RESEARCH PAPER

Spinocerebellar ataxia type 36 exists in diverse populations and can be caused by a short hexanucleotide GGCCTG repeat expansion

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ABSTRACT

Objective Spinocerebellar ataxia 36 (SCA36) is an autosomal-dominant neurodegenerative disorder caused by a large (>650) hexanucleotide GGCCTG repeat expansion in the first intron of the NOP56 gene. The aim of this study is to clarify the prevalence, clinical and genetic features of SCA36.

Methods The expansion was tested in 676 unrelated SCA index cases and 727 controls from France, Germany and Japan. Clinical and neuropathological features were investigated in available family members.

Results Normal alleles ranged between 5 and 14 hexanucleotide repeat expansions. Expansions were detected in 12 families in France (prevalence: 1.9% of all French SCAs) including one family each with Spanish, Portuguese or Chinese ancestry, in five families in Japan (1.5% of all Japanese SCAs), but were absent in German patients. All the 17 SCA36 families shared one common haplotype for a 7.5 kb pairs region flanking the expansion. While 27 individuals had typically long expansions, three affected individuals harboured small hexanucleotide expansions of 25, 30 and 31 hexanucleotide repeat-units, demonstrating that such a small expansion could cause the disease. All patients showed slowly progressive cerebellar ataxia frequently accompanied by hearing and cognitive impairments, tremor, ptosis and reduced vibration sense, with the age at onset ranging between 39 and 65 years, and clinical features were indistinguishable between individuals with short and typically long expansions. Neuropathology in a presymptomatic case disclosed that Purkinje cells and hypoglossal neurons are affected.

Conclusions SCA36 is rare with a worldwide distribution. It can be caused by a short GGCCTG expansion and associates various extracerebellar symptoms.

INTRODUCTION

Spinocerebellar ataxia (SCA) is a group of autosomal dominant neurodegenerative disorders clinically showing adult-onset progressive cerebellar ataxia often complicated with various extracerebellar signs or symptoms.1–3 SCA36 is caused by a hexanucleotide GGCCTG repeat expansion in the first intron of the NOP56 gene located on human chromosome 20.4 In normal individuals, the repeat is a polymorphic and complex sequence containing slightly different hexanucleotides [AGCCTG], [GGCCTG] repeat and [CGCCTG], and the entire hexanucleotide repeats range in length between 3 and 14 units.4–5 In contrast, this GGCCTG repeat was previously described as being highly expanded in SCA36 patients, reaching between 6.8 kb and ~18 kb pairs in Southern blot analysis, which was roughly estimated to be between 650 and 2500 repeat-units.4–6 Given that the expansion lies in the first intron (i.e., a non-coding region) of NOP56, SCA36 is regarded as one of the neurological diseases caused by non-coding microsatellite repeat expansion.7–10 Clinically, individuals with SCA36 show progressive cerebellar ataxia complicated by motor neuron dysfunction, which is particularly prominent in their tongues.4,8 So far, SCA36 has been described only in western regions of Japan4,6 and the Costa da Morte region of Spain,9 suggesting that there could be strong founder effects.

We conducted this study to clarify whether SCA36 is seen beyond these founder populations by investigating the SCA36 hexanucleotide repeat in a cohort of 676 families comprising mostly French, German and Japanese SCA index cases collected across these countries. Besides genetic analysis, we investigated clinical and neuropathological features in available family members to clarify how this disease differs from other SCAs.

MATERIALS AND METHODS

Patient and control enrollment

We enrolled 676 index patients with SCA comprising 270 French, 175 German and 231 Japanese families. Eighty-seven per cent of the French families were of French Caucasian origin, while the remaining 13% were the descendants of other ethnic populations. The German cohort was Caucasian, and the Japanese cases were of single ethnicity. These index patients were all excluded for previously known SCA mutations, which...
accounted for about 50% of all SCAs in their cohorts. All patients were collected from diverse areas of their respective countries: Pitié-Salpêtrière University Hospital and through the SPATAX network (France; http://spatax.wordpress.com/), Ludwig-Maximilians-University Munich (Germany), University of Tübingen (Germany) and Tokyo Medical and Dental University (Japan). A half of the Japanese SCA cohort was collected from the Tokyo Metropolitan area, and thus the present Japanese cohort is based on a population distinct from the previous studies. Seven hundred and twenty-seven healthy controls comprising 186 French, 304 German and 237 Japanese were also recruited.

