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（分担）研究報告書
大規模孤発性 ALS 患者前向きコホートの遺伝子・不死化細胞リソースを用いた
病態解明、治療法開発研究

大規模 ALS 患者コホートのゲノム遺伝子検体を用いた遺伝子多型解析

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研究要旨 孤発性筋萎縮性側索硬化症 (ALS) の根治的治療法開発のためには、発症、経過、予後等に関与する遺伝子 (群) を同定する必要がある。本研究では、これまで JaCALS (Japanese Consortium for Amyotrophic Lateral Sclerosis research) と共同で一塩基多型 (SNP) を用いたゲノム解析を実施してきた。昨年度は、643 例の孤発性 ALS 検体を用いてゲノムワイド SNP タイピングを行った。現在、臨床情報との関連を解析中である。本年度は、新たに ALS 検体 340 例、対照 7,784 例についてゲノムワイド関連解析 (GWAS) を行い、現在までに孤発性 ALS と有意な相関を示す 33 種の SNP を同定した。その中には、以前 ALS 発症・進展に関わる遺伝子として同定されたものも含まれていた。

A.研究目的

孤発性 ALS は、遺伝因子と環境因子の相互作用によって発症する多因子遺伝性疾患である。これまで、欧米人 ALS 症例を用いた GWAS により 8 種以上の候補遺伝子が報告されている。しかしながら、その後の追試で関連が認められるものは殆どない。現在のところ、欧米人における *C9ORF72* 内の 6 塩基異常伸長だけである。ただし、その関連には人種特異性がある。

一方、本邦においては、厚労省神経変性班を基に全国 28 施設が参加する JaCALS (Japanese Consortium for Amyotrophic Lateral Sclerosis research) が、孤発性 ALS 患者 700 症例以上の前向き臨床情報、ゲノム DNA を収集、保管している。

我々は、孤発性 ALS の遺伝的背景を明らかにするために、これまで大規模ゲノム関連解析を行い、新規の孤発性 ALS 易罹患性遺伝子 *ZNF512B* を同定してきた。また、61 万種の SNP を搭載した SNP アレイを用いた GWAS を行った。昨年度は 643 例の孤発性 ALS 検体の SNP タイピングを行った。現在、経過、予後、病型などの病態に関わる SNP を探索中である。さらに、本年度は、

引き続き、追加検体をタイピングし、GWAS から新規孤発性 ALS 易罹患性遺伝子を探索した。

B.研究方法

本年度は、日本人 ALS 検体 340 例についてタイピングを行った。対照には、日本人一般集団 7,784 例を用いた。まず、対象 ALS 検体において、家族性 ALS の原因遺伝子 (*SOD1*、*OPTN* 等) の変異が無いことを確認した。その後、SNP タイピングを行った。SNP アレイは、Illumina 社製 Human OmniExpressExome BeadChip を使用した。全例について、データの品質管理を行った (Hardy-Weinberg 平衡検定値、call rate 等)。さらに identity-by-state 法により近親者がいないことを確認した。QQ プロット (quantile-quantile plot) と主成分分析で、集団階層化がないことを確認した。遺伝統計学的解析の際において、imputation 解析を行った。関連値 (P 値) を用いて SNP をランキングし、多重検定値による有意水準を満たす SNP を候補とした。

(倫理面への配慮を含む) 文部科学省、厚生労働省及び経済産業省合同「ヒトゲノム・遺伝子解析研究に関する倫理指針」のもとに行った。検体

収集と研究計画は、名古屋大学、理化学研究所、その他関連機関における倫理委員会の承認を受け、実施した。検体は、書面によるインフォームド・コンセントを取得したのち、収集した。

C. 研究結果

33 SNPs が孤発性 ALS と有意な相関を認めた。それらの SNP は、全て rare バリエーションであった。また、そのうち、数種の SNP は ALS 関連遺伝子領域にマップされた。欧米人において過去に見出された SNP と孤発性 ALS との関連は、本研究では認められなかった。

D. 考察

現在、我々は、JaCALS の持つ大規模前向きコホートを用いて日本人 ALS を対象にゲノム解析を行っている。昨年度は、643 例のゲノムワイド SNP タイピングを行い、発症、経過、予後との関連を検索している。そして、本年度は、GWAS 解析により孤発性 ALS と強い関連を認める新規の SNP を見出した。候補 SNP の中には、既知の ALS 関連遺伝子領域にマップされるものも含まれ、興味深い。これまで蓄積した関連解析のデータと本研究におけるデータを統合したメタ解析を行っている。

E. 結論

ALS の易罹患性に関わる新規の候補遺伝子領域を同定した。欧米人で認められた既報の SNP と孤発性 ALS の関連は認められなかった。

F. 健康危険情報

なし。

G. 研究発表

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H. 知的財産権の出願・登録状況（予定を含む）

なし。

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(分担)研究報告書
大規模孤発性 ALS 患者前向きコホートの遺伝子・不死化細胞リソースを用いた
病態解明、治療法開発研究

日本人 ALS での遺伝学的背景の解析

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研究要旨

神経難病のひとつである筋萎縮性側索硬化症(ALS)の病態は未だ明らかとなっておらず、孤発症例と同様、上位および下位運動ニューロン障害を示す家族性 ALS (FALS)の病態を解析することで、孤発症例の原因を解明しようという試みが行われている。そこで、我々はまず遺伝学的背景を明らかにすることを目的に、日本人 ALS の遺伝子バンクである JaCALS 検体を用いて、既知の ALS 原因遺伝子の解析を行った。

A.研究目的

日本人 ALS における遺伝学的背景を明らかとする。

は次々に同定していることから、ALS の病態における遺伝学的な要因はさらに高いものと推察される。

B.研究方法

日本人 ALS の遺伝子バンクである JaCALS より供与を受けた 592 例、および 177 例の正常対照群を対象とした。いずれの症例からも、遺伝子解析について文書で同意を得ている。

