Genomic investigation of α-Synuclein multiplication and parkinsonism

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Abstract

Objective—Copy number variation is a common polymorphic phenomenon within the human genome. While the majority of these events are non-deleterious they can also be highly pathogenic. Herein we characterize five families with parkinsonism that have been identified to harbor multiplication of the chromosomal 4q21 locus containing the *α-synuclein* gene (*SNCA*).

Methods—A methodological approach employing fluorescent *in situ* hybridization (FISH) and Affymetrix 250K SNP microarrays (CHIPs) was used to characterize the multiplication in each family and identify the genes encoded within the region. The telomeric and centromeric breakpoints of each family were further narrowed using semi-quantitative PCR with microsatellite markers and then screened for transposable repeat elements.

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Results—The severity of clinical presentation is correlated with *SNCA* dosage and does not appear to be overtly effected by the presence of other genes in the multiplicated region. With the exception of the Lister kindred, in each family the multiplication event appears *de novo*. The type and position of Alu/LINE repeats are also different at each breakpoint. Microsatellite analysis demonstrates two genomic mechanisms are responsible for chromosome 4q21 multiplications, including both *SNCA* duplication and triplication.

Interpretation— *SNCA* dosage is responsible for parkinsonism, autonomic dysfunction and dementia observed within each family. We hypothesize dysregulated expression of wild-type α-synuclein results in parkinsonism and may explain the recent association of common *SNCA* variants in sporadic Parkinson's disease. *SNCA* genomic duplication results from intra-allelic (segmental duplication) or inter-allelic recombination with unequal crossing-over, whereas both mechanisms appear to be required for genomic *SNCA* triplication.

Keywords

Parkinsonism; *SNCA*; Genomic multiplication; Alu repeat; Parkinson's disease

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Introduction

The human genome displays a considerable level of inter-individual variability from simple single nucleotide polymorphisms (SNPs) and short repeats to large-scale deletions, multiplications and rearrangements. Recent studies have demonstrated that large gene copy number variations (CNVs) occur frequently in the general population with no determinable disadvantage to carriers. However this phenomenon can also be pathogenic and result in severe disease phenotypes [1–3].

In 2003, Singleton and colleagues reported a triplication on one allele of the chromosomal locus (4q21) containing the *α-synuclein* gene (*SNCA*) in affected members of a family with parkinsonism known as the Iowan kindred [4]. Although a relatively rare event, several families have since been described that carry multiplications of this region including both triplications and duplications which segregate with disease [5–11]. The severity of the clinical phenotype of *SNCA* duplication and triplication families appears to be associated with gene dosage and mRNA/protein expression levels in brain [6]. The *SNCA* duplication families are generally reminiscent of typical, late-onset Parkinson's disease (PD) [5, 7, 9], while the two families (Iowan & Swedish-American) with monoallelic triplication of *SNCA* present with a severe form of early-onset parkinsonism with autonomic dysfunction and subsequent dementia [6, 12].

In the Iowan kindred, the region triplicated is reported to contain seventeen gene transcripts (1.6–2.1Mb) whereas in both French and Japanese patients much smaller genomic intervals are duplicated (~0.5Mb) [4, 5, 7, 9]. Although *SNCA* multiplication appears necessary for parkinsonism, whether increased dosage of adjacent genes contributes to the phenotype is unclear. The mechanism underlying chromosome 4q21 genomic multiplication has also to be elucidated. The region appears to be evolutionarily fragile given the spontaneous deletion of *SNCA* within an inbred strain of C57BL/6J (OlaHsd) mice, albeit with no apparent deleterious effects [13, 14].

Herein we compare the phenotypes of *SNCA* multiplication families and present data on the genomic copy number, size and breakpoints for each 4q21 multiplication mutation, using a combination of fluorescent *in situ* hybridization (FISH) and Affymetrix 250k SNP microarrays (CHIPS). Within each interval/family, we detail the genes with aberrant copy number and expression. We characterize the transposable repeat elements at each breakpoint

and provide a mechanistic hypothesis for the genomic instability, rearrangement and multiplication of this locus.