From each participant, peripheral blood was drawn after obtaining informed consent following bioethics guidelines from each country. When index patients were found to be positive for SCA36 expansions, other available family members were further investigated. The study conformed to the tenets of the Declaration of Helsinki, and was ethically approved by each institutional review board.

**Mutation screening**

The hexanucleotide repeat expansion was screened by both repeat-primed PCR and PCR-fragment analyses using primer pairs flanking the hexanucleotide repeat. The conditions of these reactions were determined using a DNA sample from an original patient with SCA36 (Pedigree B, II-3). Primer sequences are shown online (see online supplementary table). The forward primer used for the repeat-primed PCR and PCR-fragment analyses (5′-TTTCCGGCTGCGTTCGGG-3′) was set between the 6 bp (base-pairs) CGGGCG insertion/deletion polymorphism (rs28970277) and the GGCCTG repeat sequence, allowing us to detect only the hexanucleotide repeat complex containing the GGCCTG repeat. The numbers of repeat-units in selected individuals, both controls and SCA36 individuals with short expansions, were determined by cloning in pCR-TOPO (Invitrogen, California, USA) followed by sequencing analysis. PCR products were finally separated and analysed in an ABI PRISM 3100 (Applied Biosystems) and 2% agarose gel. Segregation of genotype and phenotype was analysed in all available family members. For every individual discovered to harbour the expansion, the source of transmission was analysed by tracing and identifying the parent who had transmitted the mutation. Then the bias of parental gender in massive contraction was determined.

Southern blot analysis was carried out in 10 selected SCA36 samples using Avr II (New England Biolabs, Ipswich, Massachusetts, USA) and 0.8% agarose gel. A 900 bp probe was synthesised from genomic DNA by PCR, as shown online (see online supplementary table). Using this probe, normal controls show a single 3.5 kb band.

**Haplotype analysis**

We investigated all available Japanese and French SCA36 individuals for the microsatellite markers D20S906, D20S179, D20S113, D20S198, D20S842, AFMa049yd1, D20S181 and D20S193. The genotypes were expressed with an allele numbering consistent with that of a previously published individual (II-3 in Pedigree 3). In addition, eight informative single-nucleotide polymorphism (SNP) markers within NOP56 were further tested to assess the founder haplotype. Six SNPs were in the 5′-untranslated region (5′-UTR) and two were in intron 3. No informative SNPs were found in intron 2. We could reconstruct SNP haplotypes in every SCA36 individual with long expansion by using a forward primer ‘NOP56-5′-UTR-F’ in the 5′-UTR and a reverse primer ‘NOP56 intron 3-R’ in intron 3 (see online supplementary table). This was because the normal allele was specifically amplified in the presence of long hexanucleotide repeat expansions. Allele frequencies were investigated in Japanese (n=9) and French (n=10) control individuals.

**Clinical investigations**

Data on clinical features were collected by neurologists in charge of each participant with SCA36. The clinical features were retrospectively reviewed for three Japanese patients (Chubu #1, #2 and Chugoku) and four French participants (AAD-508 #5 and #7, AAD-681 #7 and #11), as they had been examined long before the identification of the SCA36 mutation. The rest of the Japanese and all the French SCA36 patients were clinically re-analysed and summarised in one common format. The correlation between the clinical features and the length of the expansion was statistically analysed by Welch’s test.

**Neuropathological analysis**

An autopsy was undertaken in the individual AAD-508 #7, who died at the age of 83. This participant did not show obvious neurological dysfunction. After formaldehyde fixation, multiple samples of the brain were embedded in paraffin and sectioned at a thickness of 5 μm. The spinal cord was not available. The sections were stained with H&E. Immunohistochemistry was performed using the following primary antibodies: antibiiquitin (Dako, rabbit polyclonal, diluted in phosphate buffered saline: 1/500), anti-TAR DNA-binding protein 43 (TDP-43) (Protein Tech Group, rabbit polyclonal, 1/2000), antifused in sarcoma (FUS) (Sigma, rabbit polyclonal, 10 μg/mL) and anti-p62 (MBL, mouse monoclonal, 1/300). Appropriate positive-control and negative-control specimens were also stained to check the staining conditions. The primary rabbit antibodies were detected with appropriate secondary antibodies using the XT Ultraview DAB system (Ventana, Oro Valley, Arizona, USA). The anti-p62 antibody was detected with the Vectastain ABC mouse IgG kit (Vector Laboratories, Burlingame, California, USA), and visualised by using Histofine Simple Stain DAB (Nichirei Bioscience, Tokyo, Japan) according to the manufacturer’s protocol.

