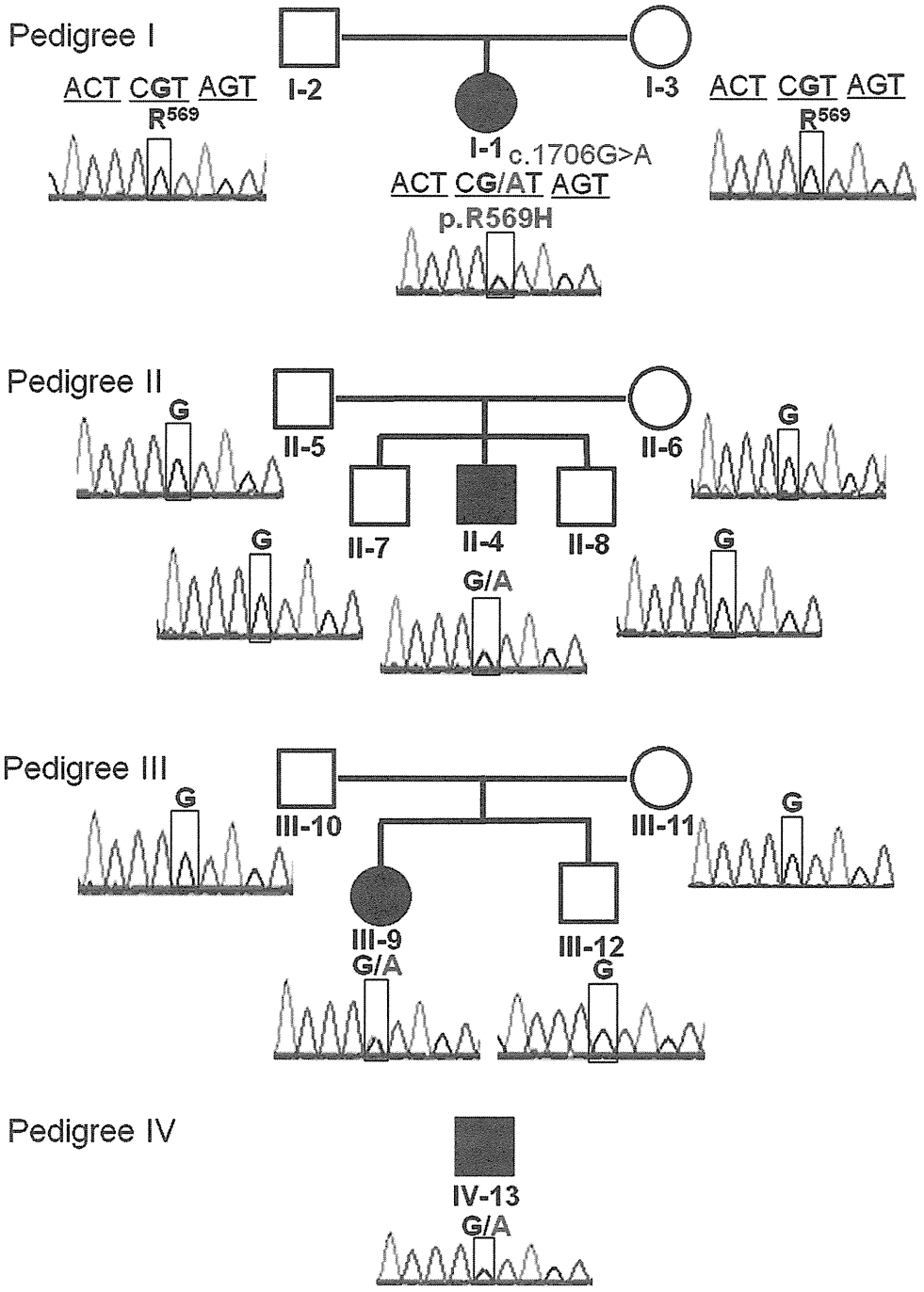

Fig. 1. Four pedigrees analyzed in this study, showing the chromatograms of Sanger sequencing reactions of the *FAM111A* mutation in patients and family members. Data were obtained by Sanger sequencing during the confirmation process. All mutations were checked by bidirectional sequencing. In each pedigree, a black symbol represents the proband, a square indicates a male, and a circle shows a female. In the chromatogram, black letters indicate the wild-type nucleotide sequence. Nucleotides in red indicate mutations. R569H was identified in all probands but not in any of the unaffected family members.

were collected, and conducted in accordance with the Declaration of Helsinki. Genomic DNA was extracted from peripheral white blood cells of the patients and family members using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Healthy Japanese volunteers were recruited, and DNA was extracted with informed consent.

Exome sequencing

Exome sequences were enriched using a TruSeq Exome Enrichment Kit (Illumina, San Diego, CA, USA) from 1 μg of genomic DNA, according to the manufacturer's instructions. The captured DNA samples were subjected to massively parallel