Subjects and Methods

Frequency, Clinical manifestations and Neuropathology

The frequency of *SNCA* multiplication is low and appears to be a relatively rare event [15]. World-wide seven families have been identified that harbor *SNCA* multiplication; one triplication (Iowa, US) [4, 6, 12], five duplication kindreds (2 French, 2 Japanese and 1 Italian) [5, 7, 9, 11], and one kindred with individuals with either duplication or triplication mutations (Swedish-US, now recognized as a branch of the 'Lister family complex') [6, 11]. The clinical presentation and available pathological findings for five of these families are summarized in Table 1. In the preparation of this manuscript Ahn and colleagues reported the first *SNCA* duplication patients in Korea[16]. Intriguingly, of the three PD patients identified only one is described with a family history of parkinsonism. This familial *SNCA* duplication patient presented with symptoms at age 40 years and initially had a good response to l-DOPA therapy, however the disease course progressed rapidly with postural hypotension, personality changes and dementia by the age of 46 years. The two sporadic patients presented with typical PD with ages at onset of 50 and 65 years. These alternate clinical presentations demonstrate the phenotypic range of *SNCA* multiplication symptoms.

The clinical phenotype in the *SNCA* triplication families is rapid, progressive parkinsonism with onset in the third and fourth decades. Movement disorder (resting tremor, bradykinesia and rigidity) occurs early in the course with autonomic dysfunction (including hyposmia and orthostatic hypotension) and neuropsychological impairments (hallucinations, anxiety, paranoia and depression), with subsequent cognitive decline and dementia. The neuropathology of *SNCA* triplication patients is reminiscent of diffuse Lewy body disease with numerous α-synuclein-positive Lewy bodies, Lewy neurites and glial cyoplasmic inclusions, with neuronal cell loss in the *substantia nigra* and *locus ceruleus*. Extensive neuronal loss is also observed in the hippocampus CA2/3 region and is a feature of both missense and multiplication *SNCA* mutations [6, 17].

In contrast, most patients in *SNCA* duplication families present with signs and symptoms that closely resemble idiopathic PD. Onset of motor symptoms is in the fifth to sixth decades, neither cognitive decline nor dementia are prominent and generally disease progression is slow with a sustained response to l-DOPA therapy [5, 7, 9]. However, with each report, clinical variability within and among *SNCA* duplication families becomes more extensive. For example, Japanese A and B families are noted for reduced penetrance among carriers; patients may have a long duration of disease, may exhibit signs of cognitive decline and dementia, and have either a mild or excellent response to l-DOPA therapy [9]. In contrast, affected carriers within Branch J of the Swedish Lister family initially present with dysautonomia (orthostatic hypotension and syncope) rather than motor problems but quickly develop rapidly progressive parkinsonism that is only poorly responsive to l-DOPA [11]. Neuropathological examination of *SNCA* duplication highlights α-synuclein pathology reminiscent of diffuse Lewy body disease comparable to that observed in *SNCA* triplication patients [18]. It is evident that disease onset in *SNCA* duplication carriers is several decades later than in *SNCA* triplication families [11]. While *SNCA* dosage appears responsible and sufficient for disease, clinical variability may reflect the size of the duplicated segment and the aberrant expression of the additional genes [9, 11].

Genetic analysis: FISH and CHIPS

Fluorescent *in situ* hybridization (FISH) was performed on Epstein-Barr virus (EBV)-immortalized lymphocytes from one affected member of each family as previously described, with *SNCA* PAC 27M07 (146 kb; AF163864) labeled using FITC and *SNCA* promoter and intron 4 fragments (13 and 21 kb) labeled with rhodamine 4. Samples were considered duplicated/triplicated if they had 3/4 FISH probe signals in greater than 20% of interphase cells scored, from 100 interphase nuclei examined. To exclude the possibility of an artifact of EBV-immortalization, semi-quantitative PCR was performed on genomic DNA extracted from blood and confirmed a multiplication of the region of chromosome 4 containing *SNCA* in all families. Affymetrix 250k SNP microarrays (CHIPS) genotyping and SNP dosage analysis was then performed on 250ng of total genomic DNA samples for the probands of each family as previously described11. Copy number was estimated using dChipSNP software with GTYPE exported genotype calls and signal intensities (.cel files) 19–21. This algorithm uses a rigorous 'within and between array' normalization method to compute estimates of the normal signal values for genotype calls observed with a set of arrays. Deviations from the normal signal values seen for any particular genotype in the set of abnormal DNA samples were compared with values observed for a set of 10 samples with normal 2N copy numbers throughout chromosome 4. Copy number changes in the probands and families were then inferred by median smoothing with a Hidden Markov Model applied. In our study, we present results based on a sliding window approach to average the inferred copy numbers across a continuous 250 kb stretch centered on each SNP, and for simplicity, only a single proband is shown for each nuclear family.

