Mol Neurobiol
DOI 10.1007/s12035-017-0535-1

A Clinical and Molecular Genetic Study of 50 Families with Autosomal Recessive Parkinsonism Revealed Known and Novel Gene Mutations

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Received: 4 January 2017 / Accepted: 7 April 2017

Abstract In this study, the role of known Parkinson's disease (PD) genes was examined in families with autosomal recessive (AR) parkinsonism to assist with the differential diagnosis of PD. Some families without mutations in known genes were also subject to whole genome sequencing with the objective to identify novel parkinsonism-related genes. Families were selected from 4000 clinical files of patients with PD or parkinsonism. AR inheritance pattern, consanguinity, and a minimum of two affected individuals per family were used as inclusion criteria. For disease gene/mutation

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Published online: 13 May 2017

identification, multiplex ligation-dependent probe amplification, quantitative PCR, linkage, and *Sanger* and whole genome sequencing assays were carried out. A total of 116 patients (50 families) were examined. Fifty-four patients (46.55%; 22 families) were found to carry pathogenic mutations in known genes while a novel gene, not previously associated with parkinsonism, was found mutated in a single family (2 patients). Pathogenic mutations, including missense, nonsense, frameshift, and exon rearrangements, were found in *Parkin*, *PINK1*, *DJ-1*, *SYNJ1*, and *VAC14* genes. In conclusion, variable phenotypic expressivity was seen across all families.

Keywords Early-onset · Parkinson's disease · Pathogenic mutations · Genotype-phenotype correlations

Introduction

Parkinson's disease (PD; MIM# 168600) is the second most common neurodegenerative disorder, with the prevalence of about 1% in people over 60 years of age [1]. PD results from degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of proteinaceous inclusions called Lewy bodies (LBs) in the surviving neurons [2]. PD is characterized by the presence of motor symptoms, including resting tremor, bradykinesia, rigidity, postural instability, stooped posture, and freezing, as well as non-motor symptoms, such as fatigue, cognitive, behavioral and sensory phenotypes, sleep disorders, and autonomic dysfunction [3, 4]. Despite that the majority of PD is sporadic and is thought to be caused by a combination of genetic and environmental risk factors, approximately 5–10% of patients have monogenic forms of the disease with either an autosomal dominant (AD) or autosomal recessive (AR) Mendelian pattern of inheritance [5, 6]. To date, several genes have been found to be mutated in monogenic PD, with nine genes being involved in the pathogenesis of AR PD (ARPD) and/or juvenile parkinsonism (ARJP). These include *Parkin* [6q26; MIM# 600116], *PINK1* [1p36.12; MIM# 605909], *DJ-1* [1p36; MIM# 606324], *ATP13A2* [1p36; MIM# 606693], *FBXO7* [22q12.3; MIM# 260300], *PLA2G6* [22q12.3; MIM# 612953], *DNAJC6* [1p31.3; MIM# 608375], *SYNJ1* [21q22.2; MIM# 615530], and *VPS13C* [15q.22.2; MIM# 616840] [7–16]. Overall, missense, nonsense, splice site, frameshift mutations, and whole exon and gene deletions/duplications have been identified in all forms of PD, including AD, AR, and sporadic PD/parkinsonism [17–19].

In this study, we investigated the known autosomal recessive PD/parkinsonism genes in 50 Iranian consanguineous families with ARPD or ARJP and identified *VAC14* as a novel gene for hereditary progressive dystonic tremor and disabling dystonia.

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Materials and Methods

Subjects

As part of a large multi-center study, we investigated 4000 clinical files, which belonged to Iranian patients with a diagnosis of parkinsonism. Tables 1 and 2 include information about the age at onset, pattern of inheritance, and the presence of consanguinity of all clinical files examined. Early-onset was considered when the disease symptoms begun before age 46, while patients with late-onset disease developed their symptoms at the age of 46 or later [20]. A total of 50 recessive families (116 patients) with at least two patients being born to consanguineous parents were selected to be examined. Parents did not show any sign of parkinsonism or other movement disorder. Selected families were from various parts of Iran, and they belonged to different ethnicities. Expert neurologists from different clinical centers examined selected families and confirmed their diagnosis. The local ethics committees at each participating medical center approved this study, and informed consent, according to the Declaration of Helsinki, was obtained from all participants. DNA samples from ethnicity-matched neurologically normal individuals were also available ($n = 96$). DNA samples from all participants were isolated from whole blood, using standard procedures.

Genetic Analysis

Multiplex Ligation-Dependent Probe Amplification and Quantitative PCR Assays

The salsa multiplex ligation-dependent probe amplification (MPLA) kit P051-c1/P52-c1 (MRC-Holland, Amsterdam, The Netherlands) was used to detect large deletions or duplications, which were later confirmed by using gap PCR or relative quantification of implicated exons. The relative quantification was performed and analyzed by using the Eco Real-

Table 1 Distribution of patients according to the age of onset and recurrence in the family

Age of onset	Familial (%)		Sporadic (%)	Total (%)
	AD	AR		
Early onset	22 (2.2)	215 (22.2)	736 (75.6)	973 (100)
Late onset	272 (9)	160 (5.3)	2595 (85.7)	3027 (100)
Total	294 (7.3)	375 (9.4)	3331 (83.3)	4000 (100)

AD autosomal dominant, *AR* autosomal recessive

Time PCR System following the $2^{-\Delta\Delta Ct}$ method and as described elsewhere [21, 22].

Linkage Analysis

Families who showed no mutations in the MLPA assays were subject to linkage analyses. Linkage analyses were carried out by using short tandem repeat (STR) markers, covering the nine known genes involved in ARPD or AJP. At least six different markers were selected for each gene to be examined. STR markers were amplified by PCR, and the products were analyzed by polyacrylamide gel electrophoresis (8%).