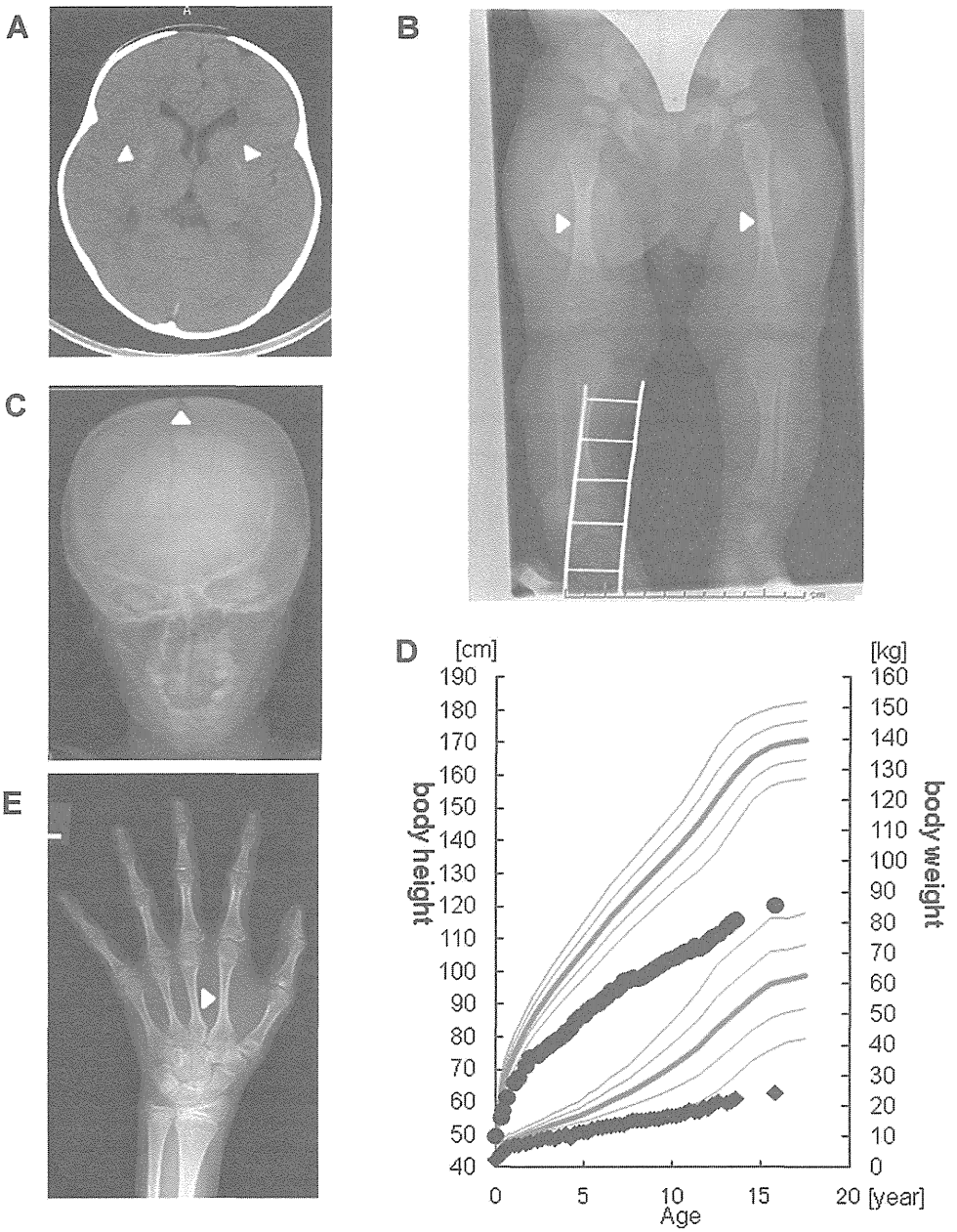

Fig. 2. Radiographic studies and growth charts of probands. (*A*) Brain computed tomography of patient I-1. The arrowheads indicate calcification in the basal ganglia. (*B*) Radiograph of patient I-1 at diagnosis. Cortical thickening and medullary stenosis are evident. The object shown in the right leg is used for fixing a peripheral catheter. (*C*) Radiograph of patient II-4 at age 9 years. It is of note that the anterior fontanelle is open. (*D*) Growth chart of patient II-4 superimposed on the standard growth chart for a Japanese boy. Black circles indicate the patient's height, and black squares indicate his weight. (*E*) Radiograph of patient III-9. Cortical thickening and medullary stenosis can be observed.

sequencing (100-bp paired-end reads) on an Illumina HiSeq2000 sequencing system (Illumina). An average of 95 million reads of the sequence data was obtained for each individual. On an average, 98.50% of the total bases were mapped to the reference genome with a mean coverage of 140.5 ×, which encompassed 91.94% of the targeted regions with coverage >10× (Supplemental Table S2).{TBL S2} The Burrows-Wheeler Aligner (BWA) package[14] and SAMtools[15] were used as default settings for alignment of raw reads and detection of single-nucleotide variants (SNVs) and indels. Subsequently, SNVs and indels were filtered with three trio samples (ie, pedigrees I, II, and III) (Supplemental Fig. S1). We extracted both homo/heterozygous nonsynonymous coding variants, which were called in the proband, and filtered these candidates using the following three steps:

Step 1: Using candidate de novo mutations that are homozygous references in both parents and are supported by 10 or more high-quality reads at the mutated sites for every trio member.

Step 2: Using reliable homozygous references in each parent such that the likelihood of heterozygosis, ${}_nC_i\,(1/2)^i\,(1/2)^{n-i}$, is less than that of homozygosis, ${}_nC_i\,(999/1000)^i(1/1000)^{n-i}$, where the average error rate is assumed to be 1/1000, n represents the number of total reads, and i is the number of reads consistent with the reference. One may have an impression that this condition does not often hold true; however, we often observed cases that violated this condition, especially when the reference base was mutated into one of the other three bases with an almost equal probability.

Step 3: Using reliable de novo mutations of the proband such that the number of alternative allele reads was at least 30% among the total reads, which is the condition proposed in a recent report.[16]

Sanger sequencing

Sanger sequencing was performed to detect *TBCE* (KCS1) and validate the presence of each variant detected by exome sequencing in patients with KCS2 and the absence of each in the genomes of the parents and siblings. The entire coding region and exon–intron boundaries of *TBCE* and *FAM111A* were amplified from genomic DNA by polymerase chain reaction (PCR) using the designed PCR primers (Supplemental Table S3). {TBL S3} Subsequently, PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and the forward and reverse primers used for PCR amplification. Direct sequencing in both directions was performed on an autosequencer (PE Applied Biosystems 3130 × 1, Genetic Analyzer).

FAM111A mRNA expression analysis

Total RNA was prepared using ISOGEN reagent (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions, from peripheral white blood cells of the patients and family members. Total RNA (4 μg) was used to synthesize cDNA with the SuperScript Preamplification System for first-strand cDNA synthesis (Life Technologies, Rockville, MD, USA). mRNA levels were measured using an ECO real-time PCR system (Illumina) and KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA, USA) using the following primer pairs: FAM111Ae5-2F and FAM111Ae5-2R; FAM111Ae5-3F and 5′-CCTCATCACTCATCATTTCTACATCC-3′; GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ (F) and 5′-GAAGATGGTGATGGGATTTC-3′ (R). The relative mRNA level was calculated using an arithmetic formula based on the difference between the threshold cycle of a given target cDNA and that of an endogenous reference cDNA. Direct sequencing of the RT-PCR products was performed by Sanger sequencing as for DNA samples.

Results

We first confirmed by Sanger sequencing that none of the 4 patients had *TBCE* mutations. This finding, together with the fact that all the patients were of normal intelligence, distinguishes these patients from patients with KCS1.

We hypothesized that these sporadic cases may be caused by de novo mutations in novel nonsynonymous coding variants. Whole exome sequencing was performed for 3 patients (I-1, II-4, and III-9; Fig. 1) and their parents (I-2, I-3, II-5, II-6, III-10, and III-11; Fig. 1). Statistical data of exome sequencing experiments are shown in Supplemental Table S2. The candidate variants were selected according to the processes described in Materials and Methods based on the de novo mutation detection pipeline designed in the present study (Supplemental Fig. S1). Supplemental Table S4{TBL S4} summarizes the results of filtering to detect candidate genes for KCS2. To select variants as candidate mutations for KCS2, variations that caused amino acid substitution were extracted, which resulted in 11,024 (pedigree I), 10,828 (pedigree II), and 11,020 (pedigree III) SNVs and indels. After three filtering steps, 5 (pedigree I), 5 (pedigree II), and 6 (pedigree III) SNVs were identified. Among the candidate genes filtered using the three aforementioned filtering steps, only one single gene, *FAM111A* (NM_001142519.1), was shared among all 3 families. Sanger sequence analysis of all exons of *FAM111A* confirmed an identical c.1706G > A heterozygous mutation in exon 5 in all 3 patients (Fig. 1). This mutation is predicted to result in substitution of arginine to histidine in codon 569 (R569H). None of the unaffected family members had this mutation, indicating that R569H was a de novo mutation. This mutation was also found in an additional unrelated patient (IV-13).

R569H is not present in 373 Japanese healthy control subjects of an in-house exome database, and not in another 100 alleles from 50 unrelated healthy Japanese individuals by Sanger sequencing. It was also not found in the Japanese SNP control database established by the National Bioscience Data Base Center that has 1 million genome-wide SNPs of 700 samples (http://gwas.biosciencedbc.jp/snpdb/snp_top.php), nor among 6500 samples listed on the exome variant server (http://evs.gs.washington.edu/EVS/), implying that the minor allele frequency is less than 0.01% in these data. However, one SNP was found in the 1000 Genomes database at R569 (rs184251651), which results in substitution to "cysteine" (minor allele frequency 0.1%).

We assessed the functionality of the R569H mutation using the Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org) and Polymorphism Phenotyping 2 (PolyPhen2) (http://genetics.bwh.harvard.edu/pph2) tools, by homology modeling and threading. These in silico studies predicted R569H as "tolerated" and "benign," respectively.

We analyzed the expression levels of *FAM111A* mRNA in peripheral white blood cells by real-time PCR. *FAM111A* expression levels in the patients were comparable with those in unaffected family members and normal controls (data not shown). We also found that mutant and wild-type *FAM111A* were equivalently expressed in the patients, which were identified by sequencing the reverse-transcribed PCR products.