FALS の原因遺伝子である、*SOD1*、*FUS/TLS*、*TDP-43*、*OPTN*、*ANG* および Hereditary motor and sensory neuropathy with proximal dominant involvement (HMNS-P)の原因遺伝子 *TFG* の変異の有無を HRM(high resolution melting)法でスクリーニングし、direct sequence 法にて確認を行った。

C.研究結果

正常対照群がなく、患者群のみで認められたアミノ酸置換を伴う変異を *SOD1*(23 例)、*TDP-43*(3 例)、*ANG*(4 例)、*FUS/TLS*(7 例)、*OPTN*(4 例)、*TFG*(2 例)にそれぞれ確認した。

D.考察

今回の我々の解析では、ALS 群の約 7.3%(43/592)に遺伝子変異を見出した。解析した遺伝子は、既知の遺伝子の一部であること、また ALS に関連する遺伝子

E.結論

日本人 ALS 群での遺伝子変異では *SOD1* の頻度が最も多く、次に *FUS/TLS*であった。

ALS の病態における遺伝学的な影響は大きいと思われる。

F.健康危険情報

なし

G.研究発表

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H.知的財産権の出願・登録状況(予定を含む)

なし

Ⅲ. 研究成果の刊行に関する一覧表

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雑誌

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IV. 研究成果の刊行物・別刷

Evaluation of *SLC20A2* mutations that cause idiopathic basal ganglia calcification in Japan

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ABSTRACT

Objective: To investigate the clinical, genetic, and neuroradiologic presentations of idiopathic basal ganglia calcification (IBGC) in a nationwide study in Japan.

Methods: We documented clinical and neuroimaging data of a total of 69 subjects including 23 subjects from 10 families and 46 subjects in sporadic cases of IBGC in Japan. Mutational analysis of *SLC20A2* was performed.

Results: Six new mutations in *SLC20A2* were found in patients with IBGC: 4 missense mutations, 1 nonsense mutation, and 1 frameshift mutation. Four of them were familial cases and 2 were sporadic cases in our survey. The frequency of families with mutations in *SLC20A2* in Japan was 50%, which was as high as in a previous report on other regions. The clinical features varied widely among the patients with *SLC20A2* mutations. However, 2 distinct families have the same mutation of S637R in *SLC20A2* and they have similar characteristics in the clinical course, symptoms, neurologic findings, and neuroimaging. In our study, all the patients with *SLC20A2* mutations showed calcification. In familial cases, there were symptomatic and asymptomatic patients in the same family.

Conclusion: *SLC20A2* mutations are a major cause of familial IBGC in Japan. The members in the families with the same mutation had similar patterns of calcification in the brain and the affected members showed similar clinical manifestations. *Neurology*® 2014;82:1-8

GLOSSARY

DNTC = diffuse neurofibrillary tangles with calcification; **FIBGC** = familial idiopathic basal ganglia calcification; **IBGC** = idiopathic basal ganglia calcification; **MMSE** = Mini-Mental State Examination; **PDGF** = platelet-derived growth factor; **PDGFRB** = platelet-derived growth factor receptor- β ; **Pi** = inorganic phosphate; **PIB** = Pittsburgh compound B; **PiT** = type III sodium-dependent phosphate transporter; **PKC** = paroxysmal kinesigenic choreoathetosis.

Idiopathic basal ganglia calcification (IBGC), also known as Fahr disease, is thought to be a rare neuropsychiatric disorder characterized by symmetrical calcification in the basal ganglia and other brain regions. Clinical manifestations range widely from asymptomatic to variable symptoms including headaches, psychosis, and dementia.¹ The diagnosis of IBGC generally relies on the visualization of bilateral calcification mainly in the basal ganglia by neuroimaging and the absence of metabolic, infectious, toxic, or traumatic causes.^{2,3}

The mode of inheritance of familial IBGC (FIBGC) has been thought to be autosomal dominant and, to date, 4 responsible chromosomal regions have been identified, namely 14q (IBGC1), 2q37 (IBGC2), 8p11.21 (IBGC3), and 5q32 (IBGC4).³⁻¹⁴ The causative gene at the IBGC3 locus was identified as *SLC20A2* encoding type III sodium-dependent phosphate transporter 2 (PiT-2). Screening of a large series of patients with IBGC revealed that mutations in *SLC20A2* are a major cause of FIBGC¹⁰; moreover, other mutations in *SLC20A2* have recently been reported in China and Brazil.¹¹⁻¹³ The mutations of *PDGFRB* encoding platelet-derived growth factor

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(PDGF) receptor- β (PDGFRB) and *PDGFB* have recently been reported to cause calcification in the brain.^{14,15}

We have collected clinical information of patients with IBGC in a nationwide survey in Japan. Here, on the basis of a mutational analysis of *SLC20A2*, we aim to establish the molecular epidemiology of IBGC and evaluate clinically and genetically *SLC20A2* mutations in Japan.

METHODS Subjects and samples. We collected clinical information on patients with IBGC in a nationwide study. The criteria for the selection of patients in the initial survey were as follows: 1) conspicuous calcification is observed in the basal ganglia and/or dentate nucleus by CT scan; 2) calcification is bilateral and symmetrical; and 3) idiopathic (absence of biochemical abnormalities, and an infectious, toxic, or traumatic cause).^{2,3} Neurologists enrolled patients in the survey. They examined the medical charts and performed the neurologic examinations again if necessary. The survey was approved by the Ethics Committee of the Gifu University Graduate School of Medicine. During the survey, some patients were found to have hypoparathyroidism, Aicardi-Goutières syndrome, and Cockayne syndrome, and these patients were excluded. For the genetic study, a total of 69 subjects from 41 hospitals provided written informed consent and were enrolled in the project. Of these patients, 46 came from families with a single affected member, and the other 23 came from 10 families with multiple affected members. We defined the former as sporadic patients and the latter as familial patients. The patients' mean age \pm SD was 41.3 ± 23.6 years at registration. The patients comprised 32 males and 37 females.

Standard protocol approvals, registrations, and patient consents. All experiments on human DNA were approved by the Ethics Committees of both Gifu University and the University of Tokyo. After written informed consent was obtained, peripheral blood samples were collected.

