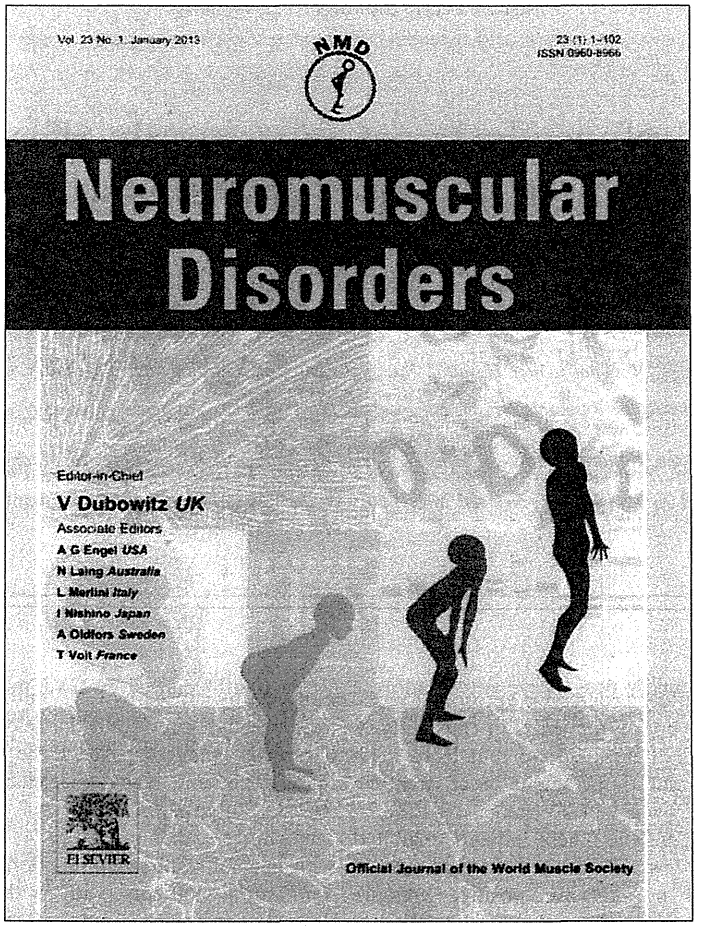

myopathy, then, physicians should punctually monitor their respiratory function with pulmonary function tests to look for early signs of respiratory dysfunction, perform respiratory training, coup with airway infection using a mechanical in-exsufflator (MI-E), and induce mechanical ventilation if required, as they do for patients with neuromuscular disease who exhibit respiratory failure.

The aim of this study is to evaluate past and present clinical respiratory function test parameters of GNE myopathy patients, and analyze factors that correlate with disease severity.

## 2. Patients and methods

### 2.1. Study population

Medical records of all genetically confirmed GNE myopathy patients who underwent pulmonary function tests at the National Center Hospital, National Center of Neurology and Psychiatry, were retrospectively reviewed. We collected data on genetic diagnosis, respiratory function (% vital capacity [%VC], % force vital capacity [FVC], cough peak flow [CPF]), creatine kinase (CK), chest X-ray and/or CT scan and body mass index (BMI) for analysis.

### 2.2. Data handling and analysis

Data were summarized using descriptive statistics, and each variable was compared against age, sex, respiratory dysfunction (whether their %FVC was up to or over 80%), and domain mutation (i.e., within the UDP-GlcNAc 2-epimerase domain: ED or *N*-acetylmannosamine kinase domain: KD). The *t*-test was used to compare the means of each group. Data for the two study populations were calculated using chi-square contingency table analysis. Multivariate regression analysis was performed with %FVC as the dependent variable. Explanatory variables included age at disease onset, CK and BMI. We found that the variables age, duration from onset to present, age upon wheelchair use, age at loss of ambulation, were highly correlated (over 0.5) with age at disease onset. As such, we eliminated these three due to multicolinearity in the multivariate regression analysis. When past %FVC data were available, the present data were compared with serial changes in respiratory function during the preceding 5–7 years, and changes in %FVC over time were determined by calculating the difference between past and present data. All analyses were performed using SPSS for Macintosh (Version 18; SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. General characteristics

A total of 39 Japanese patients (13 men, 26 women) were recruited. The mean age at the time of data collection was 43.1 (11.3) years (mean [standard deviation, SD]) (Table 1). The mean age at first appearance of symptoms was 26.8 (9.0) years (range, 15–58 years; median, 25 years). Present age, age at disease onset, age at wheelchair use, and present ambulation status were not significantly different between men and women; 20.5% (8/39) had symptom onset before age 20. Of the 39 patients, 51.3% (20/39) could walk but needed assistance, and 69.2% (27/39) were wheelchair-bound (8/27 and 19/27 were partially and totally wheelchair-bound, respectively). Age at first use of a wheelchair was 33.3 (10.8) years (range, 18–59 years; median, 31.5 years) and that for loss of ambulation was 36.9 (11.9) years (Table 1).