Using polymorphic microsatellite markers the centromeric and telomeric ends of the breakpoints were confirmed and further refined. Internal control peak height of heterozygous individuals were calculated and compared between patient samples, diploid, *SNCA* duplication and triplication samples to give copy numbers. These analyses confirmed the Affymetrix 250K SNP microarray results showing a different length of the multiplicated region in each family (Suppl Table 1).

Results

In each proband, all three *SNCA* FISH probes gave results that were consistent with Affymetrix dosage and microsatellite genotype analyses. Affymetrix CHIP dosage data was obtained from between 62 – 363 SNPs, within the chromosome 4q21 region of multiplication in each family. Illustrative results are shown for both *SNCA* duplication and triplication cell lines (FISH) and the proband of each family (CHIPS) (Figure 1). The longest region (4.93–4.97 Mb) is present in the French duplication family (also reported as FPD-131) and encompasses 31 transcripts, including genes associated with epileptic encephalopathy (*MAPK10*), type II dentinogensis imperfecta (*DMP1 & SPP1*), and polycystic kidney disease II (*PKD2*) (http://www.ncbi.nlm.nih.gov)(Figure 2). Five transcripts including *SNCA* are expressed at high levels in the brain (microarray expression data retrieved from UCSC website; http://genome.ucsc.edu/). In contrast, the shortest region (0.4 Mb) was observed in the Japanese B family with duplication of only *SNCA* and the 5' region of the *MMRN1* gene.

Microsatellite genotype analysis revealed *SNCA* genomic duplication results from intra-allelic (segmental duplication) or inter-allelic recombination with unequal crossing-over, whereas both mechanisms are required for genomic *SNCA* triplication (Figure 3). The reason for genomic instability and chromosome 4q21 rearrangement remains unclear. Thus VISTA software 22 was employed for comparison of the DNA sequences, short and long interspersed repeats (SINE/LINE) at the centromeric and telomeric ends of the multiplicated region in each family. It is reported that Alu repeats constitute ~10% of the human genome

23, 24, and mobile elements make up over 45% of the human genome 23. Although our analysis identified a number of transposable repeat elements with over 70% conservation, the proportion of sequence occupied by SINE or LINE repeats in the breakpoint regions was not greater than observed within flanking sequence. Rather than one specific repeat there were a variety of Alu sub-types at the 5' and 3'-ends of the *SNCA* multiplication regions (Suppl Table 2). Nevertheless, the presence of these genetically mobile elements can lead to genomic instability, unequal recombination and rearrangements that result in CNVs including multiplication or deletion 23, 25.

Discussion

Multiplication of the *SNCA* locus is now reported to account for a greater number of families with autosomal dominant parkinsonism than the known pathogenic α-synuclein missense substitutions (A30P, E46K and A53T) 26. However this is still a small number of familial patients, given that ~10–15% of patients with PD report a family history of disease.

Only multiplication of *α-synuclein* (*SNCA*) appears necessary for parkinsonism as Japanese kindred B has only full-length *SNCA* and the 5' end of *multimerin1* (*MMRN1*). Deficiency of *MMRN1*, a specific platelet Factor V/Va binding protein is associated with an inherited bleeding disorder, Factor V Quebec, although haploinsufficiency does not appear to be associated with any phenotype 27. *MMRN1* is increased in copy number in all other *SNCA* multiplication kindreds. It may be noteworthy that *γ-synuclein* (*SNCG*) and *multimerin 2* (*MMRN2*) lie in the same orientation to each other on human chromosome 10 (murine chromosome 14) suggesting that these paralogs arose due to an evolutionary duplication event. Gamma-synuclein, also known as breast cancer-specific protein 1, is elevated in cancer and may play a role in disease 28, 29.