Mutational analysis. Genomic DNA was extracted from the whole blood samples. *SLC20A2* analysis was performed by Sanger sequencing of all coding regions, as described in detail in e-Methods and table e-1, A and B, on the *Neurology*[®] Web site at www.

neurology.org. The pathologic potential of the identified variants was predicted using PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph/>).¹⁶

RESULTS Mutational analysis. We screened a total of 69 subjects including 23 subjects from 10 families in which multiple affected subjects were observed and 46 subjects in sporadic cases, all of whom were Japanese. Six new mutations in *SLC20A2* were found: 4 missense mutations, 1 nonsense mutation, and 1 frameshift mutation (figure 1). Electropherograms showed the individual heterozygous mutations (figure e-1). None of them were present in an in-house exome sequencing data set (358 Japanese control subjects), dbSNP 137 (www.ncbi.nlm.nih.gov/snp/), or the National Heart, Lung, and Blood Institute "Grand Opportunity" Exome Sequencing Project (ESP6500SI-V2). In silico analysis predicted deleterious consequences, as determined from the residue changes in figures 1 and e-1. When confined to the IBGC patients, 5 of the 10 families (50.0%) showed mutations in *SLC20A2*. In contrast, 2 of the 46 patients (4.3%) with sporadic IBGC carried mutations in *SLC20A2* in this study.

Clinical manifestations. The clinical manifestations are summarized in table 1. A positive family history of IBGC was present in 5 families. Families 1 and 2 had the same mutation.

Familial cases. Case 1 (in family 1). The proband in family 1 was a 64-year-old woman who had dysarthria and gait disturbance for 5 years. She showed no dementia. Her neurologic examination revealed dysarthria, small steppage gait, rigidity at bilateral wrist joints, bradykinesia, and a pyramidal sign. Her CT images revealed severe calcification at the bilateral globus pallidus, caudate nuclei, thalamus, subcortical white matter, and dentate nuclei (figure 2C). Her son's CT showed similar brain calcification (figure 2D), although he was clinically asymptomatic. His DNA study revealed the

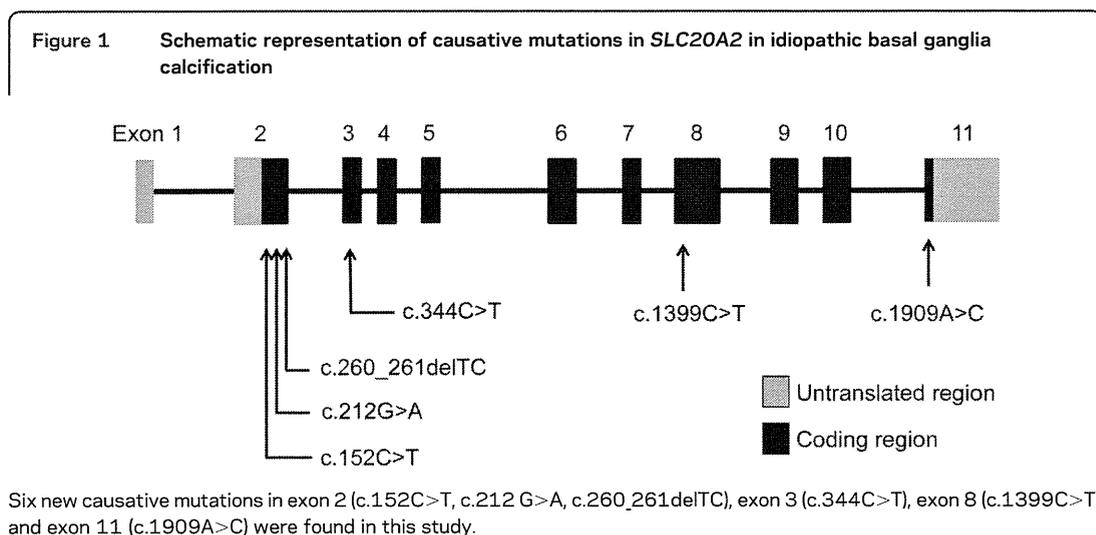


Table 1 Clinical features of 6 individuals (proband) with SLC20A2 mutations

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Mutation	c.1909A>C	c.1909A>C	c.344C>T	c.212G>A	c.1399C>T	c.152C>T	c.260_261delTC
	S637R	S637R	T115M	R71H	R467X	A51V	L87Hfs*6
Zygosity	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
Exon	11	11	3	2	8	2	2
Proband information							
Age at detection of calcification, y	60	51	60	73	23	71	74
Age at onset, y	58	50	60	71	15	71	57
Onset symptom	Dysarthria	Dysarthria	Dementia	Parkinsonism	PKC	Dementia	Athetosis
Neurologic findings							
Cognitive impairment (MMSE)	27	24	20	16	30	22	22
Pyramidal sign	+	+	-	-	-	-	-
Extrapyramidal sign	+	+	-	+	-	-	+
Family information (except the proband)							
No. of other individuals with calcification	1	2	5	1	1	0 ^a	0 ^a
No. of other individuals with confirmed mutations	1	NE	5	NE	1	NA	NA
No. of other symptomatic individuals	0	0	2	0	0	NA	NA
Other symptoms (no.) in the family	—	—	Mental disorder (1), alcoholism (1)	—	—	NA	NA

Abbreviations: MMSE = Mini-Mental State Examination; NA = not applicable; NE = not examined; PKC = paroxysmal kinesigenic choreoathetosis.

^aBecause there was no other family member who had any neurologic symptoms, brain CT screening of other family members was not performed.

same mutation in exon 11 that had been found in his mother.