### 3.2. GNE mutations

Of the 39 patients, 30.7% (12/39) carried homozygous mutations, while 69.2% (27/39) harbored compound heterozygous mutations (Supplementary Table 1). Among the homozygous patients, 66.7% (8/12) harbored the p.V572L mutation. Among the compound heterozygous patients, 25.9% (7/27) exhibited the p.D176V/p.V572L genotype, while the other patients each had a different mutation. With respect to the location of the mutation (i.e., protein domain), 28.2% (11/39) homozygous patients carried mutations only in ED (ED/ED), 46.2% patients (18/39) were compound heterozygotes with 1 mutation each in the ED and KD (ED/KD), and 25.6% patients (10/39) had a mutation in the KD of both genes (KD/KD) (Table 2). The allelic frequencies of p.V572L, p.D165V, p.C13S, and p.R129Q were 35.9% (28/78), 28.2% (22/78), 11.5% (9/78), and 2.6% (2/78), respectively, while all other mutations had only 1 allele each (Supplementary Table 1).

### 3.3. Respiratory function

None of the patients had lung and/or thoracic diseases that could affect their respiratory function in chest X-ray and/or chest computed tomography. The %VC and %FVC in patients with GNE myopathy were 91.9 (26.9) (range, 18.2–126.3; median, 100.3) and 92.0 (25.8) (range, 16.4–128.5; median, 100.5; Table 1), respectively.

### 3.4. Patients with respiratory dysfunction

In 30.7% of patients (12/39), %FVC was <80. Of these 12 patients, 91.6% (11/12) were wheelchair-dependent and 83.3% (10/12) had already lost ambulation. Their onset was significantly earlier (19.3 [4.4] vs. 30.3 [8.4], $p < 0.001$) and mean CK level was significantly lower (55.8 [71.6] vs. 279.0 [184.7], $p = 0.004$) than those of patients with normal respiratory function. Four patients exhibited advanced respiratory dysfunction (%FVC < 50% and cough peak flow [CPF] < 160 L/min) (Table 2). All 4 patients had experienced recurrent pneumonia, and 2 patients required nocturnal NPPV. They were all early onset (before 20 years old) and non-ambu-

Table 1
Patient characteristics by respiratory function.

| | Total | %FVC ⩾ 80% | %FVC < 80% | |
|---|---|---|---|---|
| *n* | *39* | 27 | 12 | *p* |
| Age (years) | 43.0 ± 11.3 | 44.3 ± 11.7 | 39.9 ± 10.3 | 0.267 |
| Age at onset (years) | 26.8 ± 9.0 | 30.2 ± 8.4 | 19.2 ± 4.4 | <0.001 |
| GNE/GNE | 10 (25.6%) | 7 (70.0%) | 3 (30.0%) | 0.640 |
| GNE/MNK | 18 (46.2%) | 16 (88.9%) | 2 (11.1%) | 0.018 |
| MNK/MNK | 11 (28.2%) | 4 (36.4%) | 7 (63.6%) | 0.009 |
| Duration from onset of disease to present | 16.2 ± 8.4 | 14.1 ± 7.8 | 20.8 ± 8.2 | 0.021 |
| Wheelchair use (%) | 27 (69.2%) | 16 (59.3%) | 11 (40.7%) | 0.141 |
| Wheelchair use since (years) | 33.3 ± 10.8 | 37.9 ± 11.3 | 26.6 ± 5.1 | 0.002 |
| Lost ambulation | 19 (48.7%) | 8 (42.1%) | 11 (57.9%) | 0.014 |
| Age at lost ambulation (years) | 36.9 ± 11.9 | 41.2 ± 11.7 | 28.2 ± 6.4 | 0.018 |
| CK (IU/L) | 201.3 ± 187.5 | 279.0 ± 184.7 | 55.8 ± 71.6 | 0.004 |
| BMI | 21.1 ± 4.2 | 20.8 ± 3.2 | 21.9 ± 5.8 | 0.457 |
| FVC (%) | 91.9 ± 26.9 | 106.9 ± 12.5 | 58.2 ± 18.7 | <0.001 |
| VC (%) | 92.0 ± 25.8 | 106.4 ± 11.6 | 59.5 ± 17.6 | <0.001 |
| CPF (L/min) | 334.2 ± 139.5 | 378.0 ± 105.7 | 250.2 ± 161.5 | 0.008 |

Most patients with reduced respiratory function had already lost ambulation and were entirely wheelchair-dependent. Their onset was significantly earlier and CK levels significantly lower than those of patients with normal respiratory function. FVC: forced vital capacity, VC: vital capacity, CPF: cough peak flow, BMI: body mass index, CK: creatine kinase.