**RESULTS**

**Molecular results**

The PCR-fragment analysis revealed that normal repeats ranged from 5 to 14 complex hexanucleotide repeat-units in our cohorts of 727 control individuals. The distribution of normal repeats was basically identical among French, German and Japanese controls (see online supplementary figure 1). The most common allele carried 9 complex hexanucleotide repeat-units and the normal upper limit of our cohort was 14 complex hexanucleotide repeats as described by García-Murias et al.

The repeat-primed PCR analysis disclosed GGCCTG repeat expansions in 17 index cases from 17 families (12 from the French cohort, including one individual each from Chinese, Portuguese and Spanish families living in France and five Japanese; figures 1 and 2A). Thus, SCA36 accounted for 1.9% of all SCAs of the French cohort and 1.5% of all Japanese SCAs, both including the families with already known mutations in other SCA genes. No expansions were found in the German cohort. Further investigations additionally revealed hexanucleotide expansions in a total of 30 individuals with the SCA36 GGCCTG expansion.

On the PCR-fragment analysis, normal participants often showed two different peaks (figure 2B). On the other hand,
Figure 1 Twelve French and 4 Japanese SCA36 families. The French families consisted of 9 originally French kindred, one for each of Portuguese, Spanish and Chinese descendants (the origins are not shown to protect from personal identification). In the family AAD-352, an individual with short expansion (#6) and his nephew (#18) with typically long expansion are observed. Note that all three individuals with short expansions (AAD-709 #6, French AAD-709 #15 and Japanese Kanto-1) had inherited the disease from their mothers. Owing to ethical reasons, pedigree structures are simplified and genders of some participants are anonymised. The pedigree information on the Japanese family was not available and thus not shown in this figure.

*Genetically tested; grey symbols: individuals without any complaints of neurological disturbances, or those not examined by the authors.

27 of the 30 SCA36 individuals showed single peaks within the normal range, suggesting that these patients harbour typically long expansions that hinder amplification by ordinary PCR (figure 2C). The remaining three (two French [AAD 709 #15 and AAD 352 #6] and one Japanese [Kanto-1]) showed two peaks on the PCR-fragment analysis, the larger one of which was always mosaic and exceeded the normal repeat range (figure 2D, E). Cloning and subsequent sequence analysis revealed that the longer repeats ranged between 25 and 31 complex hexanucleotide repeats containing 21 to 28 GGCCCTG repeats: AAD 709 #15 (with 25 hexanucleotide repeats): 5'-[AGCCCTG]-

(JGCCCTG)3-3; AAD 352 #6 (with 31 hexanucleotide repeats): 5'-[AGCCCTG]-

(GGCCCTG)3-24([GCCCTG]2-3; Kanto-1 (with 26 hexanucleotide repeats): 5'-[AGGCCC]-

(GGCCCTG)3-25([GCCCG]-[GCCCTG]3). There was no affected individual without the expansion as far as the available DNA samples were tested, supporting the theory that the expansion segregated with the disease in all families examined. In addition, an individual with a typically long expansion and another affected individual with a short expansion were present in the same French family AAD352 (figure 1). Southern blot analysis disclosed that the typically long expansion ranged between 800 and 2000 repeats (figure 2F). In addition, a broad band was demonstrated from individuals harbouring short expansions, supporting the theory that the expansions in these three participants were indeed very small. All three individuals with short expansions (AAD-709 #15, AAD-352 #6 and Kanto-1) had received the disease from their mothers (figure 1). However, it was not certain whether the short expansions in the three individuals were from contractions, as we could not directly test the transmission in a parent-offspring basis.