To reduce the number of candidate genes in one family without mutations in known PD genes (F23), all available family members were subject to genome-wide SNP genotyping (HumanOmniExpress Exome arrays v1.3; Illumina Inc., San Diego, CA, USA) and genotyping data was used to perform homozygosity mapping as previously described [15, 23].

Whole Genome Sequencing

Two affected individuals from three different families ($n = 6$) without mutations in known PD genes were subject to whole genome sequencing (WGS) analyses. WGS was carried out at the New York Genome Center (NYGC). Sequencing libraries were constructed with the TruSeq PCR-Free Library kit (Illumina) following the manufacturer's recommended protocol. Libraries were sequenced on the Illumina HiSeq X instruments, with 2 × 150 bp paired reads, to a minimum coverage of >30×. Sequencing data was processed with NYGC's automated analysis pipeline, which includes alignment to GRCh37 using BWA-MEM (v0.78) [24], and further processing with GATK Best Practices, including the marking of duplicates with Picard (v1.83, http://picard.sourceforge.net) and GATK (v3.2.2) [25]. Single-nucleotide variations (SNVs) and indels were called by using the GATK HaplotypeCaller and were jointly genotyped. Deletions were called by using Genome STRiP (v2.0) [26] and were jointly called by using 17 HapMap individuals (CEPH Platinum Genomes pedigree). All deletions annotated as PASS in the Genome STRiP results were further filtered by using custom scripts to remove redundant calls and breakpoints overlapping repeat regions, or with extensive mapping ambiguity. SplazerS, which identifies and split-aligns reads that cross structural variant breakpoints, was further used to determine the breakpoints of candidate deletions [27]. First, all reads mapping to the candidate region were extracted, and then by using SplazerS, they were mapped back to the region to identify and confirm the breakpoint locations. Annotations of variants included predictions of the effect of nucleotide change on protein sequence using SnpEff; variant frequencies in different populations from the 1000 Genomes Project, the NHLBI GO Exome Sequencing Project, cross-species conservation scores from PhyloP, Genomic Evolutionary Rate Profiling (GERP), and PhastCons; functional prediction scores from PolyPhen-2, SIFT, and Combined Annotation Dependent Depletion (CADD) [28]; variant disease associations from OMIM, ClinVar, and Genetic Association Database (GAD); regulatory annotations from ENCODE, RegulomeDB, ORegAnno, and KEGG pathway annotations; transcription factor binding sites from the TRANSFAC database; and Gene Ontology (GO) annotations for biological process, cellular component, and molecular function.

Sanger Sequencing

Direct *Sanger* sequencing was used to examine the PD genes found to be associated with disease in one or more families

Table 2 Distribution of patients according to parents' consanguinity and number of patients in the family

Marriage	One affected (%)			More than one affected (%)		
	Late	Early	Sum	Late	Early	Sum
Consanguineous	151 (22.8)	512 (77.2)	663 (100)	45 (19.3)	188 (80.7)	233 (100)
Non-consanguineous	2444 (91.6)	224 (8.4)	2668 (100)	387 (88.8)	49 (11.2)	436 (100)
Total	2595 (77.9)	736 (22.1)	3331 (100)	432 (64.6)	237 (35.4)	669 (100)

and to validate the mutations identified through WGS. Primers, covering all exons and intron-exon boundaries of the genes of interest (*Parkin*, *PINK1*, *SYNJ1*, *VAC14*), or flanking the identified mutations in *Parkin* and *DJ-1* genes, were designed by using a public primer design website (http://ihg.gsf.de/ihg/ExonPrimer.html; primer sequences available upon request). PCR products were then purified, sequenced, and analyzed as previously described [15, 23].

Computational Prediction of Mutation Pathogenicity

The pathogenicity of the novel missense mutations identified within the *SYNJ1* and *VAC14* genes was predicted by several computational methods, including MutPred (http://mutpred.mutdb.org/) and SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/), previously evaluated as most efficient [29], as well as MutationTaster (http://www.mutationtaster.org/), SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and CADD [28]. The allele frequency of novel mutations was also investigated in the recent released "The Genome Aggregation Database" (gnomAD; http://gnomad.broadinstitute.org/).

Results

Genetic Analyses

Twenty-two out of 50 (44%) consanguineous families with parkinsonism were found to have PD due to mutations in known genes. The most prevalent mutated gene was *Parkin*, being found mutated in 18 different families. Exon deletions within the *Parkin* gene were the most common mutations, being present in 15 different PD families (Table 3). MLPA assays identified large *Parkin* deletions in 14 different PD families while a homozygous *Parkin* exon 5 deletion was identified through WGS in a single family (Family F3; Fig. 1a). Deletions in exons 3 and 5, respectively identified in four and three different families, were the most common deletions. The breakpoints of the *Parkin* exon 5 deletion identified through WGS were determined by using both Genome STRiP [26] and SplazerS [27]. A large deletion of 128 kb in size was identified in all affected family members. Validation of this deletion through Sanger sequencing additionally revealed the presence of a small 12-bp insertion within the deletion breakpoints in all affected individuals (Fig. 1b). QPCR analyses confirmed its segregation with disease status (Fig. 1c). Additional families with mutations in *Parkin* include two families with the same missense mutation (p.Arg42Pro) and one family with a novel single nucleotide deletion (p.Thr414Profs*20; Table 3). The p.Arg42Pro mutation was already reported to be pathogenic [30, 31], while the novel p.Thr414Profs*20 mutation causes a premature stop codon, further supporting its pathogenicity. All *Parkin* mutations were found to segregate with disease status, being found in homozygous state in the affected patients and in heterozygous state or absent in the unaffected family members.