Table 2
Patients with FVC < 50% and CPF < 160 L/min.

| Case | Age | Sex | Mutation | Mutant domain | Ambulation status | Disease onset | Disease duration | Age at lost ambulation | %VC | %FVC | CPF (L/min) | Reccurent pneumonea | NPPV | CK (IU/L) | BMI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 51 | Man | p.C13S homozygote | ED/ED | Non-ambulant | 17 | 34 | 25 | 18.2 | 16.4 | 48.0 | Yes | Nocturnal | 13 | 18.6 |
| 2 | 42 | Woman | p.V572L homozygote | KD/KD | Non-ambulant | 16 | 26 | 23 | 37.6 | 34.4 | 141.6 | Yes | Nocturnal | 13 | 22.2 |
| 3 | 45 | Woman | p.V572L homozygote | KD/KD | Non-ambulant | 17 | 28 | 31 | 49.0 | 48.3 | 147.6 | Yes | No | 8 | 31.6 |
| 4 | 37 | Woman | p.V572L homozygote | KD/KD | Non-ambulant | 16 | 21 | 24 | 53.7 | 48.6 | 118.8 | Yes | No | No data | 20.4 |

Table 3
Multivariate regression analysis of predictive factors for respiratory dysfunction.

| | Regression coefficient | *p* | Lower limit of 95% confidence interval | Upper limit of 95% CI |
|---|---|---|---|---|
| Age at onset | 0.949 | 0.042 | 0.038 | 1.86 |
| CK | 0.068 | 0.008 | 0.02 | 0.115 |
| BMI | −1.8 | 0.09 | −3.811 | 0.302 |

Multivariate linear regression analysis was performed to evaluate the relationship between %FVC and other clinical parameters. Age at onset and CK were significantly correlated with %FVC.

lant. The majority (7/12) of patients had KD/KD mutations, whereas significantly fewer patients with respiratory dysfunction had ED/KD mutations.

In order to identify predictive factors for respiratory dysfunction in GNE myopathy, we performed multivariate analysis to determine the relationship with %FVC. This revealed age at onset ($p = 0.042$) and CK ($p = 0.008$) as significantly correlated to %FVC (Table 3, Fig. 1).

Past (5–7 years ago) data were available for 9 patients. The %FVC decrements in 5 patients with respiratory dysfunction were significantly greater than those of patients without dysfunction (20.9 [6.0] vs. 0.8 [9.7], $p = 0.004$; Supplementary Table 2).

## 4. Discussion

To our knowledge, we are the first to report respiratory dysfunction in GNE myopathy. Our study demonstrates that (1) certain GNE myopathy patients in Japan exhibit respiratory dysfunction, and (2) early onset and lower CK levels resulting from severe muscle atrophy and weakness, and KD/KD mutations can be risk factors for respiratory dysfunction.

Malicdan et al. reported that pathological changes in the diaphragms of the GNE (−/−) hGNED176V-Tg model mice were variable and ranged from almost normal to the presence of marked fibrosis and rimmed vacuoles. On the other hand, the gastrocnemius muscles of all mice exhibited myopathic features [5]. The features in these mice correspond to individual differences observed in the patients of our study. The fact that not all cases in our study exhibited respiratory dysfunction as observed in the GNE (−/−)

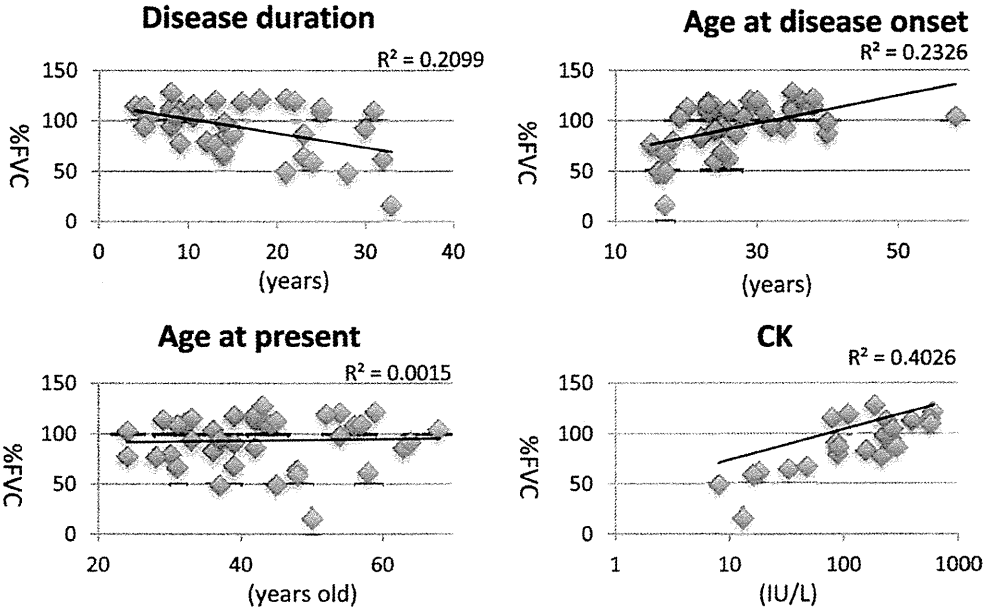


Fig. 1. Scatterplots of %FVC as functions of age, age at disease onset, disease duration, and creatine kinase (CK) level. Age at disease onset, disease duration, and CK level were correlated with %FVC.

hGNED176V-Tg mice indicates that severe respiratory muscle involvement is not a constant feature of GNE myopathy. Yet, since about 30% of patients had decreased %FVC and severe respiratory dysfunction was overlooked by neurologists or physicians, clinicians should be made more aware of the possibility of respiratory dysfunction, particularly in patients with advanced GNE myopathy. If %VC decreases to 70%, patients should be taught air stacking as with other neuromuscular disorders [4,6]. CPF should be routinely measured in patients with GNE myopathy, given that its decrement was associated with recurrent pneumonia in our study. Early induction of assisted CPF and/or MI-E is required if patients with reduced CPF have an airway infection. Serial data suggest that %FVC decreased from the normal range to %FVC < 80, indicating that continuous monitoring is required even in patients with normal respiratory function. Moreover, respiratory function parameters may provide quantitatively useful data for clinical trials, particularly those directed to non-ambulant patients.