As we found SCA36 families from diverse ethnic origins, we tested if SCA36 families harboured different haplotypes. Genotype data from all available participants are summarised in table 1. We found a common haplotype in all SCA36 individuals irrespective of the ethnicity (Japanese, Chinese, Portuguese and French) for the SNP markers flanking the GGCCCTG repeat in NOP56 and for D20S198, only 7 kb away from the repeat. On the other hand, haplotypes diverged significantly among the families when we tested distant microsatellite DNA markers. For example, D20S842, which showed a conserved allele among Spanish SCA36 families, was discordant within the Japanese as well as the French SCA36 families. These data imply that SCA36, even with different ethnic origins, is associated with a common haplotype close to the repeat. However, the SNP haplotype common to all SCA36 families was also found in 26% of control chromosomes, and the frequency of allele 3 for D20S198 was 28% in Japanese controls and 55% in French controls.

Clinical results
Clinical information was available from 28 individuals with the expansion: 20 French and 8 Japanese (table 2). Among these, three French individuals (AAD-508 #5, #7 and SAL-334 #9) did not complain of any neurological disturbances, and were thus excluded from the evaluation of clinical features. The age at onset defined by the time when patients started to notice cerebellar signs was 50.4±7.2 (SD) years, with a range of 39–65 years in the remaining 25 individuals. The cardinal clinical feature was progressive cerebellar ataxia in all 25 symptomatic individuals. Other frequent involvements included (1) hearing impairment (6 French, 1 Chinese, 1 Portuguese and 7 Japanese, a frequency of 60% among the 25 individuals), (2) postural tremor (5 French and 2 Japanese; 28%), (3) ptosis (2 French and 4 Japanese; 24%) and (4) cognitive impairment (3 French, 1 Spanish and 2 Japanese; 24%). Reduced vibration sense was seen in 13 (6 French, 1 Portuguese and 6 Japanese; 52%).

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Evidence of lower motor neuron signs, such as tongue atrophy and bulbar signs, was present in two French symptomatic participants and five Japanese individuals (28%). On ancillary investigations, eight patients (4 French and 4 Japanese) who were tested by peripheral nerve conduction study revealed reduced sensory action potentials (SNAPs) with an overall frequency of 32%, suggesting that peripheral nerves can be affected by this disease. Among the 14 participants examined by MRI, all showed cerebellar atrophy (100%), with some cases also exhibiting additional atrophy in the cerebrum (n=2; 14.3%) and in the brainstem (n=4; 28.6%).

Regarding the genotype-phenotype correlations, the average age at onset in the three individuals with short expansions (57.3 years) tended to be later than that of patients with long expansions (49.4 years, n=22). However, this difference was not significant (p=0.408, Welch’s test).

Neuropathological findings
The neuropathological examination of patient AAD508#7 revealed diffuse cortical cerebellar atrophy. Histology demonstrated a mild Purkinje cell loss with Bergmann’s gliosis (Figure 3A, black arrow), distorted dendrites and atrophic cell body of the Purkinje cells (Figure 3B, white arrows) and swellings of Purkinje cell axons called ‘torpedo’ (Figure 3B, a black arrow). The hypoglossal nucleus (outlined by four arrows in Figure 3C) showed a mild neuronal loss and gliosis (Figure 3D). No alteration was evident in the cochlear and pontine nuclei. Ubiquitin, TDP43, FUS and p62 immunohistochemistry were all negative. Neuropathological changes compatible with Alzheimer’s disease, such as numerous amyloid plaques of the Braak and Braak Stage IV,14 were also seen.