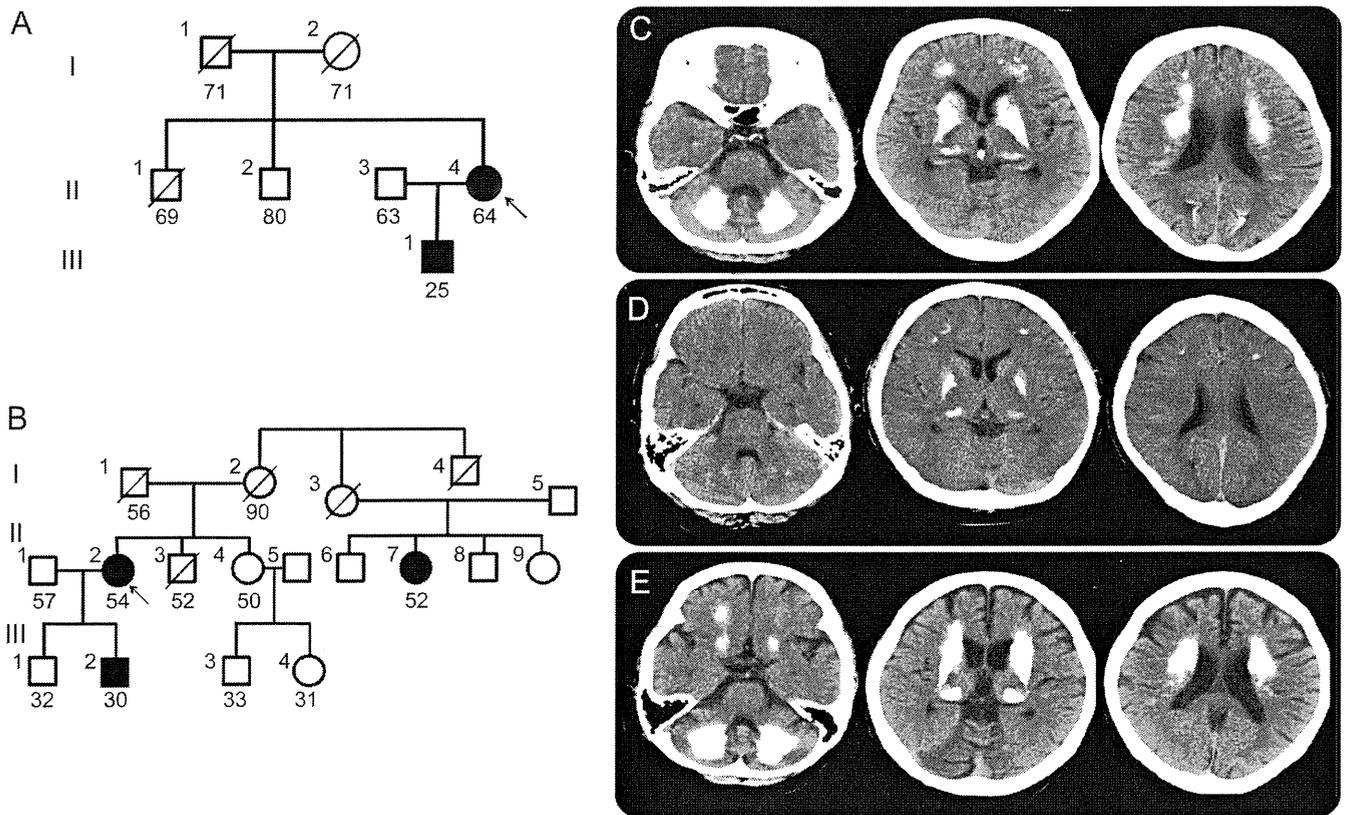
Case 2 (in family 2). The proband in family 2 was a 54-year-old woman who had dysarthria and gait disturbance for 4 years. She showed mild mental deterioration in Mini-Mental State Examination (MMSE) score of 24 points, frontal signs, dysarthria, mild parkinsonism (rigidity of bilateral wrist joints and bradykinesia), adiadochokinesis, spasticity, and small steppage gait. Her CT images revealed severe calcification at the bilateral globus pallidus, caudate nuclei, thalamus, subcortical white matter, and dentate nuclei (figure 2E). Although her son and cousin also showed calcification in CT images, they were asymptomatic. Her DNA analysis revealed the same mutation as that in family 1.

Case 3 and other symptomatic individuals (in family 3). The proband was a 69-year-old woman (II-1 in the pedigree in figure 3). She was admitted to a hospital at the age of 65 because of forgetfulness since the age of 60 years. Her MMSE score was 20, which indicated a possibility of dementia (MMSE score below 22). Decreased blood flow was detected in the bilateral basal ganglia and thalamus and the right frontal lobe in particular by SPECT. She had a positive family history of brain calcification, as shown in figure 3A. The initial clinical diagnosis had been diffuse neurofibrillary tangles with calcification (DNTC),¹⁷

although to our knowledge familial cases of DNTC have not been reported. Her son had psychological disorders including violent behavior; unfortunately, no brain CT had yet been performed on him. In the patients in family 3, the degree of calcification was mild compared with that observed in the other families (figure 3, B–G). Her brother with calcification in the brain (II-7) had a mental disorder and another (II-8) presented with alcoholism. The 3 other relatives with calcification were asymptomatic (II-5, II-9, and III-3). The symptomatic patients (II-1, II-7, and II-8) showed more apparent brain atrophy than the others (figure 3, B, D, and E, respectively). The individuals with calcification on the CT images (II-1, II-5, II-7, II-8, II-9, and III-3) had the same mutation in exon 3 in *SLC20A2*. However, the individuals with no calcification (III-2, III-5, and IV-1) revealed no mutation in *SLC20A2*. In summary, 6 patients had calcification among the 10 individuals examined by CT scan in family 3 and all of them carrying the *SLC20A2* mutation exhibited similar calcification on CT images. However, persons without the mutation did not show calcification.

Case 4 (in family 4). Family 4 had a mutation in exon 2. The proband developed clumsiness of her hands and gait unsteadiness at the age of 71 years, and she was diagnosed as having Parkinson disease. Visual

Figure 2 CT images and family trees of families 1 and 2



(A) Family tree of family 1. (B) Family tree of family 2. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. (C) CT images of proband (II-4 in pedigree of family 1, part A). (D) CT images of the proband's son (III-1 in pedigree of family 1, part A). (E) CT images of the proband (II-2 in pedigree of family 2, part B). All have mutation of S637R.