All 4 patients with severe respiratory dysfunction exhibited early onset, homozygous mutations, and advanced muscle weakness. However, not all early onset, homozygous, or non-ambulant patients exhibited severe respiratory dysfunction. Although the underlying reasons are unclear, we also found that ED/KD mutations were less associated with decreased respiratory function, while many patients with KD/KD mutations showed respiratory dysfunction. A large scale, cross-sectional study could better identify key factors responsible for respiratory dysfunction and genotype-phenotype correlations.

We are aware that the recruitment of patients from NCNP, highly specialized for muscle disease, is a potential source of selection bias, because they may be particularly more severely affected than the general patient population. Therefore, our study may not correctly reflect the general patient population. Investigations of small populations may underestimate the statistical significance as well. However, our previous GNE myopathy questionnaire study revealed a similar correlation between genotypes and phenotypes [7]. We are currently in the process of establishing a Japanese national GNE myopathy patient registry called Registration of Muscular Dystrophy (REMUDY, http://www.remudy.jp) to perform a broader epidemic investigation of associated conditions, including respiratory dysfunction. To clarify the relationship between respiratory dysfunction and other clinical/laboratory factors, we have initiated a prospective observational study on GNE myopathy.

Three of 4 patients with severe respiratory dysfunction had homozygous p.V572L mutations. Given the frequency of the p.V572L mutation in the Japanese population, it will be interesting to determine whether non-Japanese individuals harboring this mutation also exhibit respiratory dysfunction.

In conclusion, advanced GNE myopathy patients are at risk for respiratory dysfunction. The KD/KD genotype, early onset, loss of ambulation/wheelchair use, and low CK level resulted in advanced muscle atrophy may be associated with respiratory dysfunction.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2012.09.007.

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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.)


The members of the Multiple-System Atrophy Research Collaboration are listed in the Appendix. Address reprint requests to Dr. Shoji Tsuji, Department of Neurology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, or at tsuji@m.u-tokyo.ac.jp.

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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]).