Given the instability of the *SNCA-MMRN1* region, *SNCG-MMRN2* multiplications/deletions may yet be identified. Limited expression and functional data is available on other genes within regions of chromosome 4q21 multiplication. Nevertheless, only *SNCA* dosage appears to specifically contribute to the variability in clinical observations among families. Genetic and genealogic studies recently identified a Swedish family with *SNCA* duplication and a US family of Swedish descent with a *SNCA* triplication as branches of the "Lister kindred" 11 30, 31. Within the families examined, this was the only example of copy number changes from one generation to another. Earlier onset, faster progression and more fulminant disease are associated with increasing *SNCA* copy number, suggestive of 'genetic anticipation', a clinical phenomenon usually confined to small simple repeats such as in spinocerebellar ataxias 32.

It is remarkable that both segmental intra-allelic duplication and inter-allelic recombination with unequal crossing-over appear to be responsible for *SNCA* multiplications. Microsatellelite genotyping clearly demonstrates both mechanisms operate; duplication does not necessarily precede unequal crossing-over and the opposite may occur. While our study identified a number of large repeat elements at either ends of the multiplicated regions, no single repeat was consistently identified at the breakpoints of all multiplications (Suppl Table 2). Thus a variety of transposable repeat elements including Alu and LINE repeats may promote instability causing irregular gene duplication and recombination events. Cloning the exact multiplication breakpoints across repeat elements may yet be insightful. Rovelet-Lecrux et al. have reported similar multiplication events on chromosome 21, that involve the amyloid precursor protein gene and result in Alzheimer disease 33. The regions duplicated in five of these families ranged from 0.58 to 6.37Mb in size and differed in their haplotypic structure suggesting these multiplication events are also independent.

Ahn and colleagues recently reported two sporadic patients with *SNCA* duplication suggestive of age-related penetrance, as observed for other mutations causing parkinsonism16. The frequency and the direct relevance of *SNCA* multiplication to the vast majority of PD patients remains to be determined. The hypothesis that α-synuclein overexpression contributes to disease susceptibility predates the discovery of *SNCA* multiplications. A number of classical association studies have examined the Rep1 microsatellite (D4S3481) in the *SNCA* promoter, a region implicated in transcript expression by *in vitro* luciferase assays 34–37. Combined, pooled analysis by the Genetic Epidemiology of Parkinson's Disease (GEO-PD) Consortium observed a significant association with increasing Rep1 allele size, 259<261<263 base-pairs 38. On-line meta-analysis of all published studies also highlight a SNP (rs356165) in the 3' untranslated mRNA (www.pdgene.org). In contrast, genome-wide SNP association studies of PD have not highlighted common variants in *SNCA* suggesting their power to identify susceptibility genes is limited 39, 40. A reanalysis of genome-wide data highlighted copy number variants in *PARKIN* 41. The identification of heterozygous carriers and one homozygous early-onset patient demonstrates the method can detect both multiplications and deletions.

In vivo findings with respect to *SNCA* mRNA expression are inconsistent 42. We find a decrease in *SNCA* transcript levels in specific brain regions such as the surviving neurons of the *substantia nigra*, as well as the putamen and frontal cortex in subjects with PD (unpublished data) 43. Changes in mRNA expression in end-stage disease may compensate for the accumulation of α-synuclein protein but mRNA and protein expression levels have yet to be correlated within the same samples. Whether alternately spliced *SNCA* mRNA, predicted to lead to smaller isoforms (α-synuclein 98, 112 and 126) may also contribute to disease has yet to be determined.