Discussion

In the present study, we identified *FAM111A* as the gene responsible for KCS2 by applying an exome sequencing strategy, and we identified a heterozygous identical de novo *FAM111A* mutation, R569H, in 4 Japanese patients with KCS2. While preparing this article, another independent research group from Switzerland reported similar findings following whole exome sequencing of the patients.[13] They reported that all 5 clinically diagnosed KCS2 patients had de novo *FAM111A* mutations. Most interestingly, 4 of the 5 patients from different countries had the same R569H mutation as detected in our patients. Our 4 pedigrees are unrelated to each other and live in different areas in Japan. Moreover, the parents of the 3 patients did not have the mutation, suggesting that this recurrent mutation was caused by sporadic mutation. Taken together, these two independent

studies confirm that *FAM111A* is the causative gene for KCS2, and R569H is the hot spot mutation of KCS2.

FAM111A encodes a previously uncharacterized protein consisting of 611 amino acids. The carboxy-terminal half of the protein has homology to trypsin-like peptidases, and the catalytic triad specific to such peptidases is conserved.[17] Transcriptional expression of *FAM111A* is ubiquitous according to the human protein atlas (http://www.proteinatlas.org/ENSG00000166801/normal). It is expressed in the parathyroid gland and bone, but the expression levels are similar to those in other tissues. FAM111A has 35% amino acid homology to FAM111B, a paralog located on 11q12.1 at a distance of only 16 kb from FAM111A. The functions of FAM111A and FAM111B are largely unknown. A recent report showed that FAM111A functions as a host range restriction factor and is required for viral replication and gene expression by specifically interacting with Simian Virus 40 large T antigen (LT).[17] In addition, *FAM111A* mRNA and protein levels have been shown to be regulated in a cell cycle-dependent manner with the lowest expression during the G0 or quiescent phase and peak expression during the G2/M phase.[17] Another recent report revealed that variants in the region including *FAM111A* and *FAM111B* were associated with prostate cancer.[18] However, the clinical course of disease in our 4 patients revealed neither increased viral infections nor carcinogenesis up to early adulthood.

In silico analyses suggested that the de novo mutation (R569H) would not significantly affect the function of FAM111A. We also found that the mutant *FAM111A* mRNA was expressed similarly to the wild type in peripheral blood cells. This raises the question of how this mutation causes KCS2. One hypothesis is that this mutation does not cause loss of function of the protein but rather modulates its peptidase activity for a particular target peptide in a mutant-specific way. Another possibility is that FAM111A functions with some physiological partner(s) and the disease occurs as a result of specific modulation of this putative network. This may fit the observation that FAM111A is regulated in a cell-dependent manner and interacts with the LT C-terminal region.[17] We speculate that one of the candidate partner proteins is TBCE because KCS1 and KCS2 share distinctive phenotypic features: skeletal dysmorphic features and primary hypoparathyroidism.

Some diseases are caused by specific mutations of a single gene. Some mutations may cause a gain-of-function effect, as in achondroplasia or McCune–Albright syndrome,[19,20] whereas others have an unknown function, as in Caffey syndrome caused by mutations in *COL1A1*[21] or in several diseases related to *FGFR3*. In this study, we found that a specific mutation (R569H) of *FAM111A* would lead to KCS2. Intriguingly, one SNP was found in the 1000 Genomes database at R569 (rs184251651), which results in substitution to "cysteine." This SNP has been reported to have minor allele frequency of 0.1% (only one allele) and is not validated. Moreover, the absence of the SNP in 6500 samples in the exome variant server suggests a possibility of sequencing error in the database. Nevertheless, it might be speculated that a specific change to "histidine" may lead to an unidentified function of this protein resulting in KCS2, which is not caused by other amino acids. This hypothesis will be supported by the fact that this amino acid is not well conserved among various species (Fig. 3).

It is reported that 95% and 97% of KCS1 cases had prenatal and postnatal growth retardation, and mental retardation, respectively.[22] In contrast, most of the reported KCS2 patients, including our patients with *FAM111A* mutations, had normal

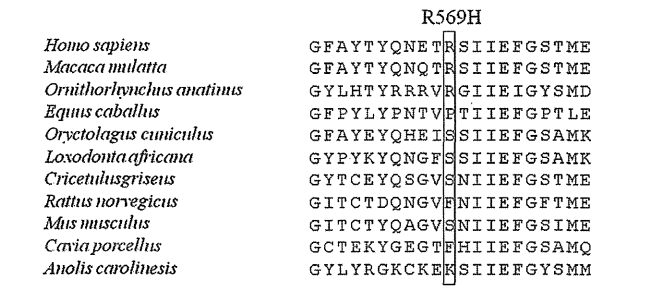

Fig. 3. Homologous comparison of the altered protein. Letters in the rectangular box indicate the human *FAM111A* R569 residue. It is of note that R569 is not well conserved among various species.

birth weight and length and normal intelligence (Supplemental Table S1).[13] These phenotypic differences between KCS1 and KCS2 suggest that the *FAM111A* mutation does not affect bone development and height gain in the fetus but becomes important postnatally. It also suggests that the *FAM111A* mutation does not affect mental development. Now that *FAM111A* has been identified as a causative gene for KCS2, further studies on the physiological function of FAM111A and TBCE should be performed to uncover the phenotypic differences between these two types.

There are several human diseases, as well as mouse models of hypoparathyroidism, caused by aberrations in the cascade of genes indispensable for the development and regulation of the parathyroid gland.[23,24] To date, FAM111A is not known to relate to any of these genes. There have been only a few reports describing the pathophysiology of hypoparathyroidism in KCS. Absence of the parathyroid glands has been reported in some patients with KCS2 and KCS1.[25,26] In contrast, some patients do not have hypoparathyroidism from early infancy, suggesting the presence of some parathyroid gland as in our patient I-1.[4,27] Furthermore, hypoparathyroidism may be secondary to hypomagnesemia as in our patient IV-13. Considering the fact that all of our 4 patients as well as another reported KCS2 case had hypomagnesemia,[4] FAM111A might be involved in magnesium homeostasis. Although further investigation is necessary to reveal the cause of hypoparathyroidism in KCS2, this study shows that a new gene, *FAM111A*, is indispensable for PTH development and/or regulation.

In conclusion, our finding that all 4 Japanese KCS2 patients we tested have the same de novo mutation (R569H) of *FAM111A* indicates that KCS2 is caused by a heterozygous mutation of *FAM111A*, and R569H is the hot spot mutation in patients with KCS2. Although the function of FAM111A is largely unknown, this study provides evidence that FAM111A is a key molecule for normal bone development, height gain, and PTH development and/or regulation. Our finding further creates a new research area in the fields associated with shared phenotypic features in KCS and different phenotypes between KCS1 and KCS2.

Disclosures

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Pfizer, Eli Lilly, and JCR Pharmaceuticals and has received speaker's fees from Chugai Pharmaceutical. All other authors state that they have no conflicts of interest.

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ORIGINAL ARTICLE

Mutations in *COQ2* in Familial and Sporadic Multiple-System Atrophy

The Multiple-System Atrophy Research Collaboration

ABSTRACT

BACKGROUND

Multiple-system atrophy is an intractable neurodegenerative disease characterized by autonomic failure in addition to various combinations of parkinsonism, cerebellar ataxia, and pyramidal dysfunction. Although multiple-system atrophy is widely considered to be a nongenetic disorder, we previously identified multiplex families with this disease, which indicates the involvement of genetic components.