We also identified two families, respectively carrying previously reported nonsense *PINK1* mutations (p.Arg246Stop; p.Gln456Stop), one family carrying a novel frameshift *DJ-1* mutation (p.Asp24Metfs*3) and another family carrying a novel *SYNJ1* mutation (p.Arg795/800/839His) (Table 3). Both *PINK1* mutations segregate with disease status: the p.Arg246Stop mutation was found in homozygous state in the six patients and in heterozygous state in both parents as well as two unaffected siblings, while the p.Gln456Stop mutation was found in homozygosis in the three affected members, in heterozygosis in the unaffected mother, and absent in the only unaffected sibling. The novel *DJ-1* mutation, located in exon 1 and resulting in a premature stop codon, was identified by WGS. It did segregate with disease status, being found in homozygosis in both the affected patients, heterozygosis in unaffected mother and one unaffected sibling, and absent in another unaffected sibling. The novel *SYNJ1* mutation did segregate with disease status, was found to be absent in 192 ethnicity-matched control chromosomes, and was shown to be conserved among other orthologs (Fig. 2a, b). It is not described in public databases, such as the NHLBI GO Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) and gnomAD, and it was predicted to be pathogenic by various computational methods (MutPred score: 0.818; SNPs&GO effect: disease; MutationTaster: disease causing; SIFT: deleterious; PolyPhen-2: probably damaging; and CADD_phred: 35). It is located in exon 19 of the *SYNJ1* gene and lies within the inositol-5-phosphatase domain of Synaptojanin 1 (Fig. 2c) which is known to dephosphorylate a variety of lipids, such as $PI(4,5)P_2$ and $PI(3,4,5)P_3$ [15, 32].

Lastly, a novel gene, *VAC14* (MIM #604632), was found to be mutated in a single family by combining both WGS and HM approaches. Briefly, WGS performed on the two affected siblings (Fig. 2d) identified 10 different homozygous genetic variants, not reported or present with low frequency in public databases, in both affected individuals. No common compound heterozygous variants were identified. Genetic variants were located on chromosomes 3, 16, and 19. To assist with disease gene identification and reduce the number of candidate variants, HM was then performed and eight different homozygous segments, on chromosomes 5, 7, 11 ($n = 3$), 16, 18, and 21 (data not shown), were identified to be shared exclusively by both affected siblings. Four genetic variants were

Table 3 Detailed clinical description of patients with mutations in PD/parkinsonism genes

Patient (gender)	AAO	Age	Gene mutation	Start location	First symptom	Tremor	Progression	Parkinsonism	Rigidity	Postural instability	Dystonia
F1P1 (F)	26	32	PINK1	Hands	Resting tremor	Rest, intention, limbs, head, chin	Fast	Sy	N	N	N
F1P2 (F)	15	55	c.736C>T;	Hands	Resting tremor	Rest	Slow	As	N	N	N
F1P3 (F)	22	40	p.R246X	Hands	Resting tremor	Whole body	Slow	Sy	Y	La	Y
F1P4 (F)	33	35		Right hand	Resting tremor	Limbs, head, chin, voice	Fast	As	N	La	Y
F1P5 (M)	30	38		Hands	Resting tremor	Whole body	Fast	Sy	Y	N	N
F1P6 (M)	40	46		Hands	Resting tremor	Intention, limbs	Slow	Sy	N	N	N
F2P1 (F)	16	52	PINK1	Hands	Resting tremor	Rest	Slow	Sy	Y	Ea	Y
F2P2 (M)	24	50	c.1366C>T;	Hands	Resting tremor	Rest	Slow	As	N	La	N
F2P3 (F)	24	42	p.Q456X	Hands	Resting tremor	Rest	Slow	As	Y	La	N
F2P4 (M)	39	45		Hands	Resting tremor	Rest	Slow	As	Y	La	N
F3P1 (M)	13	62	Parkin	Limbs	Resting tremor	Rest, intention, limbs	Slow	Sy	Y	La	Y
F3P2 (M)	13	54	Deletion	Limbs	Resting tremor	Intention, limbs, chin	Slow	Sy	N	La	N
F3P3 (M)	13	50	exon 5	Whole body	Whole body tremor	Rest, intention, limbs	Slow	Sy	Y	La	N
F4P1 (M)	24	29	Parkin	Leg	Resting tremor	Limbs, head, neck	Fast	Sy	N	N	N
F4P2 (F)	23	35	Deletion	Leg	Resting tremor	Limbs, voice	Slow	Sy	Y	La	N
F4P3 (F)	27	42	exon 3	Limbs	Resting tremor	Rest, intention, limbs, chin, voice	Slow	N	N	La	N
F5P1 (F)	34	42	Parkin	Hands	Resting tremor	Intention, limbs	Slow	Sy	Y	La	N
F5P2 (F)	17	37	Deletion	Hands	Resting tremor	Intention, limbs	Fast	Sy	N	La	N
F5P3 (M)	20	49	Exons 5 and 6	Whole body	Whole body tremor	Rest, intention, limbs, voice	Fast	Sy	Y	La	N
F6P1 (M)	23	30	Parkin	Limbs	Resting tremor	Rest, limbs, head, chin	Slow	As	Y	La	N
F6P2 (M)	24	34	Deletion	Limbs	Resting tremor	Rest, limbs, head, chin	Slow	As	Y	La	N
F6P3 (F)	35	38	Exon 5	Limbs	Resting tremor	Rest, limbs, head, chin	Slow	As	Y	La	N
F7P1 (F)	27	42	Parkin	Limbs	Resting tremor	Limbs	Slow	Sy	N	La	N
F7P2 (M)	27	60	Deletion Exons 3 and 4	Left leg	Resting tremor	Limbs	Slow	Sy	Y	La	N
F8P1 (F)	28	31	Parkin	Legs	Resting tremor	Rest, intention, limbs	Fast	N	N	N	N
F8P2 (M)	30	33	Deletion Exon 3	Left hand	Resting tremor	Rest, intention, limbs	Fast	N	Y	N	N
F9P1 (M)	44	47	Parkin	Legs	Resting tremor	Rest	Fast	Sy	Y	La	N
F9P2 (M)	37	44	Deletion Exon 4	Left upper limb	Myoclonus	Other	Moderate	As	Y	La	Y
F10P1 (F)	27	32	Parkin	Legs	Resting tremor	Rest	Slow	As	N	Ea	Y
F10P2 (F)	35	45	Deletion Exon 7	Legs	Resting tremor	Rest	Slow	As	N	Ea	N
F11P1 (F)	45	50	Parkin	Hands	Resting tremor	Rest, limbs	Slow	Sy	Y	Ea	Y
F11P2 (F)	45	52	Deletion Exon 5	Hands	Resting tremor	Rest	Slow	Sy	Y	Ea	Y
F12P1 (M)	35	47	Parkin	Left hand	Resting tremor	Limbs	Slow	As	Y	La	N
F12P2 (F)	32	45	c.1240delA; p.T414PfsX20	Lower limbs	Stiffness of the lower limbs	Non	Very Slow	Sy	Y	N	N
F13P1 (M)	22	41	Parkin	Un	Bradykinesia	Rest, limbs	Slow	As	Y	La	N
F13P2 (M)	22	40	Deletion Exons 5 and 6	Un	Slowness	Non	Slow	Sy	N	N	N
F14P1 (F)	14	42	Parkin	Hands	Resting tremor	Rest	Slow	Sy	Y	Ea	Y