The discovery of *SNCA* multiplication demonstrates aberrant α-synuclein expression is sufficient for parkinsonism and highlights a direct, dose response with age of onset, progression and symptom severity. Whether *SNCA* multiplication is a distinct entity, or a more aggressive form of typical PD, both are part of a spectrum of Lewy body disorders. The challenge is now to functionally translate genetic insights focused on *SNCA* into patient therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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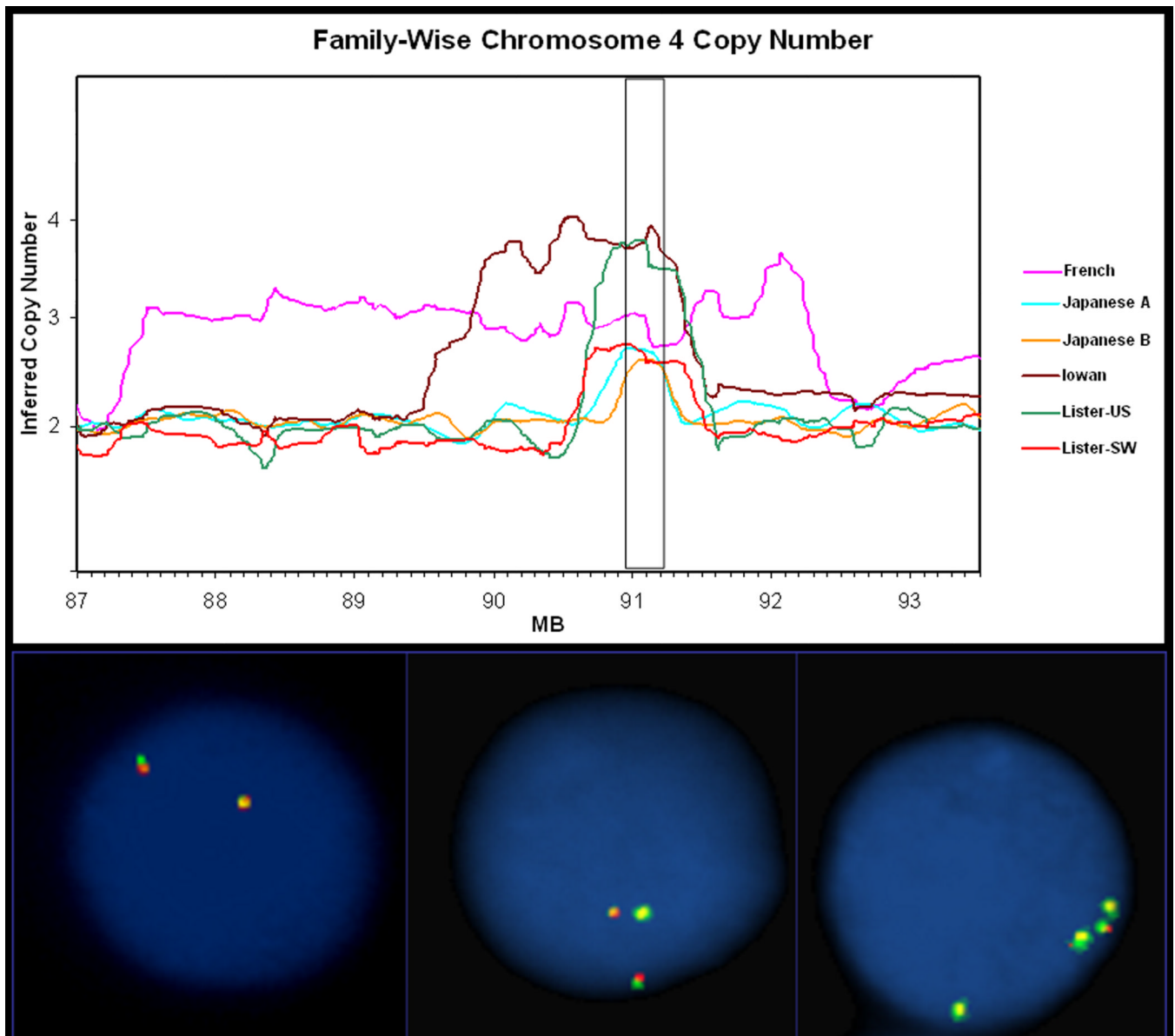
Figure 1. FISH and CHIPs