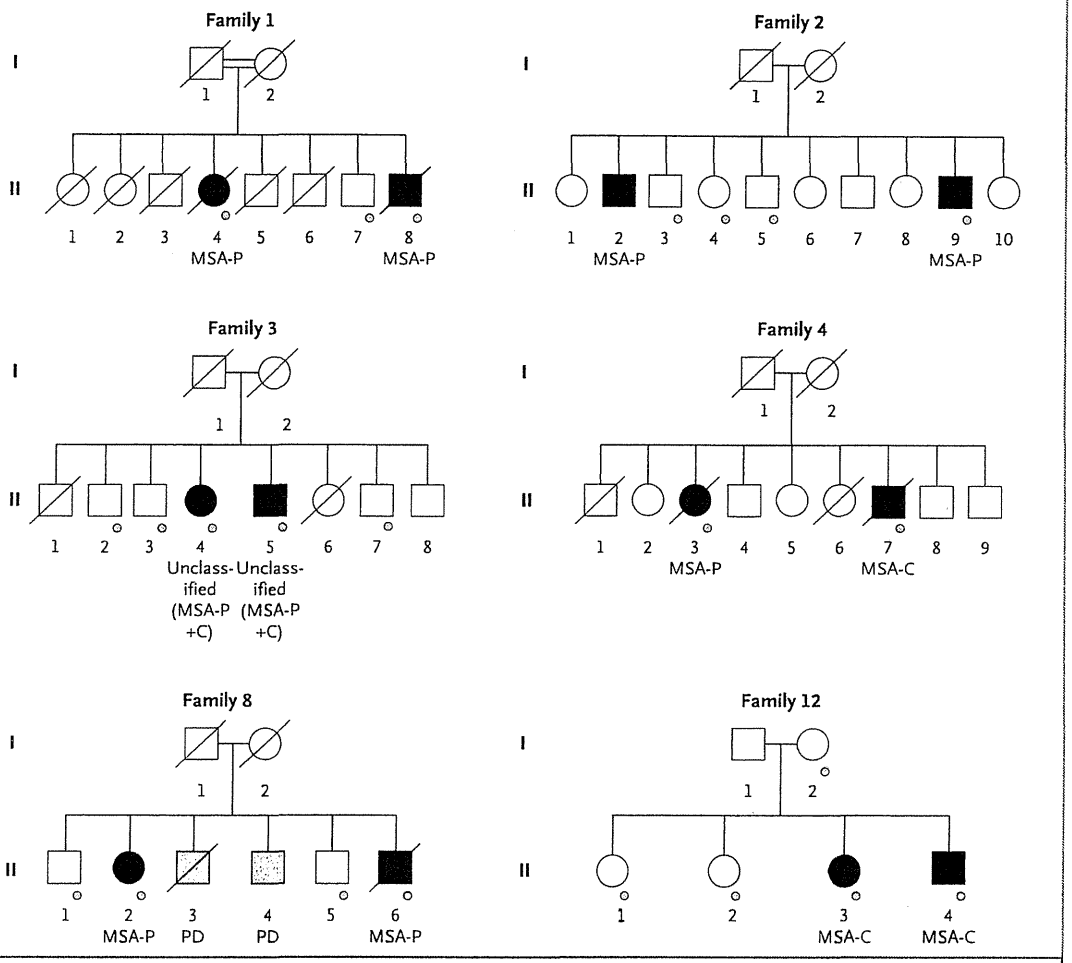


Figure 1. **Pedigrees of Six Multiplex Families with Multiple-System Atrophy.**

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 | | | | | | Patients with Other Neurologic Diseases | | |
|---|---|---|---|---|---|---|---|---|---|
| | Patients (N=363) | Tier 1 Controls (N=520) | Comparison with Tier 1 | | Tier 2 Controls (N=2383) | Comparison with Tier 2 | 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) | $1.5\times10^{-4}$ | 106/4766 (2.2) | 2.23 (1.46–3.32) | $6.0\times10^{-5}$ | 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

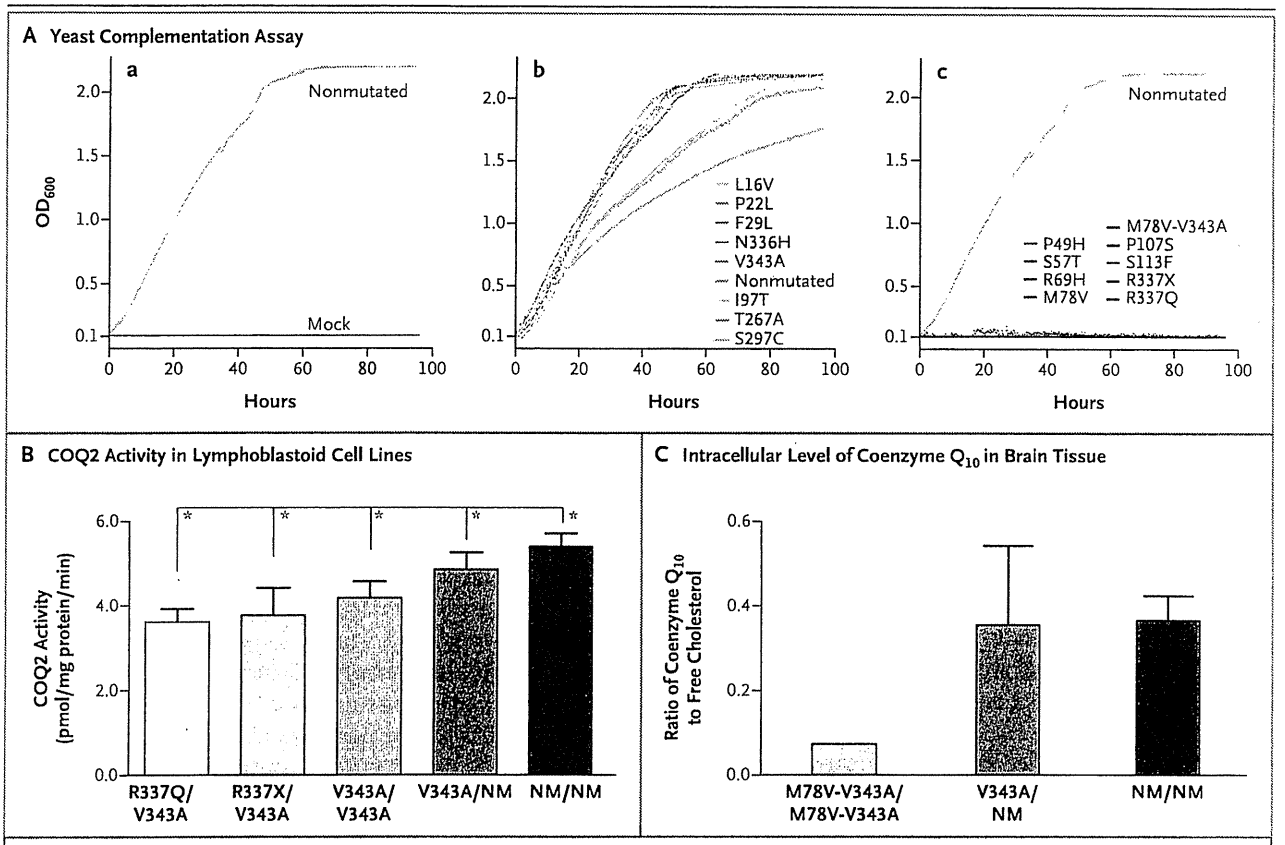


Figure 2. **Functional Analysis of *COQ2* Variants.**

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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Case Report