Table 3 (continued)

Patient (gender)	AAO	Age	Gene mutation	Start location	First symptom	Tremor	Progression	Parkinsonism	Rigidity	Postural instability	Dystonia
F14P2 (F)	40	54	c.125G>C; p.R42P	Hands	Resting tremor	Rest	Slow	Sy	Y	La	N
F15P1 (F)	14	28	Parkin	Un	Bradykinesia	Rest, limbs	Slow	As	Y	La	Y
F15P2 (M)	12	30	Deletion Exons 3 to 7	Un	Bradykinesia	Rest, limbs	Fast	As	Y	Ea	Y
F16P1 (M)	19	23	Parkin c.125G>C;	Left limbs	Resting tremor	Intention, limbs, neck, chin, voice, tongue	Fast	Sy	Y	N	N
F16P2 (M)	15	32	p.R42P	Hands	Resting tremor	Rest, limbs, chin, voice	Fast	Sy	Y	Ea	Y
F17P1 (M)	14	35	Parkin	Un	Bradykinesia	Rest, limbs	Slow	As	Y	La	Y
F17P2 (M)	15	46	Deletion Exons 5 to 7	Un	Bradykinesia	Rest, limbs	Fast	As	Y	Ea	Y
F18P1 (F)	25	35	Parkin	Left hand	Resting tremor	Intention, limbs, head, voice	Slow	As	Y	La	Y
F18P2 (M)	24	48	Deletion Exon 9	Un	Bradykinesia	Intention, limbs, chin, voice	Fast	As	Y	Ea	Y
F19P1 (M)	30	38	Parkin	Left hand	Resting tremor	Intention, limbs, voice	Fast	Sy	N	La	N
F19P2 (M)	21	42	Deletion Exon 3	Whole body	Resting tremor	Intention, limbs, chin, voice	Fast	Sy	Y	La	N
F20P1 (M)	23	52	Parkin	Un	Bradykinesia	Non	Slow	Sy	Y	Y	Y
F20P2 (M)	24	47	Deletion Exon 3	Hands	Resting tremor	Rest	Slow	Sy	Y	Y	N
F21P1 (M)	27	42	DJ-1 c.70delA;	Un	Oromandibular dystonia then parkinsonism	Non	Slow	Sy	Y	La	Y
F21P2 (M)	27	35	p.D24MfsX3	Un	Oromandibular dystonia then parkinsonism	Non	Slow	Sy	Y	La	Y
F22P1 (M)	24	30	SYNJ1	Right hand	Tremor, rigidity	Rest	Fast	As	Y	Y	N
F22P2 (F)	27	47	c.2515C>T; p.R839C	Whole body	Tremor, rigidity	Rest, limbs	Fast	As	Y	Y	N
F23P1 (M)	13	24	VAC14	Lower limbs and gait	Dystonic gait	Whole body dystonic tremor	Fast	Sy	Y	n/a	Y
F23P2(M)	8	13		Lower limbs and gait	Dystonic gait	No	Fast	Sy	N	N	Y

Patient (gender)	Bradykinesia	Hypokinesia	Autonomic dysfunction	Pyramidal signs	Stride and sleep apnea	REM sleep behavior disorder	Falling	Response to levodopa	Other symptoms
F1P1 (F)	Y	Y	Y	N	Y	N	N	Y	Eyelid apraxia or blepharospasm, amnesia, insomnia, impaired smell
F1P2 (F)	Y	Y	Y	N	Y	N	Y	Y	Wheelchair dependency, impaired smell
F1P3 (F)	Y	Y	N	Sp	Y	N	Y	Y	Incontinence, wheelchair dependency, insomnia
F1P4 (F)	Y	Y	N	Sp	N	Y	N	Y	Amnesia, insomnia
F1P5 (M)	Y	Y	Y	N	N	N	Y	Y	Amnesia, insomnia
F1P6 (M)	Y	Y	N	N	N	Y	N	Y	Amnesia
F2P1 (F)	Y	Y	N	N	N	N	N	Y	–