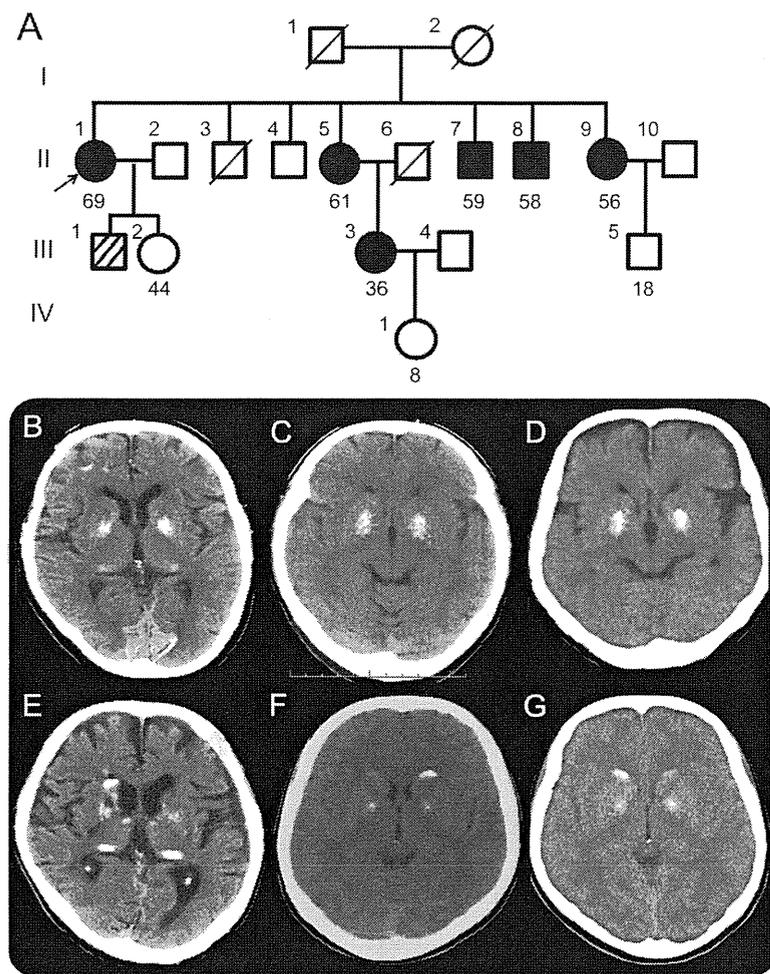
hallucinations started with the initiation of medication. She showed parkinsonism (rigidity, bradykinesia, and postural instability), which responded to levodopa. Her MMSE score was 16. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, and dentate nuclei, and her daughter, who was asymptomatic, also had intracranial calcification (figure e-2C). Brain CT was not performed in her other children. Her SPECT images showed decreased perfusion in the bilateral frontal, temporal, and parietal regions of the brain. She died of pneumonia at the age of 79. Neuropathologic examination revealed neuronal loss and Lewy bodies in the substantia nigra, locus ceruleus, amygdala, and parahippocampal gyrus indicative of Parkinson disease, and prominent deposition of calcium in the parenchyma and the wall of arteries in the globus pallidum and dentate nuclei compatible with the pathologic findings of IBGC.

Case 5 (in family 5). The proband was a 24-year-old man who had paroxysmal kinesigenic choreoathetosis (PKC). His laboratory data were normal except for CT findings. He presented with an attack of PKC after exercise and his symptom responded well to carbamazepine. His CT images revealed calcification at

the globus pallidus, thalamus, subcortical white matter, and dentate nuclei (figure e-2B [A]). We had an opportunity to examine his parents, who had no symptoms or signs. Mutational analysis of *SLC20A2* of his parents with their informed consent revealed the same mutation in exon 8 in his mother as he had. Brain CT scan of his mother confirmed calcification at the globus pallidus, subcortical white matter, and dentate nuclei.

Sporadic cases. Case 6. The patient had a mutation in exon 2. She was a 72-year-old woman who noticed forgetfulness at the age of 71. She had no motor deficits. Her MMSE score was 22, and her score on the revised Hasegawa Dementia Scale was 24. Her Frontal Assessment Battery score at bedside was 5, indicating a frontal lobe deficit (cutoff score, 11/12). The index scores of the revised Wechsler Memory Scale were as follows: attention and concentration, 86; verbal memory, 89; general memory, 85; attention/concentration, 71; and delayed recall, 75. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, thalamus, subcortical and periventricular white matter, and dentate nuclei (figure e-2B [B]). Her SPECT images showed decreased perfusion in the left frontal,

Figure 3 Pedigree and CT images of family 3



(A) Pedigree of family 3. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. The striped symbol represents a symptomatic patient, although his CT image and DNA sample were not available for the study. (B) CT image of the proband (III-1 in pedigree of family 3). (C) CT image of asymptomatic II-5. (D) CT image of symptomatic II-7. (E) CT image of symptomatic II-8. (F) CT image of asymptomatic II-9. (G) CT image of asymptomatic III-3. All have mutation of T115M.

temporal, and parietal regions of the cerebrum and bilateral cerebellum. [^{11}C] Pittsburgh compound B (PiB) retention was not observed by [^{11}C]PiB PET. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

Case 7. The patient was a 78-year-old man who had a frameshift in exon 2. Involuntary movement of the left thumb and index finger like “pill-rolling” began in his sixth decade. His family first noticed memory impairment at the age of 75. Gait disturbance appeared at the age of 77 and oral dyskinesia and left shoulder shrugging appeared at the age of 78. His scores on the MMSE and Frontal Assessment Battery were 22 and 10, respectively. His brain CT images showed calcification at the globus pallidus, thalamus,

subcortical and periventricular white matter, and dentate nuclei (figure e-2B [C]). His SPECT images showed decreased perfusion in the bilateral (predominantly in the left) frontal and temporal regions of the cerebrum and bilateral cerebellum. [^{11}C]PiB retention was not observed by [^{11}C]PiB PET, which was performed at the age of 81. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