METHODS

In combination with linkage analysis, we performed whole-genome sequencing of a sample obtained from a member of a multiplex family in whom multiple-system atrophy had been diagnosed on autopsy. We also performed mutational analysis of samples from members of five other multiplex families and from a Japanese series (363 patients and two sets of controls, one of 520 persons and one of 2383 persons), a European series (223 patients and 315 controls), and a North American series (172 patients and 294 controls). On the basis of these analyses, we used a yeast complementation assay and measured enzyme activity of parahydroxybenzoate-polyprenyl transferase. This enzyme is encoded by the gene *COQ2* and is essential for the biosynthesis of coenzyme Q_{10}. Levels of coenzyme Q_{10} in lymphoblastoid cells and brain tissue were measured on high-performance liquid chromatography.

RESULTS

We identified a homozygous mutation (M78V-V343A/M78V-V343A) and compound heterozygous mutations (R337X/V343A) in *COQ2* in two multiplex families. Furthermore, we found that a common variant (V343A) and multiple rare variants in *COQ2*, all of which are functionally impaired, are associated with sporadic multiple-system atrophy. The V343A variant was exclusively observed in the Japanese population.

CONCLUSIONS

Functionally impaired variants of *COQ2* were associated with an increased risk of multiple-system atrophy in multiplex families and patients with sporadic disease, providing evidence of a role of impaired COQ2 activities in the pathogenesis of this disease. (Funded by the Japan Society for the Promotion of Science and others.)

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MULTIPLE-SYSTEM ATROPHY IS A PROgressive neurodegenerative disease that is clinically characterized by autonomic failure in addition to various combinations of parkinsonism, cerebellar ataxia, and pyramidal dysfunction. The term multiple-system atrophy was introduced in 1969 to encompass the disease entities of olivopontocerebellar ataxia, striatonigral degeneration, and the Shy–Drager syndrome, on the basis of neuropathological findings in these disorders.[1] Multiple-system atrophy is characterized by the development of cytoplasmic aggregates of α-synuclein, primarily in oligodendroglia.[2-7] However, the pathogenic mechanisms underlying this disease remain unknown, making it difficult to develop effective therapies.

The disorder is classified into two subtypes: subtype C, characterized predominantly by cerebellar ataxia, and subtype P, characterized predominantly by parkinsonism.[8] Among patients with multiple-system atrophy, subtype C has been reported to be more prevalent than subtype P in the Japanese population (65 to 67% vs. 33 to 35%),[9,10] whereas subtype P has been reported to be more prevalent than subtype C in Europe (63% vs. 34%)[11] and North America (60% vs. 13%, with 27% of cases unclassified).[12] Although multiple-system atrophy has been defined as a nongenetic disorder until recently, several multiplex families with the disease have been described, indicating that strong genetic factors confer susceptibility to the disease.[13-15]

METHODS

PATIENTS AND MULTIPLEX FAMILIES

Patients with multiple-system atrophy were enrolled in the study on the basis of research protocols that were approved by the institutional review board at each participating center. Written informed consent was obtained from all participants.

The diagnosis of multiple-system atrophy was made on the basis of the current consensus criteria for the disease.[8] Four Japanese families (Families 1 through 4, whose members have been described previously[13]) and two additional Japanese families (Family 8 and Family 12) were enrolled in this study (Fig. 1). In Family 1, the parents were first-degree cousins, which is consistent with autosomal recessive inheritance. The clinical features of these families are summarized in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Autopsy findings for Participants II-4[13] and II-8 in Family 1 and Participant II-6 in Family 8 showed widespread and abundant cytoplasmic aggregates of α-synuclein, primarily in oligodendroglia, in association with neurodegeneration in striatonigral and olivopontocerebellar structures. These findings confirmed the diagnosis of multiple-system atrophy.

PATIENTS WITH SPORADIC DISEASE AND CONTROLS

As with the multiplex families, the diagnosis of sporadic multiple-system atrophy was made on the basis of the current consensus criteria.[8] A total of 363 patients with multiple-system atrophy and 520 controls were included in the Japanese series, 223 patients and 315 controls in the European series, and 172 patients and 294 controls in the North American series (persons of European or Hispanic descent living in North America) (Text S2 and Table S2 in the Supplementary Appendix). Ancestry was determined by self-report on a multiple-choice questionnaire. We also enrolled an independent series of 2383 Japanese controls.

ASSOCIATION WITH OTHER NEURODEGENERATIVE DISEASES

To determine the specificity of the association between variants in candidate genes and multiple-system atrophy, we enrolled 2728 Japanese patients with Alzheimer's disease, 659 with Parkinson's disease, and 634 with amyotrophic lateral sclerosis (ALS). Their demographic characteristics are provided in Text S2 in the Supplementary Appendix.

LINKAGE ANALYSIS AND WHOLE-GENOME SEQUENCING

We performed parametric and nonparametric linkage analyses using Affymetrix SNP 6.0 arrays and software for linkage analysis.[16,17] The genomic DNA from Participant II-4 in Family 1 was subjected to four runs in an Illumina Genome Analyzer IIx (100-bp-long paired ends). We used BWA software[18] and SAMtools sequence-alignment mapping[19] with the default settings for alignment and variation detection against the human reference genome (National Center for Biotechnology Information build 36 [also known as hg18]).

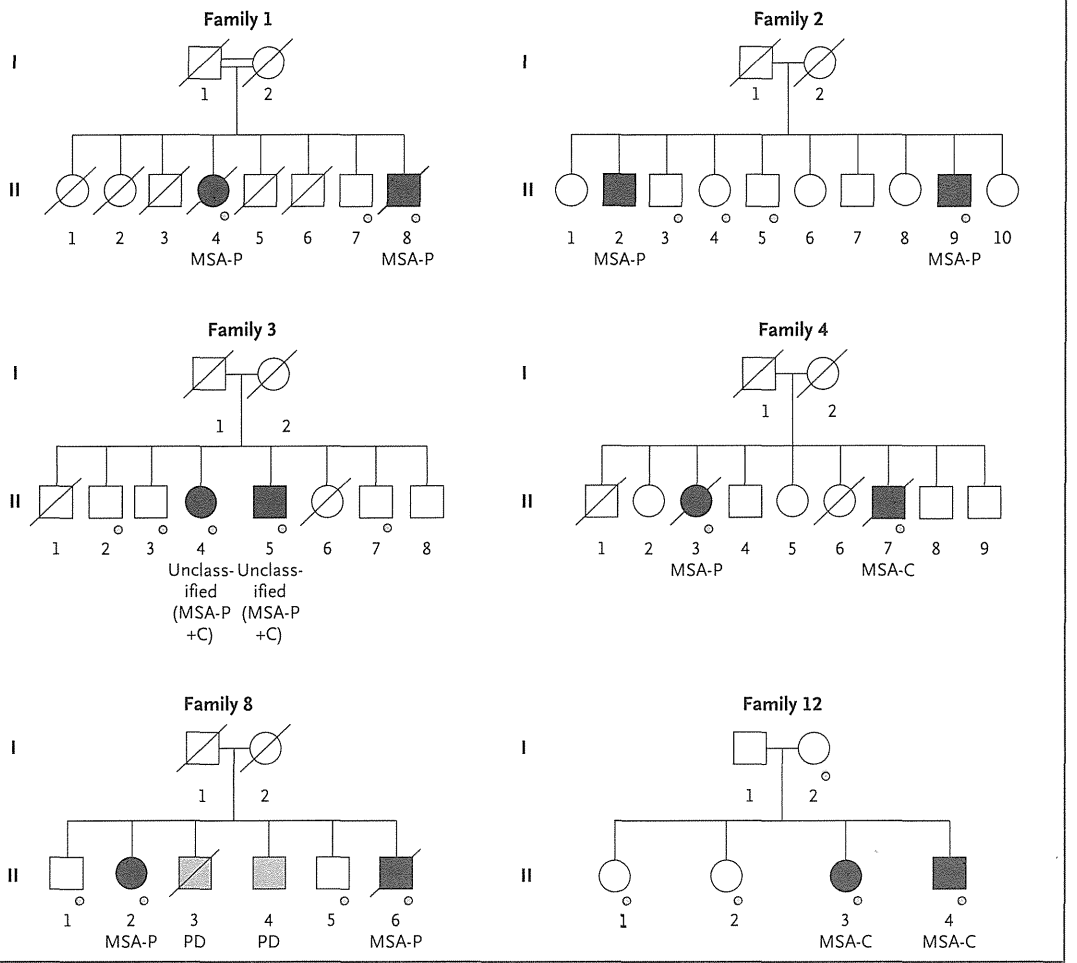

Figure 1. Pedigrees of Six Multiplex Families with Multiple-System Atrophy.