Table 3 (continued)

Patient (gender)	Bradykinesia	Hypokinesia	Autonomic dysfunction	Pyramidal signs	Stride and sleep apnea	REM sleep behavior disorder	Falling	Response to levodopa	Other symptoms
F2P2 (M)	N	N	N	N	N	N	N	Y	–
F2P3 (F)	Y	Y	N	N	N	N	N	N	–
F2P4 (M)	Y	Y	N	N	N	N	N	Y	–
F3P1 (M)	Y	Y	Y	N	N	N	Y	Y	Eyelid apraxia or blepharospasm, incontinence, sensory polyneuropathy, impaired smell
F3P2 (M)	Y	Y	N	N	N	N	Y	Y	Incontinence, sensory polyneuropathy
F3P3 (M)	Y	Y	N	N	N	N	N	Y	Sensory polyneuropathy
F4P1 (M)	Y	Y	N	Sp	Y	N	N	Y	–
F4P2 (F)	Y	Y	Y	Sp	Y	N	N	Y	Amnesia
F4P3 (F)	N	N	Y	N	Y	N	Y	Y	Insomnia
F5P1 (F)	N	N	Y	Sp	N	N	N	N	–
F5P2 (F)	Y	Y	N	N	N	N	Y	N	Amnesia, insomnia
F5P3 (M)	N	N	Y	Sp	Y	Y	Y	Y	Amnesia, insomnia, seizure
F6P1 (M)	Y	Y	Y	Sp	Y	Y	Y	Y	Incontinence, wheelchair dependency, insomnia, impaired smell
F6P2 (M)	Y	Y	Y	Sp	Y	Y	Y	Y	Incontinence, wheelchair dependency, insomnia, impaired smell
F6P3 (F)	Y	Y	Y	Sp	Y	Y	Y	Y	Incontinence, wheelchair dependency, insomnia, impaired smell
F7P1 (F)	Y	Y	Y	N	N	N	Y	Y	Amnesia, insomnia
F7P2 (M)	Y	Y	Y	Sp	N	N	Y	Y	Incontinence, wheelchair dependency, insomnia, seizure, impaired smell
F8P1 (F)	N	N	Y	N	Y	N	N	Y	Amnesia
F8P2 (M)	N	N	Y	N	N	Y	N	Y	–
F9P1 (M)	Y	Y	Y	Sp	Y	Y	N	Y	Incontinence, amnesia, insomnia, seizure
F9P2 (M)	Y	Y	N	N	N	N	N	Y	Off dystonia
F10P1 (F)	Y	N	N	N	N	N	N	Y	–
F10P2 (F)	N	N	N	N	N	N	Y	Y	Insomnia
F11P1 (F)	N	N	N	N	N	N	N	Y	Insomnia
F11P2 (F)	Y	N	N	N	N	N	Y	Y	–
F12P1 (M)	Y	Y	N	N	N	Y	N	Y	Psychosis, insomnia, paranoid ideas with pramipexole
F12P2 (F)	Y	Y	N	Sp	N	N	N	Y	Psychiatric symptoms (depression, anxiety, and panic attacks)
F13P1 (M)	Y	Y	Y	Ba	Y	Y	Y	Y	Incontinence, depression, suicidal attempt, drug induced dyskinesias, insomnia, impaired smell
F13P2 (M)	Y	Y	N	N	N	N	N	Y	Psychosis, dyskinesias, insomnia
F14P1 (F)	Y	Y	N	N	N	Y	N	Y	Incontinence
F14P2 (F)	Y	Y	Y	N	N	N	N	Y	–

Table 3 (continued)

Patient (gender)	Bradykinesia	Hypokinesia	Autonomic dysfunction	Pyramidal signs	Stride and sleep apnea	REM sleep behavior disorder	Falling	Response to levodopa	Other symptoms
F15P1 (F)	Y	Y	N	N	N	N	N	Y	Impaired smell
F15P2 (M)	Y	Y	N	N	N	N	N	Y	Impaired smell
F16P1 (M)	Y	Y	Y	Sp	N	N	N	Y	–
F16P2 (M)	Y	Y	Y	N	Y	N	Y	Y	Wheelchair dependency, impaired smell
F17P1 (M)	Y	N	N	N	N	N	N	Y	–
F17P2 (M)	Y	Y	N	N	N	N	N	Y	Wheelchair dependency
F18P1 (F)	Y	Y	Y	Sp	N	Y	Y	Y	Eyelid apraxia or blepharospasm, psychosis, amnesia, insomnia, seizure, impaired smell
F18P2 (M)	Y	Y	Y	Sp	N	Y	Y	Y	Eyelid apraxia or blepharospasm, wheelchair dependency, psychosis, amnesia, insomnia, seizure, impaired smell
F19P1 (M)	Y	Y	Y	Sp	N	N	Y	Y	Incontinence, insomnia, impaired smell
F19P2 (M)	Y	Y	Y	Sp	N	N	Y	Y	Insomnia, impaired smell
F20P1 (M)	Y	N	N	N	N	N	Y	Y	–
F20P2 (M)	Y	N	N	N	N	N	Y	Y	–
F21P1 (M)	Y	Y	N	N	N	N	Y	Y	Wheelchair dependency, psychosis, bilateral cataract
F21P2 (M)	Y	Y	N	N	N	N	Y	Y	Psychosis
F22P1 (M)	Y	Y	N	N	N	N	N	N	Seizure, chin tremor, longitudinal fissured tongue
F22P2 (F)	Y	Y	N	N	N	N	N	N	This patient was not alive to be examined for the tongue feature
F23P1 (M)	Y	Y	N	N	N	N	N	N	Striatal toe, normal intellectual function
F23P2(M)	N	N	N	N	N	N	N	N	Normal intellectual function