DISCUSSION We have obtained clinical information of 161 patients with brain calcification in a nationwide study. We discovered that 3 patients had hypoparathyroidism, Aicardi-Goutières syndrome, and Cockayne syndrome during the survey. CT images revealed varying degrees of calcification, from marked calcification in the basal ganglia to patchy calcification in various regions, suggesting diversity in the etiologies. Some patients were incidentally found to have calcification by CT performed for head injury caused by accidents. Because our previous survey revealed a considerable frequency (1%–2%) of patchy calcification in the CT images of all patients in 2 university hospitals in Japan,¹⁸ more asymptomatic IBGC patients with patchy calcification may exist than the number that we had previously assumed to be present in the population in Japan. After the examination by neurologists, we collected 69 DNA samples from patients who met the criteria for IBGC.^{2,3} Symptoms and neurologic findings varied widely from asymptomatic to variable symptoms including headaches, psychosis, and dementia.

In this study, we investigated mutations in *SLC20A2* in 69 patients with IBGC in Japan and identified 4 new mutations in 10 familial cases (the same mutation in 2 families) and 2 other new mutations in 46 sporadic cases. The frequency of families with mutations in *SLC20A2* was 50% (5 of the 10 families), and that of sporadic patients was 4.3% (2 of the 46 patients). The frequency of the mutations in *SLC20A2* in FIBGC in Japan was as high as in other countries in a previous report.¹⁰ Case 5 indicates that it is difficult to reliably determine sporadic cases without brain CT scans and genetic studies of all members in the family.

The mutations in our study existed in exons 2, 3, 8, and 11. One of these mutations (R467X) in exon 8 resulted in a substitution to a TGA stop codon, and the other (c.260_261delTC) in exon 2 was a frameshift. None of the mutations were reported previously, indicating heterogeneities of the mutations in *SLC20A2*. Taken together with other reports, causative mutations identified in *SLC20A2* include 6 mutations in exon 2, 1 in exon 3, 3 in exon 4, 1 in exon 5, 1 in exon 7, 10 in exon 8, 2 in exon 9, 4 in exon 10, and 4 in exon 11.^{9–12} It does not seem that there

are mutation hot spots in *SLC20A2*. The in silico analysis using PolyPhen-2 for the missense mutations predicted all to be likely damaging, as determined from the residue changes. We drew the structure model of the PiT-2 protein using the TOPO2 software (<http://www.sacs.ucsf.edu/TOPO/top.html>). The schematic structure of the PiT-2 protein with the mutations is shown in figure 4.

Although the clinical features varied widely among the families with IBGC with *SLC20A2* mutations, the patients in families 1 and 2 with the same *SLC20A2* mutation exhibited similar clinical manifestations including dysarthria, mild cognitive decline, pyramidal signs, and extrapyramidal signs as well as similar ages at detection of calcification and onset of symptoms. Of note, the CT images among the affected individuals in the 2 families are similar (figure 2). In family 3, in contrast, 3 symptomatic patients presented with dementia, psychological disorder, and alcoholism, accompanied with brain atrophy in CT images. None of them showed movement disorders such as those in families 1 and 2.

Although mutational analysis and CT scan were not performed in other familial members of cases 6 and 7, concordance of the presence of mutations of *SLC20A2* and brain calcification were confirmed in 15 individuals, and we did not observe any individuals who carried the mutation and did not show brain calcification. These observations strongly support a high penetrance of the *SLC20A2* mutations regarding brain calcification.

Correlations of genotypes and neurologic phenotypes, however, have been controversial. *SLC20A2* mutations in patients with FIBGC have been

described to show variability in clinical manifestations among the families. In the present study, the 2 affected individuals in families 1 and 2, who carried the same mutation, exhibited quite similar neurologic manifestations and clinical courses, suggesting a genotype-phenotype correlation of the S637R mutation. Of note, 2 individuals aged 56 and 61 years in family 3 did not exhibit any neurologic manifestations despite carrying the mutation and having brain calcification, indicating that penetrance regarding the neurologic manifestations is incomplete.

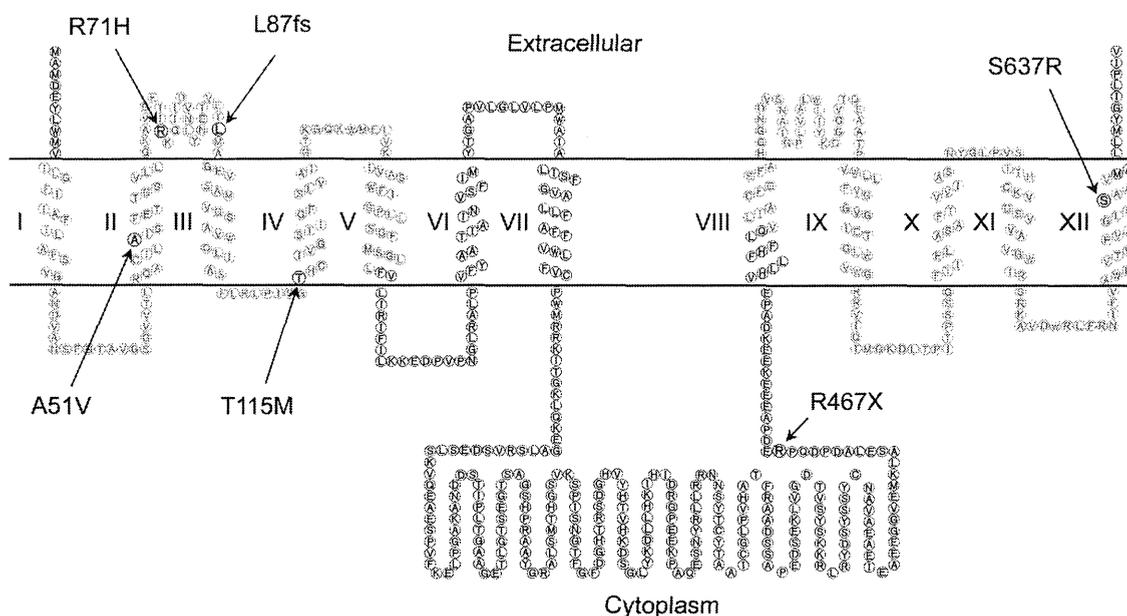
In case 4, interestingly, the proband showed pathologic findings of both IBGC and Parkinson disease. Because Parkinson disease is a common disorder in aged people, there remains a possibility that the presence of IBGC and Parkinson disease is coincidental.