# Novel neuronal cytoplasmic inclusions in a patient carrying SCA8 expansion mutation

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**It has been reported that abnormal processing of pre-mRNA is caused by abnormal triplet expansion. Non-coding triplet expansions produce toxic RNA to alter RNA splicing activities. However, there has been no report on the globular RNA aggregation in neuronal cytoplasmic inclusions (NCIs) up to now. We herein report on an autopsy case (genetically determined as spinocerebellar atrophy 8 (SCA8)) with hitherto undescribed NCIs throughout the brain. NCIs were chiefly composed of small granular particles, virtually identical to ribosomes. Neurological features are comparable to the widespread lesions of the brain, including the spinal cord. Although 1C2-positivity of NCIs might be induced by reverse transcription of the CTG expansion, it remains to be clarified how abnormal aggregations of ribosome and extensive brain degeneration are related to the reverse or forward transcripts of the expanded repeat.**

**Key words:** neuronal cytoplasmic inclusion, ribosomal aggregation, SCA8, TDP43, ultrastructure.

## INTRODUCTION

We report herein on a neuronal cytoplasmic inclusion mainly composed of ribosomal aggregations (rNCIs: ribosomal neuronal cytoplasmic inclusion), in a peculiar autopsy case carrying CTA/CTG repeat expansion in the spinocerebellar atrophy 8 (SCA8) mutation. This male patient developed psychomotor retardation in early childhood. Later, he developed cerebellar ataxia and epilepsy at school age, and finally fell into akinetic mutism at the age of 23 until he died at the age of 32. On microscopic examination, there was marked neuronal loss and gliosis and white matter degeneration in the whole brain. Peculiar hitherto undescribed rNCIs were ubiquitously observed in the brain. They were basophilic on HE stain, argyrophilic on Bodian silver impregnation, positive for ubiquitin (Ub), P62 and faintly transactivation response (TAR) DNA-binding protein 43 (TDP-43), but negative for alpha-synuclein (Syn) and phosphorylated tau (AT8). Ultrastructurally, they were composed of ribosomal aggregations devoid of filamentous structures. The absence of rough endoplasmic reticula (RER) suggests that ribosomal dysfunction may play some role on formation of this novel inclusion. Regarding the pathogenesis of the current case, the abnormal gene mutation compatible with that of SCA8 mutation might modify the disease process.

The early onset of the cerebral and cerebellar symptoms and diffuse brain devastation best characterize this case, being somewhat distinct from that of common SCA8 cases that present adult onset and restricted involvement of the cerebellum.

## CASE REPORT

The patient was a 32-year-old Japanese man. Parental consanguinity was denied and the family history was noncontributory. In spite of his motor and mental retardation in early childhood, he was ambulant and communicated verbally during childhood. Later, he developed cerebellar ataxia and epilepsy at school age when his motor and mental disability rapidly progressed. Neurological examination at the age of 11 on the initial visit to a general hospital identified mental disability, cerebellar ataxia, muscle atrophy and weakness of four extremities. Electroencephalography (EEG) showed spike waves on bilateral temporal lobes. Needle electromyography showed positive

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sharp waves and fibrillation potentials in the four extremities. Head CT scan demonstrated mild cerebellar atrophy.

Artificial ventilation was started at the age of 15 because of respiratory muscle weakness. His motor and mental disabilities slowly progressed. He fell into akinetic mutism at the age of 23. Head MRI demonstrated progressive atrophy of the whole brain. At the age of 31, there were neither responses to any external stimuli nor voluntary movements, including vocalization. Light, corneal, gag, cough and deep tendon reflexes were all lost. There was no electrical activity on EEG. He died of septic shock secondary to cholecystitis at the age of 32.

Serum creatine kinase, lactic acid and pyruvic acid were within normal limits. Other peripheral hematology and blood chemistry were within normal limits. Lysosomal enzymes examined were all in normal ranges. Genetic analysis of SCA8 showed pathogenic CTA/CTG repeat of 23/127 (normal 16–91). Genes for SCA1, 2, 3, 6, 7, dentatorubral-pallidolluysian atrophy (DRPLA) and Huntington's disease exhibited no pathological expansion. Abnormal fused in sarcoma (FUS) mutation was not confirmed. Thus we clinically diagnosed this case as marked psychomotor impairment, possibly related to the abnormal expansion of SCA8 mutation although other SCA8 cases reported up to now were quite distinct from the present case in clinical features.

## Neuropathological findings

Autopsy was done 3 h after death. The brain weighed 400 g.

Macroscopic examination revealed diffuse atrophy of the whole brain, including the cerebellum, brain stem and spinal cord. The cerebral cortex and white matter showed atrophy. The basal ganglia, thalamus, cerebellum, tegmentum of the brainstem, midbrain (Fig. 1A), pons, medulla oblongata and spinal cord were severely devastated, obscuring the details of their internal structures.

On microscopic examination, the cerebral cortex showed diffuse neuronal loss and gliosis, and white matter atrophy was comparable to that of the gray matter (Fig. 1B). The degrees of neuronal loss and gliosis (graded into mild, moderate to severe) and the frequency of rNCIs are schematized (Fig. 2).