F1P1 family 1, patient 1, *M* male, *F* female, *AAO* age at onset, *Sy* symmetric, *As* asymmetric, *N* no, *Y* yes, *Ea* early, *La* late, *Sp* spasticity, *Ba* Babinski, *Un* unknown, *n/a* not available

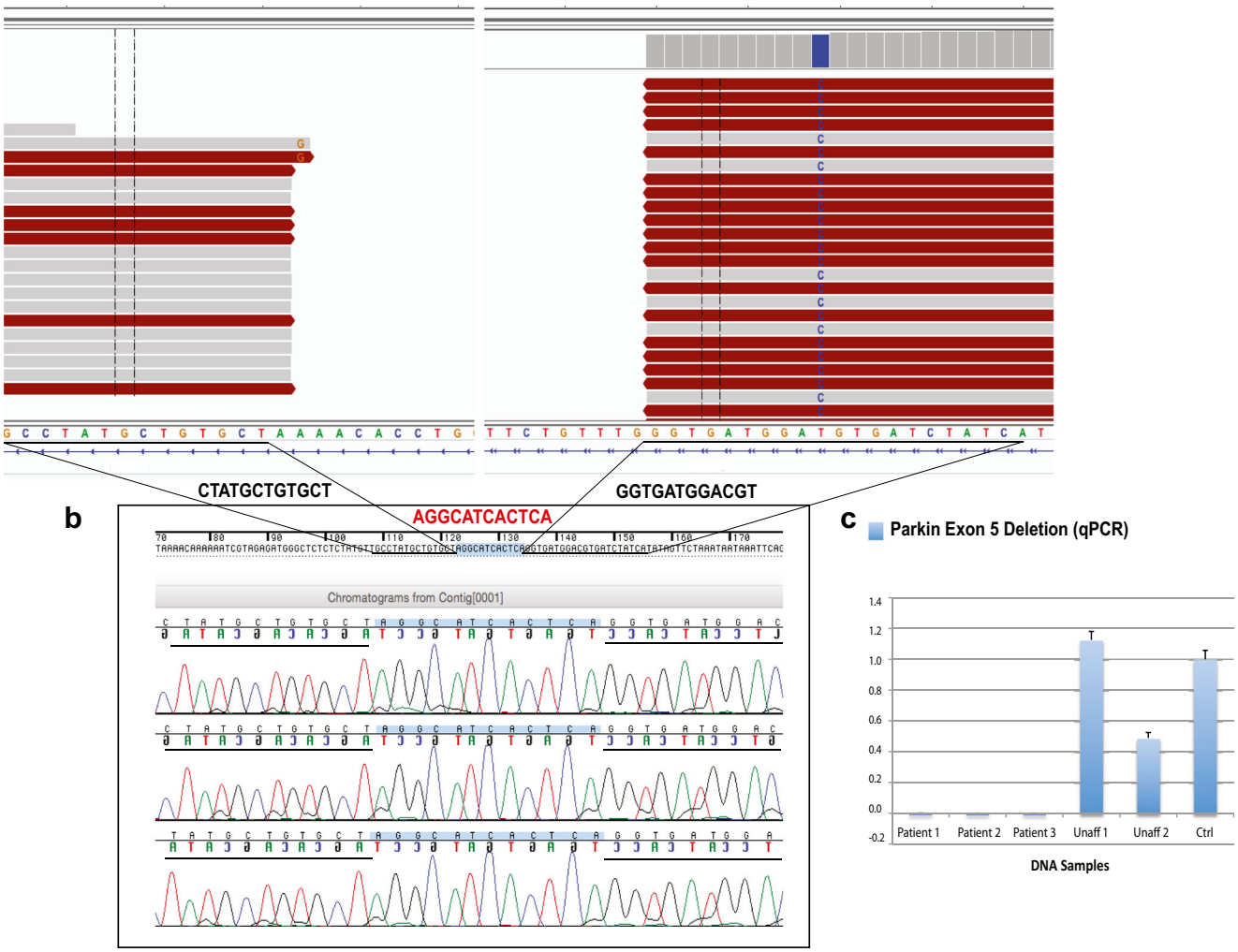

Fig. 1 *Parkin* exon 5 deletion (128-kb). **a** WGS reads were visualized by using the Integrative Genomics Viewer (IGV). Two different plots that represent the *Parkin* exon 5 deletion breakpoints are shown. **b** *Sanger* chromatograms of the three affected individuals, who carried the 128-kb *Parkin* exon 5 deletion, are shown. Deletion breakpoints are highlighted in *black* (12 bp of the flanking regions), while the 12-bp insertion identified within the deletion breakpoints is highlighted in *red*. **c** Validation of the *Parkin* exon 5 deletion through qPCR analyses. *Y-axis* represents *PRKN*/*GPR15* ratios; *x-axis*: DNA samples analyzed. Affected individuals showed no copy of *Parkin* exon 5 (ratio = 0.0), whereas unaffected individuals showed either two copies (Unaff 1; ratio = 1.0–1.2) or one copy (Unaff 2; ratio = 0.4–0.6). *Unaff* unaffected family member, *Ctrl* control DNA sample (color figure online)