Case 5 had a mutation that leads to a premature stop codon, making an incomplete structure of PiT-2. His neurologic symptom was PKC controllable by carbamazepine. Intriguingly, several patients with IBGC have been reported to present with PKC or paroxysmal nonkinesigenic dyskinesia.^{19,20} For these cases of PKC or paroxysmal nonkinesigenic dyskinesia, mutational analyses of not only *SLC20A2* but also *PRRT2* and MRI will be indispensable.^{21,22}

Herein, we have reported 5 cases of FIBGC and 2 cases of IBGC with *SLC20A2* mutations in Japan. We could not find any characteristic features of Japanese patients, although we had discovered that each case has a new mutation in *SLC20A2*, respectively.

The mechanisms of calcification and cell damage remain to be elucidated. Despite that the expression of PiT-2 encoded by *SLC20A2* is distributed widely in the human body,²³ mutations in *SLC20A2* cause

Figure 4 Schematic structure of PiT-2 (type III sodium-dependent phosphate transporter) with the mutations



calcification only in the brain. Mutations in *SLC34A2* have been reported to cause pulmonary alveolar micro-lithiasis.²⁴ Because Npt2b encoded by *SLC34A2* is the only phosphate transporter that is highly expressed in the lungs,²⁵ the mutations in *SLC34A2* are compatible with the lesion of the alveolar type II cells in the lungs.²⁴ Because the limitation of calcification to the brain cannot be explained by only the mutation in *SLC20A2* followed by abnormalities of inorganic phosphate (Pi) transport via PiT-2, there might be some other genes responsible for calcification in the brain, or the mutations in *SLC20A2* may take some toxic gain of function. The dysfunction of Pi transport can explain the accumulation of various metals in regions of the brain and the abnormal distribution of metals, which we observed in CSF²⁶ and hair in the patients with IBGC.²⁷ We have recently shown that PiT-2 immunopositivity was expressed predominantly in neurons, astrocytes, and vascular endothelial cells in the mouse brain.²⁸ PDGF-B is expressed in endothelial cells and neurons.²⁹ PDGF-B homodimer (PDGF-BB) enhanced the expression of PiT-1 mRNA encoded by *SLC20A1* in human aortic smooth muscle cells.³⁰ The hypomorph of PDGF-B in mice has recently been revealed to cause brain calcification through pericyte and blood-brain barrier impairment.¹⁵ Recently, simple knockout of *SLC20A2* has also been shown to lead to calcification in the mouse brain.³¹ PiT-2, PDGF, and as yet undetermined other molecules are considered to have pivotal roles in blood vessel-associated calcification and neuronal death in patients with IBGC. Elucidation of the molecular basis underlying IBGC will contribute to the development of therapeutic measures for patients with calcification in the brain.

AUTHOR CONTRIBUTIONS

Principal investigator: Isao Hozumi. Study supervision: Shoji Tsuji, Gen Sobue, Takashi Inuzuka, and Kortaro Tanaka. Manuscript draft preparation: Megumi Yamada and Masaki Tanaka. Acquisition and collection of data: Seiju Kobayashi, Yoshiharu Taguchi, Shutaro Takashima, Tetsuo Touge, Hiroyuki Hatsuta, and Shigeo Murayama. Analysis and interpretation: Megumi Yamada, Masaki Tanaka, Mari Takagi, Yuichi Hayashi, Masayuki Kaneko, Naoki Atsuta, Nobuyuki Shimozawa, Hiroyuki Ishiura, and Jun Mitsui.

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DISCLOSURE

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Original Investigation

Lower Motor Neuron Involvement in TAR DNA-Binding Protein of 43 kDa–Related Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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IMPORTANCE TAR DNA-binding protein of 43 kDa (TDP-43) plays a major role in the pathogenesis of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Although a pathological continuity between FTLD and ALS has been suggested, the neuropathological changes of the lower motor neuron (LMN) systems have not been assessed in TDP-43-associated FTLD (FTLD-TDP), to our knowledge.

OBJECTIVE To investigate a pathological continuity between FTLD-TDP and ALS by comparing their respective neuropathological changes in the motor neuron system.

DESIGN AND SETTING A retrospective clinical medical record review and a semiquantitative neuropathological evaluation of the cranial motor nerve nuclei and spinal cord were conducted at autopsy. We included 43 patients with sporadic FTLD-TDP, type A, B, or C, from 269 consecutively autopsied patients with TDP-43 proteinopathy. Patients were categorized as having FTLD without ALS, FTLD-ALS (onset of FTLD symptoms/signs preceded those of ALS), or ALS-FTLD (onset of ALS symptoms/signs preceded those of FTLD).

MAIN OUTCOMES AND MEASURES Neuronal TDP-43 pathological changes and neuronal loss.

RESULTS Forty-three patients were included in the clinical analysis, and 29 from whom spinal cords were obtained were included in the neuropathological analysis. Survival time was significantly shorter in the FTLD-ALS and ALS-FTLD groups than in the FTLD without ALS group ($P < .001$). At neuropathological examination, 89% of patients in the FTLD without ALS group showed aggregations of TDP-43 in the spinal motor neurons. The LMN loss was most severe in ALS-FTLD, followed by FTLD-ALS and FTLD without ALS. All the patients with type A or C FTLD-TDP were included in the FTLD without ALS group, and all those with type B pathological changes were in the FTLD-ALS or the ALS-FTLD group. Lower motor neuron loss and TDP-43-positive skeinlike inclusions were observed in all pathological subtypes.