The affected siblings in Family 1 were born to consanguineous parents (first cousins).[13] In this family, the two patients with multiple-system atrophy (Participants II-4 and II-8) also had retinitis pigmentosa, which was not present in the other siblings. The diagnosis of definite multiple-system atrophy in three patients (Participants II-4 and II-8 in Family 1 and II-6 in Family 8) was confirmed at autopsy. In Family 8, two siblings (Participants II-3 and II-4) of the affected family members had Parkinson's disease (PD). In Family 1, in which homozygous M78V-V343A mutations in *COQ2* were identified, the parents (Participants I-1 and I-2), who were obligate carriers of the mutation, showed no overt signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction, according to family report. In Family 12, in whom compound heterozygous R337X/V343A mutations were identified, Participants I-1 and I-2 (obligate carriers of the mutations) and the heterozygous carrier (Participant II-2) showed no overt signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction on examination by a neurologist. Squares represent male family members, circles female family members, black symbols family members with multiple-system atrophy, gray symbols family members with Parkinson's disease, open symbols unaffected family members, slashes deceased family members, and small circles family members for whom genomic DNA samples were available. MSA-C denotes multiple-system atrophy of the cerebellar type, MSA-P multiple-system atrophy with predominant parkinsonism, and unclassified MSA-P+C similarly predominant parkinsonian and cerebellar signs.

ANALYSIS OF *COQ2* AND OTHER GENES ASSOCIATED WITH COENZYME Q_{10}

On the basis of linkage analysis and whole-genome sequencing, we sequenced *COQ2* and the other 11 genes involved in the biosynthetic pathway for coenzyme Q_{10} (*PDSS1*, *PDSS2*, *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *ADCK3*, *COQ9*, *COQ10A*, and *COQ10B*), using the Sanger method (Table S3 in the Supplementary Appendix).

We prepared samples of mutant human *COQ2* complementary DNA (cDNA) by means of site-directed mutagenesis (Table S4 in the Supple-

mentary Appendix). A yeast *coq2*-null mutant, the BY4741Δ*coq2* strain, was transformed with pAUR123 (Takara Bio) containing the nonmutated or mutated human *COQ2* cDNA. We measured the growth rate in a medium with a nonfermentable carbon source by monitoring the optical density of a sample measured at a wavelength of 600 nm (OD_{600}). We used mitochondrial fractions prepared from lymphoblastoid cell lines with the QProteome Mitochondria Isolation Kit (Qiagen) as the enzyme source. COQ2 activity (Enzyme Commission number, 2.5.1.39) was assayed as described previously.[20]

COENZYME Q_{10} LEVEL IN TISSUES

Using high-performance liquid chromatography, we measured levels of coenzyme Q_{10} (ubiquinone-10 and ubiquinol-10) and free (unesterified) cholesterol in lymphoblastoid cell lines established from 152 patients with multiple-system atrophy and 76 controls and in cerebellum samples obtained on autopsy from 3 patients with multiple-system atrophy and 3 controls.[21]

STATISTICAL ANALYSIS

All results are presented as means and standard deviations. We used Student's t-test to evaluate the significance of differences in the mean age at disease onset between carriers and noncarriers of the *COQ2* mutation. We used Fisher's exact test to calculate the significance of the difference in allele frequencies between carriers and noncarriers, with contingency tables and standard methods used to compute odds ratios and corresponding 95% confidence intervals. We used the Kruskal–Wallis test, followed by the Steel test, to perform an analysis of variance. All statistical tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

LINKAGE ANALYSIS OF FAMILIAL DISEASE

Parametric linkage analysis of the six family pedigrees revealed no single locus showing a linkage compatible with autosomal recessive inheritance. However, in the parametric linkage analysis allowing for heterogeneity, we detected several loci showing positive scores for heterogeneity logarithm of the odds (HLOD), indicating that more than one locus was involved in the different multiplex families (Fig. S1B in the Supplementary Appendix). In particular, two regions on chromosome 4 showed the highest HLOD scores, exceeding 2.0. Results of nonparametric linkage analysis (Fig. S1C in the Supplementary Appendix) were consistent with those of parametric linkage analysis allowing for heterogeneity. Parametric linkage analysis of chromosome 4 in individual pedigrees revealed positive LOD scores in an overlapping region in four families (Family 1, Family 2, Family 4, and Family 12), with Family 1 having the highest LOD score of 1.93 (72.795 to 89.616 Mb) (Fig. S1A and S2A in the Supplementary Appendix). Thus, we selected Family 1 to undergo whole-genome sequencing.

SUSCEPTIBILITY GENE IN FAMILIAL DISEASE

Whole-genome sequencing of a sample obtained from Participant II-4, one of two affected members of Family 1, generated 187.5 Gb of short reads, with an average coverage of 58× and 3,492,429 single-nucleotide variants (SNVs) or insertions or deletions. We winnowed the 3,492,429 variants down to 4 by selecting SNVs that were located in the candidate regions defined on linkage analysis in Family 1 (regions with the highest LOD score spanning approximately 80 Mb in total), that were located in exons or splice sites, that were predicted to cause amino acid changes or changes in pre-messenger RNA splicing, and that were not registered in the database of single-nucleotide polymorphisms, build 130 (dbSNP130), indicating that the variants are extremely rare in the general population (Fig. S2B in the Supplementary Appendix). Each of these 4 SNVs is predicted to result in an amino acid substitution: K707R in SHROOM3 (Universal Protein Resource [UniProt] accession number, Q8TF72), M78V and V343A in COQ2 (UniProt accession number, Q96H96), and R231G in SCEL (UniProt accession number, O95171).

In the 180 Japanese control samples, we did not observe the SNV encoding the M78V variant but did observe SNVs encoding K706R in SHROOM3, V343A in COQ2, and R231G in SCEL, which were present on 3, 5, and 98 of 360 alleles, respectively. We therefore considered the SNP encoding M78V in *COQ2*, which encodes parahydroxybenzoate-polyprenyl transferase, an enzyme involved in the biosynthesis of coenzyme Q_{10}, as a candidate variant in conferring susceptibility to familial multiple-system atrophy.

Cosegregation analysis of samples from Family 1 revealed that the two affected family members, Participants II-4 and II-8, carried the homozygous M78V-V343A variant in *COQ2*, and the unaffected sibling who was tested (Participant II-7) did not carry this variant (Fig. S2C in the Supplementary Appendix). Mutational analysis of *COQ2* in Family 12 revealed heterozygous mutations consisting of nonsense (R337X) and missense (V343A) variants in both affected siblings (Participants II-3 and II-4). Their mother (Participant I-2) was heterozygous for V343A, one unaffected sibling (Participant II-1) lacked this variant, and the other unaffected sibling (Participant II-2) was heterozygous for R337X. R337X was not observed in the 180 Japanese controls.