localized on the largest autozygous region located on chromosome 16 (21 Mb) and flanked by rs8049176 (57,655,792 bp) and rs16949243 (79,053,995 bp) SNVs. Among these, the *ZFHX3* p.Pro2304Thr (p.Pro3218Thr) variant was also found to be present in 5 out 16,510 South Asian alleles (http://exac.broadinstitute.org/variant/16-72822523-G-T). Two variants (p.Asp1849Val, p.Pro2304Thr) located in the same gene (*ZFHX3*) were predicted non-deleterious, while the other two located in *CES2* (p.Phe127Leu) and *VAC14* (p.Ala562Val) genes were predicted to be highly pathogenic. However, *VAC14* was the only gene found to be highly expressed in brain tissues (http://gtexportal.org/home/gene/VAC14) and therefore selected as the disease candidate gene. No dystonia genes were found to be mutated in affected patients subject to WGS analyses. The novel *VAC14* mutation (c.1685C>T) was found to segregate with disease status, was absent in 188 ethnicity-matched control chromosomes, was shown to be highly conserved across other species (Fig. 2d, e), and was predicted to be pathogenic by SNPs&GO (disease), MutationTaster (disease causing), SIFT (deleterious), PolyPhen-2 (possibly damaging), and CADD_phred (29.8). Despite being absent in public databases, it was recently described with very low

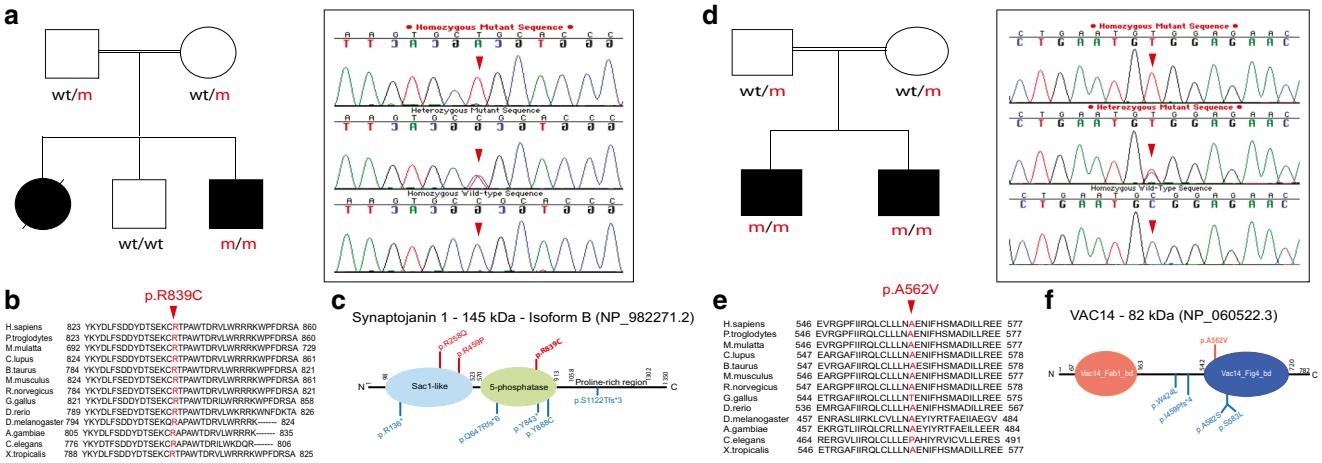

Fig. 2 *SYNJ1* p.Arg839Cys and *VAC14* p.Ala562Val mutations. **a** Pedigree structure of the family carrying the *SYNJ1* p.R839C mutation is shown on the *left side*. Affected family members are represented with *black circle* (female) or *square* (male). *Wt/m* heterozygous mutation carriers; *m/m* homozygous mutation carriers; *wt/wt* non-carriers. *Sanger* chromatogram sequences belonging to the *SYNJ1* p.R839C mutation (*red arrow*) are shown on the *right side*. **b** Conservation of the *SYNJ1* p.R839C mutation across different orthologs. **c** Diagram of the Synaptojanin 1 protein structure. The three *SYNJ1* mutations identified to date in patients with parkinsonism are represented in *red*, whereas the *SYNJ1* mutations identified in patients with seizures and severe neurodegeneration are shown in *blue*. The mutation identified in the current study is highlighted in *bold*. **d** Pedigree structure of the family carrying the *VAC14* p.A562V mutation is shown on the *left side*. Affected family members are represented with *black squares* (males). *Wt/m* heterozygous mutation carriers; *m/m* homozygous mutation carriers; *wt/wt* non-carriers. *Sanger* chromatogram sequences for the *VAC14* p.A562V mutation (*red arrow*) are shown on the *right side*. **e** Conservation of the *VAC14* p.A562V mutation across different species. **f** Diagram of the Vac14 protein structure showing the mutation identified in patients with dystonic tremor and disabling dystonia at the *top* (*bold*) and the mutations identified in patients with striatonigral neurodegeneration at the *bottom*

frequency (1/252,016) in gnomAD database. The Vac14 protein contains two binding domains, the lipid kinase PIKFYVE (MIM# 609414; Fab1) and the phosphatase Fig4 (MIM# 609390), through which it regulates the synthesis of the signaling lipid $PI(3,5)P_2$ [33, 34]. The *VAC14* p.Ala562Val mutation is located in exon 15 which encodes part of the Fig4-binding domain. The entire *VAC14* gene was additionally examined in 20 familial cases with dystonia-parkinsonism phenotypes, but no pathogenic mutation was identified.

Clinical Findings

A detailed description of all observed phenotypic features is presented in Table 3.

Families with Parkin Mutations

The mean age of onset (± standard deviation) of the patients with *Parkin* mutations was 25.3 ± 9.29 (mean age in males = 22.6, mean age in females = 29.25). The earliest AAO was 12 (F15P2) while the latest AAO was 45 years (F11P1, F11P2). The first symptom in the majority of patients was resting tremor (28/40: 70%). The progress of the disease was slow (meaning that symptoms continue and worsen over a period of years) in 27 patients (67.5%), but fast, with motor symptoms progressing very quickly, in 13 (32.5%). Rigidity was seen in 30 patients (75%). Parkin families showed variable phenotypic expressivity, including the presence of sensory polyneuropathy in one family (F3).