Many remaining neurons had round to oval rNCIs. The frequency of the neurons with rNCIs was variable between 5–30% of remaining neurons. It was low in areas with severe neuronal loss, such as the thalamus, cerebellum (Fig. 1C) and motor nucleus, such as the hypoglossal nucleus (Fig. 1D), while abundant in Ammon's horn where neuronal cells were spared. It was moderate in the frontal and parietal cortices where neuronal loss was moderate in degree. This inverse relationship between neuronal loss and rNCI was similarly evident by contrasting the deep layers of the cerebral cortex where gliosis was mild with abundant rNCIs.

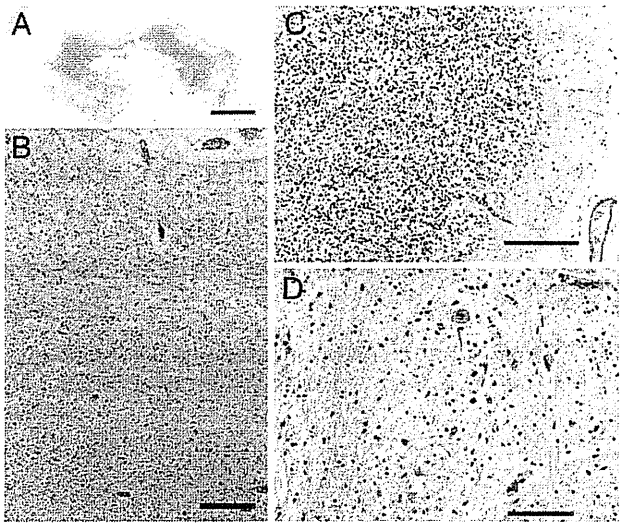


**Fig. 1** (A) The tegmentum of the brainstem, red nucleus and substantia nigra are severely atrophic; it is not easy to identify their inner details. Bar = 5 mm. (B) Diffuse neuronal loss and gliosis in the cerebral cortex, associated with spongy state of superficial layers and diffuse gliosis in the white matter (HE). Bar = 250 μm. (C) Diffuse neuronal loss and gliosis in the cerebellum (HE). Bar = 250 μm. (D) Severe neuronal loss and gliosis in the hypoglossal nucleus (HE). Bar = 100 μm.

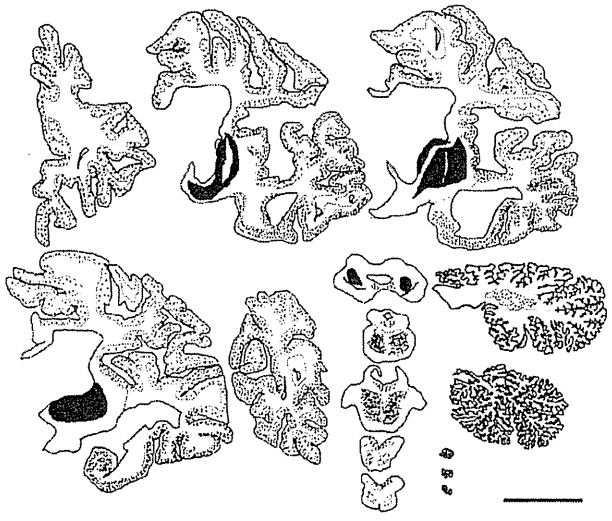

**Fig. 2** Regional variability of neuronal loss and ribosomal neuronal cytoplasmic inclusions (rNCIs). Red color stands for severe devastation of the brain parenchyma, pink for moderate degeneration, and pale pink for mild degeneration. Each dot represents five inclusions. Bar = 20 mm.

The rNCIs were basophilic on HE (Fig. 3A) and KB (Fig. 3B) and argyrophilic with Bodian silver impregnation