We did not detect variants of *COQ2* in the other four families (Families 2, 3, 4, and 8). Because *COQ2* encodes an enzyme essential for the biosynthesis of coenzyme Q_{10}, we further sequenced the other 11 genes in the biosynthetic pathway for coenzyme Q_{10} (*PDSS1*, *PDSS2*, *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *ADCK3*, *COQ9*, *COQ10A*, and *COQ10B*) in the remaining four families and in a previously described multiplex family[14] but did not observe variants that cosegregated with disease.

COQ2 VARIANTS AND SPORADIC DISEASE

To investigate the involvement of *COQ2* variants in sporadic multiple-system atrophy, we extended the mutational analysis of *COQ2* to a Japanese series consisting of 363 patients with multiple-system atrophy and 520 controls. A common *COQ2* variant (rs6818847, predicted to result in an amino acid substitution, L16V) with allele frequencies of 0.90 and 0.88 in the Japanese patients with multiple-system atrophy and controls, respectively, was not included in further analysis. Four patients with multiple-system atrophy carried two variants simultaneously (one carried an I97T and a nonmutated [NM] allele at codon 97 and V343A/NM at codon 343, one had R337Q/NM at codon 337 and V343A/NM at codon 343, and two had V343A/V343A), whereas none of the controls had two variants of *COQ2* (Table 1). Sequencing of the subcloned mutated alleles confirmed that R337Q/V343A was present in a compound heterozygous state. We were unable to determine the phase of I97T/V343A, because the distance

Table 1. *COQ2* Variants Found in Patients with Sporadic Multiple-System Atrophy in Japanese, European, and North American Series, as Compared with Controls.*

Genotype	Japanese Series		European Series		North American Series	
	Patients (N=363)	Controls (N=520)	Patients (N=223)	Controls (N=315)	Patients (N=172)	Controls (N=294)
P22L/NM	0	1	0	0	0	0
F29L/NM	0	0	1	0	0	0
P49H†/NM	0	0	0	0	1	0
S57T†/NM	0	0	1	0	0	0
R69H†/NM	0	0	0	0	0	1
I97T‡/V343A§	1	0	0	0	0	0
P107S†/NM	1	0	0	0	0	0
S113F†/NM	1	0	0	0	0	0
T267A‡/NM	0	0	1	0	0	0
S297C‡/NM	0	0	1	0	0	0
N336H/NM	0	1	0	0	0	0
R337Q†/V343A§	1	0	0	0	0	0
V343A§/NM	29	17	0	0	0	0
V343A§/V343A§	2	0	0	0	0	0

* NM denotes nonmutated.
† This variant was deemed to be severely deleterious on yeast complementation assay.
‡ This variant was deemed to be mildly deleterious on yeast complementation assay.
§ This variant had decreased COQ2 activity on enzyme assay.

Table 2. Association between the *COQ2* V343A Variant and Sporadic Multiple-System Atrophy in the Japanese Series.*

V343A Variant†	Patients with Multiple-System Atrophy				Tier 2 Controls (N=2383)	Comparison with Tier 2		Patients with Other Neurologic Diseases		
	Patients (N=363)	Tier 1 Controls (N=520)	Comparison with Tier 1					Alzheimer's Disease (N=2728)	Parkinson's Disease (N=659)	ALS (N=634)
			odds ratio (95% CI)	*P value*		*odds ratio (95% CI)*	*P value*			
Allele frequency — no./total no. (%)	35/726 (4.8)	17/1040 (1.6)	3.05 (1.65–5.85)	1.5×10^{-4}	106/4766 (2.2)	2.23 (1.46–3.32)	6.0×10^{-5}	109/5456 (2.0)	33/1318 (2.5)	31/1268 (2.4)
Heterozygous — no.	31	17			106			105	33	31
Homozygous — no.	2	0			0			2	0	0

* Odds ratios and P values are for the comparisons between patients with multiple-system atrophy and each of the two groups of controls (tier 1 and tier 2). ALS denotes amyotrophic lateral sclerosis, and CI confidence interval.

† In the combined series of Japanese, European, and North American participants, functionally deleterious variants P49H, S57T, R69H, I97T, P107S, S113F, T267A, S297C, and R337Q (as determined on yeast complementation assay) were found in 8 of 1516 alleles (0.53%) in patients with multiple-system atrophy, as compared with 1 of 2258 alleles (0.05%) in controls (odds ratio, 11.97; 95% CI, 1.60 to 531.5; P=0.004).

between I97T and V343A was too large to be amplified by means of polymerase-chain-reaction (PCR) assay in a single fragment, and samples of genomic DNA from the parents were unavailable. We found that 29 patients with multiple-system atrophy and 17 controls were heterozygous for the V343A variant. In addition, we detected four novel heterozygous variants: two in patients with multiple-system atrophy (P107S and S113F) and two in controls (P22L and N336H).

Of the *COQ2* variants, the V343A variant is relatively common in the Japanese population. As shown in Table 2, we found that the V343A allele occurred in 35 of 726 alleles (4.8%) from Japanese patients with multiple-system atrophy and in 17 of 1040 alleles (1.6%) from Japanese controls (odds ratio for patients with multiple-system atrophy, 3.05; 95% confidence interval [CI], 1.65 to 5.85; $P=1.5\times10^{-4}$). Genotyping in the second series of 2383 Japanese controls showed that the V343A variant had an allele frequency of 2.2% (106 of 4766 alleles; odds ratio, 2.23; 95% CI, 1.46 to 3.32; $P=6.0\times10^{-5}$). Genotyping Japanese persons with other neurodegenerative diseases revealed that the V343A allele frequencies were 2.0% (109 of 5456 alleles) among patients with Alzheimer's disease, 2.5% (33 of 1318 alleles) among those with Parkinson's disease, and 2.4% (31 of 1268 alleles) among those with ALS. These allele frequencies did not differ significantly from those in the first or second set of controls, confirming the specificity of the V343A variant in patients with multiple-system atrophy. Two patients with Alzheimer's disease who were found to carry homozygous V343A mutations did not show any signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction.

We then performed genotyping in the European and North American series of patients with multiple-system atrophy. In the European series, we found four singleton *COQ2* variants (encoding amino acid substitutions F29L, S57T, T267A, and S297C) among the patients, whereas none of the controls had any variants in *COQ2*. In the North American series, we found one variant (P49H) in a patient with multiple-system atrophy and one variant (R69H) in a control (Table 1). At the time of recruitment for the study, the carrier of R69H, who was 60 years old, had no signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction, but this participant was unavailable for follow-up assessment. Intriguingly, the V343A

variant, a relatively common variant in the Japanese population, was not observed in patients with multiple-system atrophy or controls in either the European or the North American series.

FUNCTIONAL ANALYSIS OF MUTANT *COQ2*

To determine the functional effect of each variant on the mitochondrial aerobic energy production in which coenzyme Q_{10} plays an essential