A representation of fluorescent *in-situ* hybridization (FISH) and Affymetrix 250k SNP microarrays (CHIPS) that were used to examine the region of multiplication in the proband of each family. (a) Relative copy number estimates were plotted against physical genomic position on chromosome 4. Raw data is shown that has not been normalized with respect to integers. (b) FISH was performed on interphase cells with three labeled *SNCA* probes directed at the entire locus (PAC 27M07 in GREEN), with promoter and intron 4 fragments (visualized in RED). *SNCA* multiplication was confirmed in all samples using both methodologies.
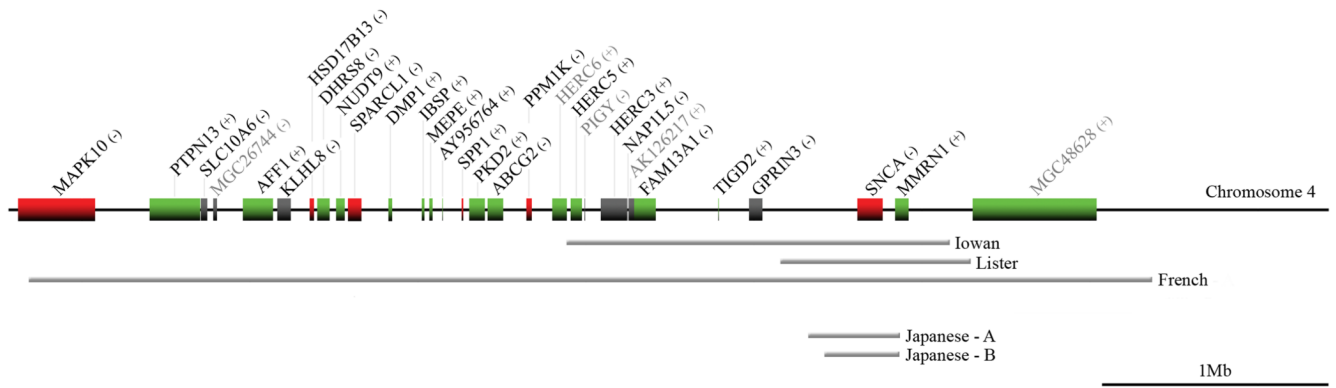
Figure 2. A representation of the genes in the multiplication region in each family
Displays the genes that are present in each of the multiplied regions of the families. The figure is drawn approximately to scale. The coding genomic DNA strand of each gene is indicated by (+) or (−). Genes are colored to represent their relative expression in brain according to the GNF Expression Atlas 2 (http://genome.ucsc.edu/), with red, black and green representing high, medium and low expression respectively. Gene symbols in gray text indicate hypothetical genes. The gray bars below the chromosome diagram show the regions of multiplication in each family.
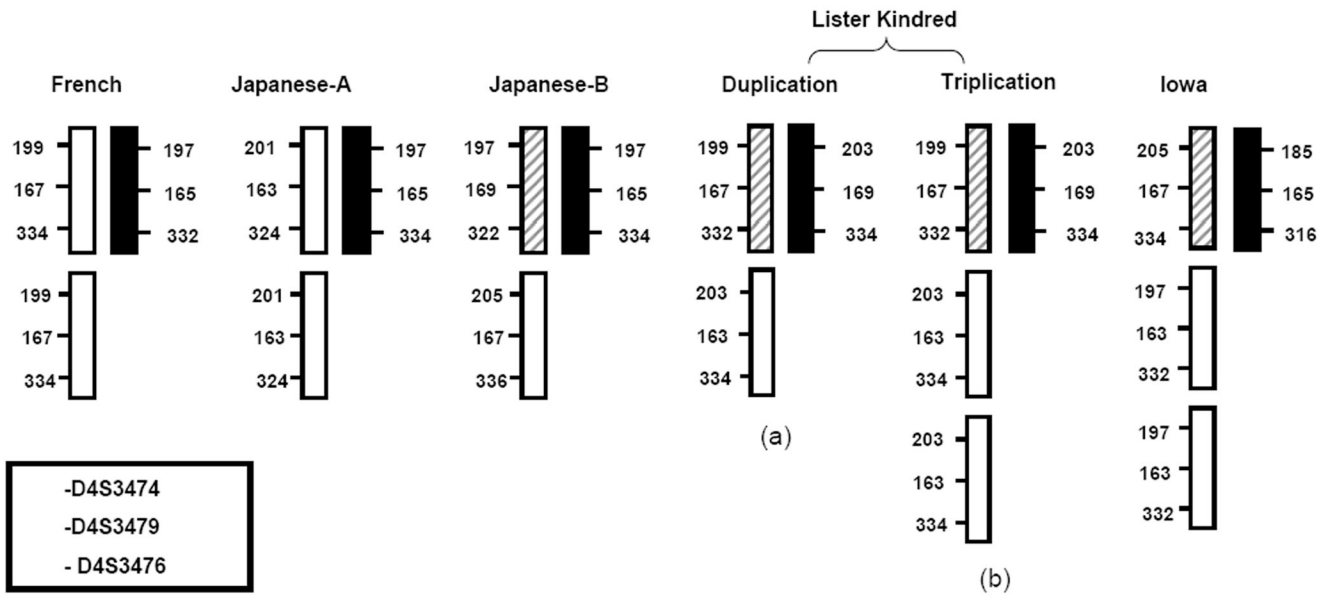
Figure 3. Legend
The allele sizes and dosage for the chromosome 4 markers D4S3476, D4S3479 and D4S3474 are shown for each family. These data demonstrate an intra-allelic, segmental duplication in the French and Japanese-A families. However inter-allelic recombination occurred initially in Japanese-B and the in Lister kindred duplication, indicated by the presence of three different alleles at marker D4S3476 (163, 167 & 169)(a). A further segmental duplication is apparent in the Lister kindred branch with *SNCA* triplication (b). It is not possible to ascertain the sequence of events for the *SNCA* triplication in the Iowa kindred but the presence of three alleles sizes at all markers demonstrates a recombination event must have occurred.