CONCLUSIONS AND RELEVANCE The LMN systems of FTLD-TDP frequently exhibit neuropathological changes corresponding to ALS. Thus, a pathological continuity between FTLD-TDP and ALS is supported at the level of the LMN system.

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Frontotemporal lobar degeneration (FTLD) is a sporadic or familial neurodegenerative disease that clinically encompasses frontotemporal dementia, language disorder, and motor symptoms.¹ Immunohistochemical profiles show that approximately half of patients with FTLD present with tau-positive disease, but the other half primarily exhibit an accumulation of TAR DNA-binding protein of 43 kDa (TDP-43), referred to as FTLD-TDP.²⁻⁵ Currently, the cortical TDP-43 pathological changes in sporadic FTLD-TDP are classified into 3 subtypes: A, B, and C.⁶⁻⁸

TAR DNA-binding protein of 43 kDa is also a major disease protein in amyotrophic lateral sclerosis (ALS), which is characterized by upper motor neuron and lower motor neuron (LMN) involvement.⁹ The pathological features of LMN involvement in ALS include neuronal loss, gliosis, TDP-43-positive neuronal inclusions with skeinlike or round shapes and glial inclusions, and Bunina bodies.¹⁰

Some patients exhibit symptoms of both ALS and FTLD, and the cerebral cortices of patients with FTLD and ALS almost always show type B TDP-43 changes.^{7,11-13} Thus, a pathological continuity between FTLD and ALS has been proposed based on brain TDP-43 pathological findings. Studies of the cerebral cortex, including the motor cortex, and subcortical gray matter have shown common TDP-43 pathological findings in FTLD, FTLD with ALS, and ALS.^{12,14-17} However, the neuropathological features of LMN systems in FTLD-TDP have not been investigated comprehensively, particularly in the spinal cord, although characterization of these features is necessary to confirm the pathological relationship between FTLD and ALS.

In this study, we investigated LMN pathological findings in patients with sporadic FTLD-TDP who clinically demonstrated FTLD, FTLD with ALS, or ALS. We also investigated the correlation between TDP-43 pathological subtypes (type A, B, and C) and LMN involvement to further elucidate the continuity of FTLD and ALS.

Methods

Study Patients

We enrolled 269 consecutively autopsied patients with sporadic and adult-onset FTLD, FTLD with ALS, or ALS in which pathological aggregation of TDP-43 was confirmed at the Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, from 1988 to 2012. All patients had been clinically evaluated by neurological experts in the affiliated hospitals of Nagoya University School of Medicine or Aichi Medical University. Permission to perform an autopsy and archive the nervous system tissues for research purposes was obtained from family members after death. The clinical data on the included patients were obtained from case notes made at diagnosis and at an advanced stage of illness. We initially excluded 216 of the 269 patients because they did not present clinical FTLD symptoms. In 53 patients, FTLD or FTLD with ALS was diagnosed according to the diagnostic criteria of FTLD and ALS.^{1,18} Moreover, we subclassified FTLD with ALS into FTLD-ALS

(onset of FTLD symptoms/signs preceded those of ALS) and ALS-FTLD (onset of ALS symptoms/signs preceded those of FTLD) groups.

The enrolled patients were categorized into 3 groups: FTLD without ALS, FTLD-ALS, and ALS-FTLD. The FTLD symptoms were categorized into 2 groups: behavior-variant frontotemporal dementia and language impairments.¹⁴ The LMN symptoms/signs were defined by progressive muscular weakness, muscular atrophy, fasciculation, or electromyographic findings. We excluded patients with Alzheimer disease-associated neurofibrillary pathological abnormalities that were more advanced than Braak stage IV,¹⁹ those with argyrophilic grain disease, and those with invalid clinical data. Finally, 43 patients were included in the clinical analysis (11 with FTLD without ALS, 9 with FTLD-ALS, and 23 with ALS-FTLD). For comparison, we prepared 13 age-matched controls (mean [SD] age at death, 68.2 [6.9] years) who had no diagnosis of any neurodegenerative disease, dementia, or cerebrovascular disease.

Clinical Analyses

The information regarding sex, age at onset, disease duration, and duration between onset of FTLD and ALS was collected from clinical notes. Causes of death were classified as respiratory failure due to respiratory muscle weakness, pneumonia, or other. The last category comprised systemic diseases other than respiratory failure or pneumonia, including cancer, ileus, infections, and renal failure. Information on the subtypes of dementia, clinical data on motor symptoms, and electromyographic results were also collected.

Pathological Evaluations

For the neuropathological analysis, we excluded patients who had received a respirator/tracheotomy ($n = 11$) or whose spinal cord was not available ($n = 3$). In total, 29 patients were included in the neuropathological analysis and divided into 3 groups: FTLD without ALS ($n = 9$), FTLD-ALS ($n = 8$), and ALS-FTLD ($n = 12$). The tissues were fixed in 20% neutral-buffered formalin. The paraffin-embedded tissue blocks were cut at a thickness of 4.5 μm . We evaluated sections from the spinal cord and whole brain. The whole spinal cord was examined at each segmental level, but only the cervical cord was available in 3 patients and the sacral cord was not available in another 3.

For routine neuropathological examinations, the sections were stained with hematoxylin-eosin and Klüver-Barrera. Immunohistochemical studies were performed using a standard polymer-based method with the EnVision Kit or anti-goat immunoglobulin (Dako). As primary antibodies, we used antibodies to the following: anti-ubiquitin (ubiquitin, monoclonal mouse, 1:250; Millipore), anti-TDP-43 (TARDBP, polyclonal rabbit, 1:2500; ProteinTech), anti-phosphorylated TDP-43 (pTDP-43 ser409/410, polyclonal rabbit, 1:2500; CosmoBio), anti-phosphorylated tau (AT-8, monoclonal mouse, 1:4000; Innogenetics), anti- β -amyloid (β -amyloid 6F/3D, monoclonal mouse, 1:100; Dako), anti-CD68 (CD68, monoclonal mouse, 1:200; Dako), anti-cystatin C (cystatin C, polyclonal rabbit, 1:200; Dako), p62 N-terminal (p62N, polyclonal