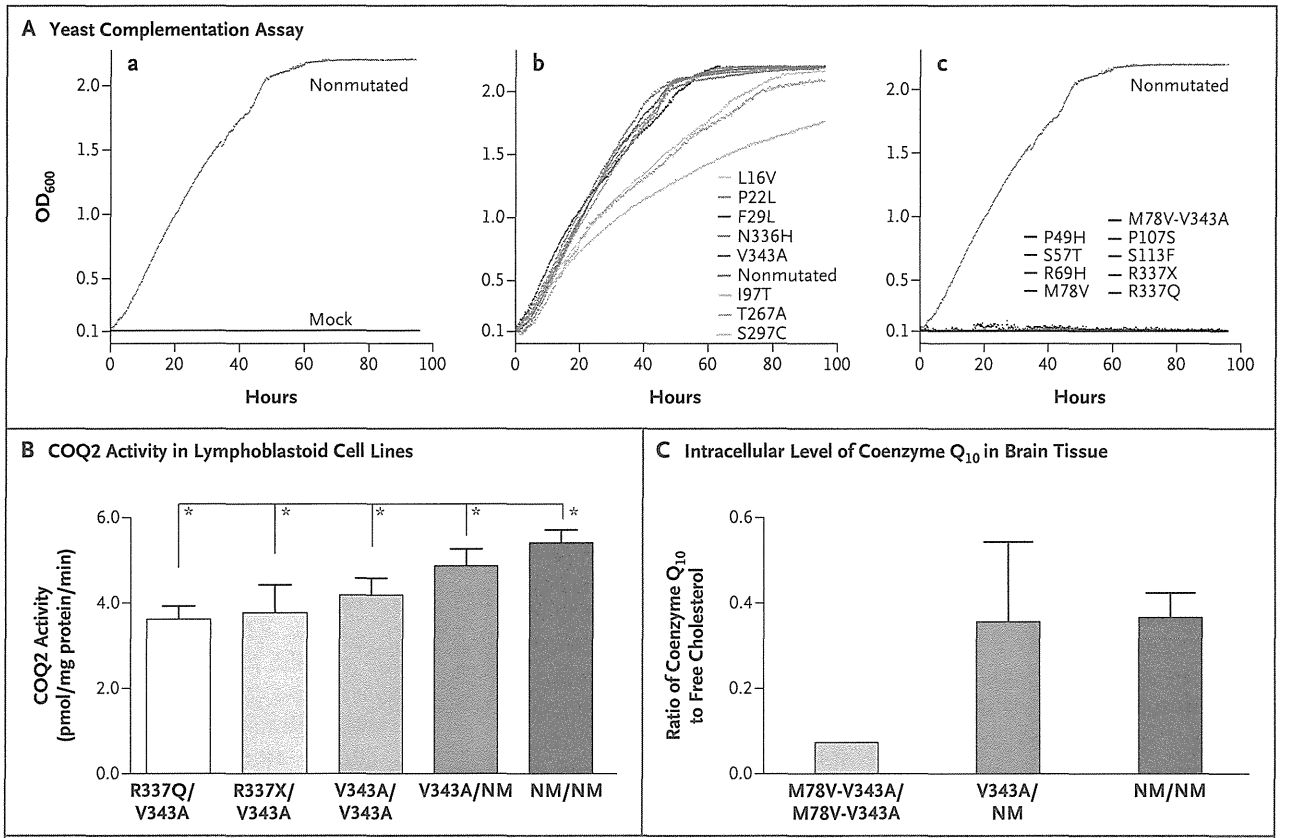

Figure 2. Functional Analysis of *COQ2* Variants.

Panel A shows the results of a yeast complementation assay, including the growth curves of the yeast *coq2*-null mutants transformed with the pAUR123 vector containing the nonmutated (NM) human *COQ2* complementary DNA (cDNA) or mock vector (subpanel a) and those transformed with the AUR123 vector containing different versions of human *COQ2* cDNA (encoding the substitutions L16V, P22L, F29L, N336H, V343A, I97T, T257A, and S297C) (subpanel b). Transformants carrying a *COQ2* allele encoding the I97T, T267A, or S297C substitution show considerably lower growth rates than nonmutated *COQ2*, which still shows a higher growth rate than the *coq2*-null strain (mildly deleterious). Transformants carrying a *COQ2* allele encoding the L16V, P22L, F29L, N336H, or V343A substitution show growth rates similar to that of nonmutated *COQ2*. The *coq2*-null mutants transformed with the pAUR123 vector containing different versions of human *COQ2* cDNA (P49H, S57T, R69H, M78V, M78V-V343A, P107S, S113F, R337X, or R337Q) show severely decreased respiration-dependent growth rates, as does the *coq2*-null strain (severely deleterious) (subpanel c). Each yeast strain was pregrown in yeast extract peptone dextrose (YPD) medium, diluted to an optical density of a sample measured at a wavelength of 600 nm (OD_{600}) of 0.1, and then incubated in yeast extract peptone glycerol (YPG) medium at 23°C with shaking at a rate of 200 rotations per minute for 4 days. OD_{600} was measured every 10 minutes. The experiments were conducted in quadruplicate, and the mean OD_{600} was plotted against the incubation time. Panel B shows COQ2 activity in lymphoblastoid cell lines, as determined by measuring the incorporation of radioactive parahydroxybenzoate (PHB) into decaprenyl-PHB. COQ2 activity was measured in lymphoblastoid cell lines from each patient with multiple-system atrophy carrying *COQ2* variants (encoding R337Q/V343A, R337X/V343A, V343A/V343A, or V343A/NM) and from a control without variants. The mean values from nine independent experiments are shown. T bars indicate standard deviations. Group comparisons were performed with the use of the Kruskal–Wallis test, followed by the Steel test. Asterisks indicate $P<0.05$ for the comparison with a control sample (nonmutated *COQ2* genotype). Panel C shows intracellular levels of coenzyme Q_{10} in frozen cerebellum samples obtained from three patients with multiple-system atrophy (one who was homozygous for M78V-V343A and two who were heterozygous for V343A) and from three controls. Brain-tissue samples weighing approximately 100 mg were homogenized in 10 volumes (volume to weight) of 10 mM TRIS–hydrochloric acid (with a pH of 7.4) containing 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20% sodium dodecyl sulfate (SDS). Coenzyme Q_{10} was then extracted with hexane and ethanol (in a 5:2 ratio according to volume), and the extract was subjected to high-performance liquid chromatography to measure the level of coenzyme Q_{10}, after adjustment for the free (unesterified) cholesterol level.[21]

role in the electron transfer, we carried out functional complementation analysis by transforming the yeast *coq2*-null strain with nonmutated or mutated human *COQ2* cDNA (Fig. 2A). Transformants of the BY4741 Δ*coq2* yeast strain with the mutated *COQ2*, including transformants separately carrying the P49H, S57T, R69H, M78V, M78V-V343A, P107S, S113F, R337Q, and R337X alleles, showed severely decreased growth rates, similar to those observed in the *coq2*-null strain. In addition, transformants with mutated *COQ2*, including those with the variants encoding the I97T, T267A, and S297C substitutions, showed substantially lower growth rates than those expressing nonmutated *COQ2*, which had a higher growth rate than the *coq2*-null strain (mildly deleterious). The transformants with mutated *COQ2*, including transformants separately carrying the L16V, P22L, F29L, N336H, and V343A alleles, showed growth rates similar to those of the transformants expressing nonmutated *COQ2*. As described above, the yeast strain with M78V-V343A identified in Family 1 showed a severely decreased growth rate, whereas the strain with V343A had a growth rate similar to that of nonmutated *COQ2*, indicating that of the two variants, M78V primarily contributed to the impairment in COQ2 function.

Focusing on the rare variants that were identified in the case–control series (Table 1), we found that nine variants (P49H, S57T, R69H, I97T, P107S, S113F, T267A, S297C, and R337Q) were mildly or severely deleterious. On combining all three series, eight variants (P49H, S57T, I97T, P107S, S113F, T267A, S297C, and R337Q) were identified in 758 patients with multiple-system atrophy, whereas only one variant (R69H) was found in 1129 controls (odds ratio, 11.97; 95% CI, 1.60 to 531.52; P=0.004) (Table 2 footnote). Yeast complementation analysis showed that the F29L variant, identified in a European patient with multiple-system atrophy, did not impair the growth rate. Lymphoblastoid cell lines from this patient were unavailable for further measurement of the activity of mutant COQ2, thus making it difficult to interpret the pathogenicity of this variant.