DISCUSSION
The present study disclosed that SCA36 is not confined to the western region of Japan and the Costa da Morte region of Spain,5 but instead shows a global distribution, including individuals of French, Portuguese and Chinese ancestry. Nevertheless, the frequency of SCA36 was low in both Japanese (1.5%) and French (1.9%) SCA cohorts: this level was much lower than the prevalence in Okayama, Japan (3.6%)6 and in Galicia, Spain (6.3%).5 The absence of SCA36 patients in the present German cohort may suggest that SCA36 has an uneven distribution in Europe. Despite their diverse ethnicity, all the present SCA36 families showed a single haplotype for the tested SNPs in D20S842, which was conserved in the Spanish families in Europe. Despite their diverse ethnicity, all the present SCA36 families showed a single haplotype for the tested SNPs in NOP56 and the nearby microsatellite D20S198, whereas their haplotypes diverged for distant microsatellite markers including D20S842, which was conserved in the Spanish families in Galicia.5 This suggests that SCA36 repeat expansions arose from one or a few founder chromosomes in ancient times. Compared to SCA10, another non-coding repeat expansion disorder with a strong founder effect particularly prevalent in Central and South American countries,15 SCA36 repeat expansion might have arisen in a much ancient era. However, we noted that the founder SNP haplotype was still common with a frequency of 26% of our control French and Japanese chromosomes. Therefore, it is necessary to find markers that are tightly linked to SCA36 individuals and then to test them on larger numbers of SCA36 families in order to draw a more definitive conclusion on founder effects.

This study also disclosed that SCA36 shows Purkinje cell dropout and neuronal loss of the hypoglossal nucleus, consistent
## Table 1  Haplotype information on Japanese and French SCA36 families

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The location of the GGCCCTG repeat is shaded in light blue. The Japanese founder haplotype in the Okayama family (see reference #4) and its extent shared in other families are shown by bold boxes. The haplotype shared by Japanese families is coloured in grey. The French haplotypes are coloured in yellow (the most common one), green and purple. The allele frequency of ‘3’ in D20S198 is 28% in the Japanese (+) control population and 55% in the French (*) control population. NA stands for single-nucleotide polymorphic markers that have not been analysed. The 5th Japanese family and the 12th French SCA36 family (SAL-367) were not able to assess their haplotype and therefore are not listed in table 1.
<table>
<thead>
<tr>
<th>Family code</th>
<th>French families</th>
<th>ID</th>
<th>Approximate repeat length sex</th>
<th>Age at examination years (onset)</th>
<th>Sign at onset</th>
<th>SARA (examined age)</th>
<th>Cerebellar gait (age when confirmed)</th>
<th>Dysarthria</th>
<th>Reflexes in lower limbs</th>
<th>Vibration at ankles</th>
<th>Ocular findings</th>
<th>Hearing impairment</th>
<th>Postural tremor</th>
<th>Ptosis</th>
<th>Cognitive impairment</th>
<th>Lower motor neuron sign</th>
<th>Electrophysiology (examined age)</th>
<th>Other features</th>
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Table 2

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<th>Stage</th>
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<th>Cerebellar gait</th>
<th>Dysarthria</th>
<th>Refexes in lower limbs</th>
<th>Vibration at ankles</th>
<th>Ocular findings</th>
<th>Hearing impairment</th>
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<th>Ptosis</th>
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<th>Lower motor neuron sign</th>
<th>Electrophysiology</th>
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<td>Fasciculation and atrophy in tongue and hypothenar region</td>
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<td>Sensory axonopathy in medial plantar nerve</td>
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</table>

The three participants without complaints (AAD-508 #5, #7 and SAL-334 #9) are shaded and are excluded for calculating frequencies of neurological signs.

Stage 0: normal; 1: no functional handicap but signs at examination; 2: mild, able to run, walking unlimited; 3: moderate, unable to run, limited walking without aid; 4: severe, walking with one stick; 5: walking with two sticks; 6: unable to walk, requiring wheelchair; 7: confined to bed.

The confirmed age is the age at examination unless described specifically in parentheses.

NA, not assessed.