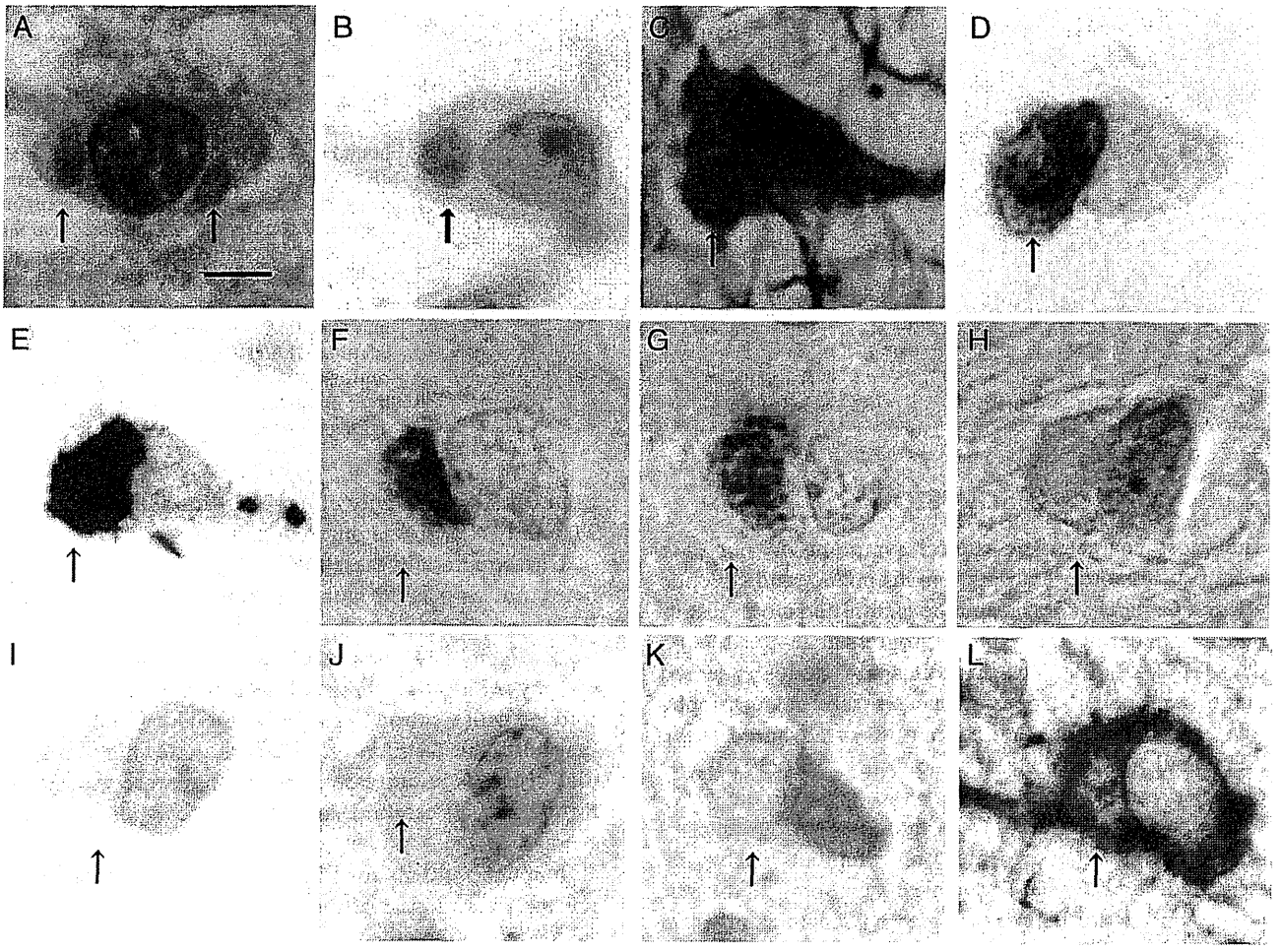


**Fig. 3** The ribosomal neuronal cytoplasmic inclusion (rNCI) is basophilic on HE (A), KB (B) and brownish black for Bodian stain (C). The rNCI are positive for ubiquitin (Ub) (D), p62 (E) and faintly for trans-activation response DNA protein 43 (TDP43) (F), partly positive for 1C2 (G), and negative for alpha-synuclein (Syn) (H), phosphorylated tau (AT8) (I), fused in sarcoma (FUS) (J), neurofilaments (K) andmitogen-activated protein 2 (MAP2) (L). Bar = 5 μm.

(Fig. 3C). The rNCIs were positive: Ub ≈ 25–35% (Fig. 3D, 1:200, Millipore, Tokyo, Japan); p62 ≈ 20–30% (Fig. 3E 1:500, Abnova, Walnut, CA, USA); and phosphorylated TDP43 ≈ 3–5% (Fig. 3F, 1:10 000, Cosmo Bio, Tokyo, Japan), then positive in a few rNCIs for expanded polyglutamine ≈ 0.5–1.0% (Fig. 3G, 1–2, 1:10 000, Millopore, Tokyo, Japan) and negative for Syn (Fig. 3H, 1:10 000, Wako, Tokyo, Japan), AT8 (Fig. 3I, 1:10 000, Innogenetics, Zwijndrecht, Belgium), FUS (Fig. 3J 1:100 gift of Dr Murayama), neurofilaments (Fig. 3K 1: 200, Dako, Tokyo, Japan) and mitogen-activated protein 2 (MAP2) (Fig. 3L, 1:1000, Sigma, St Louis, MI, USA). The rNCIs were negative for alpha-internexin (1:100, Santa Cruz Biotech, Dallas, TX, USA), T cell restricted intracellular antigen-1 (TIA-1) (1:100, Santa Cruz Biotech), and poly-(A)-binding protein-1 (PABP-1) (1:100, Santa Cruz Biotech) (data not shown). The rNCIs were stained red with methylgreen-pyronine (MGP), and these positive granules disappeared after RNA-ase digestion (data not shown). Triple fluorolabeling demonstrated coexistence of Ub and 1C2 in some rNCIs, while both Ub and TDP43 frequently coexisted in the same rNCIs.

Ultrastructurally, rNCIs were composed of aggregations of small electron-dense granular particles (20–50 nm) resembling ribosomes (Fig. 4A). These aggregated granules were not membrane-bound and only seen in the neuronal cytoplasm and not in the nucleus. Most rNCIs were closely opposed to the nucleus. Some rNCIs were globular in shape, the centers of which contained degenerative organellae, surrounded by circular aggregations of ribosomes (Fig. 4B). The RER were not found in most neurons examined. Abnormal mitochondria, lipid deposits and filamentous structures were not seen. There was no similar ribosomal aggregation in glia.