COQ2 ACTIVITIES IN LYMPHOBLASTOID CELL LINES

We measured COQ2 activities in lymphoblastoid cell lines from patients carrying *COQ2* mutations, when available. We focused on the V343A variant because it is commonly associated with multiple-system atrophy and showed an apparently normal growth rate in the yeast complementation assay. We determined COQ2 activities in lymphoblastoid cell lines with *COQ2* variants R337Q/V343A, R337X/V343A, V343A/V343A, or V343A/NM and in a control without variants. The COQ2 activities in the lymphoblastoid cell lines (V343A/NM) obtained from patients with multiple-system atrophy were significantly lower than those in the control cell lines. The COQ2 activities in the cell lines from patients with multiple-system atrophy carrying two mutated *COQ2* alleles were further decreased (Fig. 2B).

CORRELATIONS BETWEEN GENOTYPE AND PHENOTYPE

The clinical features of patients with sporadic multiple-system atrophy carrying deleterious *COQ2* variants (as determined on yeast complementation assay and COQ2-activity measurement) and those of noncarriers are summarized in Table S5 in the Supplementary Appendix. The mean age at the onset of multiple-system atrophy among carriers was older than that among noncarriers (P=0.002). Among carriers, 34 had subtype C and 5 had subtype P. Among noncarriers, 468 had subtype C and 209 had subtype P. The subtype was unclassified in 42 noncarriers. The ratio of the number of patients with subtype C to the number with subtype P was significantly higher among carriers of *COQ2* variants than among noncarriers (P=0.02).

INTRACELLULAR COENZYME Q_{10} IN LYMPHOBLASTOID CELL LINES

We measured intracellular coenzyme Q_{10} levels in lymphoblastoid cell lines from patients with multiple-system atrophy and controls. The participants were grouped as follows: 3 patients with multiple-system atrophy carrying two variants (R337Q/V343A, R337X/V343A, and V343A/V343A), 16 patients carrying heterozygous V343A, 133 patients without variants, and 76 controls without *COQ2* variants (Table 3). Intracellular levels of coenzyme Q_{10} in lymphoblastoid cell lines from patients with multiple-system atrophy who carried two variant alleles were substantially lower than levels in cell lines from controls without variants. Intracellular coenzyme Q_{10} levels in patients who were heterozygous for V343A and in those without *COQ2* variants were not significantly lower than levels in controls without *COQ2* variants.

Table 3. Intracellular Levels of Coenzyme Q_{10} in Lymphoblastoid Cell Lines, According to *COQ2* Variant.*

Variable	Patients with Multiple-System Atrophy					Controls
	R337Q/V343A	R337X/V343A	V343A/V343A	V343A/NM	NM/NM	NM/NM
No. of participants with variant	1	1	1	16	133	76
Ratio of coenzyme Q_{10} to free (unesterified) cholesterol†	2.19	2.58	1.86	3.38±0.53	3.41±0.74	3.48±0.75
Coenzyme Q_{10} level as a percentage of mean value in controls — %‡	62.9	74.1	53.4	97.1	98.0	100.0

* Plus–minus values are means ±SD. NM denotes nonmutated.

† The ratio of coenzyme Q_{10} to free (unesterified) cholesterol reflects the intracellular level of coenzyme Q_{10}. Lower values indicate decreased levels of intracellular coenzyme Q_{10}, presumably reflecting decreased biosynthesis of coenzyme Q_{10}. To calculate the ratio, coenzyme Q_{10} was measured in nanomoles per liter and free cholesterol in micromoles per liter.

‡ Lower values indicate decreased levels of intracellular coenzyme Q_{10}, as compared with the mean value in controls, presumably reflecting decreased biosynthesis of coenzyme Q_{10}.

COENZYME Q_{10} IN BRAIN TISSUE

Only a limited number of brain-tissue samples from patients with multiple-system atrophy carrying *COQ2* variants were available. Nevertheless, we measured coenzyme Q_{10} in frozen brain tissues from three patients with *COQ2* variants (one patient who was homozygous for M78V-V343A and two patients with V343A/NM) and from three controls without *COQ2* variants (Fig. 2C). The levels of coenzyme Q_{10} in patients who were homozygous for M78V-V343A were substantially lower than the levels in controls.

DISCUSSION

We identified homozygous or compound heterozygous *COQ2* mutations in two of the six multiplex families with multiple-system atrophy, a finding that suggests a role of these mutations in the pathogenesis of familial disease. We further found that functionally impaired variants in *COQ2* were associated with an increased risk of sporadic disease. In familial cases of multiple-system atrophy, linkage analysis strongly indicated locus heterogeneity in these families, and the identification of the causal variants in the remaining four families will require analyses such as whole-genome sequencing.

We found that a common variant (V343A) and multiple rare variants in *COQ2* were associated with sporadic multiple-system atrophy. The V343A variant was found exclusively in the Japanese participants, with an allele frequency of 1.6 to 2.2%. The allele frequency of V343A in patients with multiple-system atrophy (4.8%) was significantly higher than that in controls (1.6 to 2.2%) with odds ratios of 2.23 to 3.05. The modest risk of multiple-system atrophy that was associated with the common variant V343A suggests that V343A is a susceptibility factor rather than a causal factor for this disease. The odds ratio for the presence of deleterious rare variants was 11.97, which is much larger than that for V343A. Nonetheless, we should consider that these heterozygous variants in *COQ2* are not necessarily causal but rather confer a strong susceptibility to sporadic multiple-system atrophy. Members of Family 1 and Family 12 who carried deleterious variants in the heterozygous state did not have clinical signs of multiple-system atrophy.

The ratio of patients with subtype C multiple-system atrophy to those with subtype P was higher among carriers of deleterious *COQ2* variants than among noncarriers, which suggests that the cerebellum is more vulnerable to compromised COQ2 function than other regions of the central nervous system. Of the *COQ2* variants that we detected, the V343A variant was the most prevalent and was exclusively found in Japanese participants. These findings may in part explain the clinical observations that subtype C is more prevalent than subtype P in the Japanese population[9] but not in the European population[11] or the North American population.[12] However, there were only 35 carriers of deleterious *COQ2* variants among 363 patients with multiple-system atrophy in the Japanese case series. In addition, the clinical presentations of the two patients with familial

disease who had the highest mutational load were different: subtype P in the patients in Family 1 and subtype C in the patients in Family 12. Thus, the genotypes of *COQ2* do not fully explain the clinical phenotypes.

Previous studies have shown evidence of mitochondrial respiratory-chain dysfunction or oxidative injury in patients with multiple-system atrophy.[22-24] The combination of oxidative stress and overexpression of oligodendroglial α-synuclein has been reported to replicate the characteristics of this disease.[25-28] Our findings suggest that impaired COQ2 activity, which would be predicted to impair the mitochondrial respiratory chain and increase vulnerability to oxidative stress, causes susceptibility to multiple-system atrophy. A primary deficiency of coenzyme Q_{10} that is caused by *COQ2* mutations has been described as an infantile-onset multisystem disorder and a nephropathy in several families.[29,30] The clinical presentation of these affected family members, however, differed markedly from the presentations of patients with multiple-system atrophy, perhaps because the decrease in COQ2 activity associated with the mutations in patients with multiple-system atrophy appears to be milder than that observed in patients with a primary deficiency of coenzyme Q_{10}.

Previous approaches to identifying susceptibility genes have used genomewide association studies or candidate-gene approaches.[31-33] Our identification of rare *COQ2* variants was accomplished by starting with multiplex families and then extending the analysis to patients with sporadic multiple-system atrophy, reflecting an alternative approach to the elucidation of genetic variants with strong effect sizes in an apparently nongenetic disorder.[34]

From the therapeutic viewpoint, oral supplementation with coenzyme Q_{10} may be helpful in treating multiple-system atrophy, particularly for patients with susceptibility-conferring *COQ2* variants. The safety and side-effect profile of high-dose supplementation with coenzyme Q_{10} have been well established.[35,36]

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APPENDIX

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