ICARS, International Cooperative Ataxia Rating Scale; EMG, electromyogram.
with the first autopsy case of SCA36. What is important from the present case is that the patient presented evidence of neurodegeneration in the Purkinje cell and hypoglossal nucleus without showing obvious neurological signs. It would become important in future to accumulate knowledge on the extent of neurodegeneration in a pre-manifesting stage for deciding when to administer fundamental treatment. Another important finding is that there were no obvious neuronal cytoplasmic inclusions (NCIs) or nuclear inclusions (NNIs) characterising the neuropathology of amyotrophic lateral sclerosis (ALS) as far as we examined by ubiquitin, TDP43, FUS and p62 immunohistochemistry. It has been shown that the frontotemporal lobar dementia and ALS (FTD-ALS) caused by C9orf72 hexanucleotide GGGGCC repeat expansion shows the accumulation of ubiquitin-immunoreactive and p62-immunoreactive NCIs, not only in the anterior horn cells in the spinal cord, but also in the cerebellar granule cells. Large ubiquitin-positive NIs seen in fragile X-associated tremor/ataxia syndrome (FXTAS) were also not detected in this case. These findings may suggest that SCA36 pathologial features are distinct from those of non-coding repeat expansion disorders. Future investigations should search for abnormal RNA structures (‘RNA foci’) in these affected neuronal cells, as have been detected in other related diseases caused by repeat expansions in introns; myotonic dystrophy type 2 (DM2), SCA10 and SCA3.[5, 6] The most intruiging finding in this study is that the short hexanucleotide expansion of 25–31 repeat-units, slightly exceeding the upper limit in controls (14 repeat-units), is seen in some affected individuals. This indicates that such short expansions could cause the disease, although we cannot exclude a possibility that repeat expansions are much longer in the nervous system than in the blood. As the number of patients with short expansions was very small in the present cohort, the difference in the age of onset between those with short and typically long expansions did not reach a significant level. Further studies including larger numbers of individuals with short expansions are thus necessary. In all the three individuals with short expansions, it was conceivable that the disease had been transmitted from their mothers, as in a previous description. However, we could not directly investigate parent-offspring pairs to confirm maternal bias for repeat contraction. If this was the case, SCA36 would be another example of such contraction after DM1 and a mother-to-daughter repeat contraction in Huntington’s disease. Precise knowledge of such parental bias is important for clinical situations, such as genetic counselling, as well as for determining the mechanism of repeat expansion.

Interestingly, short GGGGCC repeat expansion in C9orf72 has been identified in patients with FTD and Parkinson’s disease, suggesting a common feature in C9orf72 and NOP56 repeat expansions. What seems distinct from the C9orf72-associated neurological diseases is the fact that patients with short expansions in NOP56 do not obviously differ in their phenotypes from those with long expansions, while patients associated with expansions in C9orf72 show a wide spectrum of clinical symptoms depending on the length of the expansion. As such, how can we explain that the short and long GGCCTG expansions in NOP56 cause similar phenotypes? We investigated whether a short expansion of 21 GGCCTG repeats shows any difference in the likelihood of forming hairpin structures compared with the normal 14 repeats using a computer prediction algorithm. The 21-repeat allele was indeed predicted to form a double-stranded hairpin more efficiently than the 14-repeat allele, but with only a small difference. We need to recognise that the threshold of being a
pathogenetic repeat is much lower than was previously being thought. It is also possible that the mechanism underpinning SCA36 is similar to the RAN translation mechanism proposed in DM1, SCA8 and recently confirmed in C9orf72. In summary, SCA36 is present in various ethnic backgrounds, by the sharing of a common linked haplotype. Clinical variations with regard to lower motor neuron involvement, ptosis, hearing and cognitive impairments, tremor and reduced vibration sense suggest that this disease should be tested in all cases with progressive late-onset cerebellar ataxia. To do this, the PCR-fragment analysis as well as the repeat-primed PCR test are needed. Knowledge that a short expansion of at least 25 hexanucleotide repeats containing a stretch of 21 GGCCTG can cause the disease is an important source of information regarding genetic diagnosis and for future deciphering of SCA36 pathogenesis.

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24 AV, YD, YK and YI examined the patients. CD performed the neuropathological study and partially wrote the manuscript. NS examined the patients and investigated the DNA samples with MO. NS performed the neuropathological study. CT managed the DNA samples and analysed the genetic data. H-EW collected the Munich-based German control samples with TI. TI collected the Munich-based German control samples together with H-EW. JH analysed the large share of German DNA samples. HM co-ordinated the study. LS examined the Tübingen-based patients and collected the DNA. TK collected the German samples in Munich and wrote the manuscript. AB collected the French samples with GS and AD, coordinated the whole study and wrote the manuscript. KS collected the Japanese samples, examined the patients, analysed the DNA samples with MO and NS, coordinated the whole study and wrote the manuscript. AD examined the patients, collected and arranged the French samples and wrote the manuscript. All authors have read and approved the content of the manuscript.

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Competing interests
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