Table 1

Presents a brief summary of the major clinical and neuropathological characteristics of *SNCA* multiplication families. MMSE: Mini Mental State Examination; SN: *Substantia nigra*, CTX: Cortex, HC: Hippocampus, NBM: Nucleus basalis of Meynert; LC: *Locus coeruleus*, LBs: Lewy bodies, GCIs: glial cytoplasmic inclusions; na - no data available.

Kindred	Iowan	Lister-US	Lister-Swedish	French	Japanese–A	Japanese–B
SNCA multiplication	Triplication	Triplication	Duplication	Duplication	Duplication	Duplication
Number of patients with clinical data	12	3	5	5	3	1
Average age at onset (years)	34 (20–48)	(31– early 40s)	59 (40–71)	48 (35–65)	43 (38–77)	47
Rigidity	Yes	Severe, generalized	Yes	Yes	Yes	Yes
Bradykinesia	Yes	Yes	Yes	Mild to severe	Yes	Yes
Rest tremor	Some subjects: none to pronounced	Yes	Some subjects: mild to intermittent	Some subjects: none to pronounced	No	No
Postural tremor	Yes, not segregating with triplication	Yes	No	3 of 5	No	No
Postural instability	Yes, with falls	Mild to moderate	Pronounced, with falls	3 of 5	2 of 3	Yes
Myoclonus	Late	na	Late, in distal upper extremities	na	na	na
Response to l-DOPA	Yes	Dramatic effect initially	Slight	Yes	None to good	Slight
Orthostatic hypotension	Yes, some subjects, sometimes requiring drug treatment	Moderate to severe, early in illness, partially requiring drug treatment	Early, symptomatic, required drug treatment	No	No	No
Other dysautonomia	Erectile, cardiac, and gastrointestinal dysfunctions	Urinary incontinence late in illness	Moderate urinary incontinence, dysphagia	No	No	No
Dementia / cognitive dysfunction	Memory loss, visuospatial dysfunction, decline of executive functions: may present with these features (LBD phenotype, or may be late in course (PD phenotype)	Early, severe	Not prominent (late)	Not prominent	No	Yes
Paranoia, anxiety	Not prominent	Early, pronounced	Yes	na	na	na
Depression	Yes, may precede parkinsonism by a decade or more.	History of depression between age 13 and 19, suicidal later in illness	Yes	2 of 5	Yes	na
Hallucinations	Partial: some have prominent visual when phenotype is LBD	Pronounced, visual, auditory and olfactory	Visual, olfactory and auditory	No	No	Yes
Other remarks	Weight loss may be seen. Rapidly-progressive	na	Rapidly-progressive disease	na	Psychosis in 1 of 2	na

Kindred	Iowan	Lister-US	Lister-Swedish	French	Japanese–A	Japanese–B
SNCA multiplication	Triplication	Triplication	Duplication	Duplication	Duplication	Duplication
Neuropathology	Neuronal loss in SN and LC, extensive, pleomorphic and atypical LBs, GCIs, neuritic dystrophy, neuronal loss in HC (CA 2/3)	Neuronal loss in SN and LC (few LBs), NBM, CTX (widespread LBs), and HC (CA2/3)	na	na	na	Neuronal loss in SN, LC & HC (CA 2/3), Lewy neurites in the CA2, only a few LBs in SN and LC.