Families with PINK1 Mutations

The mean age of onset (± standard deviation) for patients with *PINK1* mutations was 26.9 ± 8.06 (mean age in males = 33.25, mean age in females = 22.6). The earliest AAO was 15 (F1P2) while the latest AAO was 40 years (F1P6). Resting tremor was the first symptom in all patients. Seven patients (70%) showed slow disease progression, while the disease progressed quickly in three patients (30%). Variable phenotypic expressivity was seen in both families.

Family Carrying a Novel DJ-1 Mutation

The novel *DJ-1* mutation (c.70delA) was detected in two brothers who displayed oromandibular dystonia and profound psychosis at the age of 27. The disease progressed slowly to a marked symmetric parkinsonism with rigidity and bradykinesia. There was no significant tremor in these patients. They had frequent falling attacks and good response to levodopa therapy. One of the brothers showed bilateral cataract.

Family Carrying a Novel SYNJ1 Mutation

The novel *SYNJ1* mutation (c.2515C>T) was identified in a patient who manifested asymmetric parkinsonism and seizures at 24 years of age. The disease manifested with tremor and rigidity in the right hand, but became generalized with tremor and rigidity in all limbs within a year. The patient showed chin tremor and significant dysarthria. He received phenytoin for generalized tonic clonic seizures and showed poor response to levodopa therapy. There was no falling or autonomic dysfunction, and rapid eye movement (REM) sleep behavior disorder was absent as well. He also showed a longitudinal fissured tongue. As this seems to be an uncommon phenomenon, we re-examined our previously reported patients carrying the *SYNJ1* p.Arg258Gln mutation [15], and then observed the same feature in their tongue. There was also a female patient in the family who died from liver cancer during the early stage of this study.

Family Carrying a Novel VAC14 Mutation

Both siblings carrying the novel *VAC14* mutation were reported as having parkinsonism; however, a follow-up clinical characterization revealed that both patients presented with dystonic gait affecting both lower limbs at a young age. Moreover, the disease progression was very severe in one patient (F23P1), with dystonia spreading to upper limbs and trunk. He manifested dystonic action tremors and became bedridden 5 years after disease presentation, and now had profound hypokinesia and bradykinesia. Patients' speech was impaired due to severe dysarthria and dystonia, but their mental state was normal. In both patients, the disease progressed to marked generalized and disabling dystonia. Brain MRI was normal and there was no clinical response to levodopa treatment.

Discussion

In this study, we describe the phenotypic and genetic features of 23 consanguineous recessive families, featuring either ARPD or ARJP. In total, 56 patients were clinically examined. The majority (91.07%) of our patients with mutations reported their first symptom before the age of 40, with only five patients (8.93%) manifesting the disease at the age of 40 or later. The most common first symptom was resting tremor, being present in 67.85% of the patients with pathogenic mutations. Psychiatric features were seen only in three different families carrying either *Parkin* (F12, F13) or *DJ-1* mutations (F21). Seizures were seen in five *Parkin* and one *SYNJ1* mutation carriers. Sensory polyneuropathy, previously not reported in patients with *Parkin* mutations, was observed in three *Parkin* patients (F3). And marked generalized and disabling dystonia was the main phenotype observed in *VAC14* mutation carriers (F23).

The most common mutated gene was *Parkin*, with exon deletions being the most prevalent mutations. In concordance with previous reports, most of the exon rearrangements fell into the genomic region between exons 2 and 8 of the *Parkin* gene, further confirming this region as a mutational hotspot. Novel mutations were also reported in *DJ-1* and *SYNJ-1* genes. The *DJ-1* mutation was identified in a family presented with oromandibular dystonia, parkinsonism, and phenotypic features similar to those observed in previously reported *DJ-1* mutation carriers [8, 35]. The *SYNJ1* mutation identified in this study represents the third *SYNJ1* mutation reported to date in patients with parkinsonism and the first one reported in the inositol-5-phosphatase domain [36], further confirming the role of *SYNJ1* in the pathogenesis of parkinsonism (Fig. 2c). Like in our patient, seizures were previously reported in patients carrying the *SYNJ1* p.Arg258Gln mutation [15, 37], as well as in patients with early-onset epilepsy, progressive spastic quadriplegia, severe intellectual disability, visual impairments, and feeding problems [38, 39]. Given the variable phenotypic expressivity of *SYNJ1* mutation carriers, it has been postulated that variants with complete loss of SYNJ1 dual phosphatase activity (nonsense, frameshift mutations) lead to severe progressive neurodegeneration, while reduced SYNJ1 enzymatic activity (caused by missense mutations) leads to a milder phenotype associated with parkinsonism and increased seizure susceptibility [39].

Lastly, we identified a novel gene (*VAC14*) to be mutated in patients with progressive and disabling dystonia (F23). Although mutations in *VAC14* have recently been identified in pediatric patients with striatonigral degeneration [40], some differences in clinical presentation and course were observed in our family when compared with the previously reported *VAC14* patients. In our family, disease presentation occurred at older age, ranging from 8 to 13 years, versus 18 months and 3 years; no psychomotor regression was observed; and despite the marked abnormality in the basal ganglia reported in patients with *VAC14* mutations, our patients showed normal brain MRI with no obvious abnormality in the striatum. However, the oldest patient (F23P1) showed profound hypokinesia and bradykinesia at a later age. The *VAC14* p.Ala562Val mutation we identified is very likely to result in $PI(3,5)P_2$ deficiency, as it lies in the protein's Fig4-binding domain that is required for Vac14 dimerization and is thought to regulate Fab1 activity to maintain normal levels of PI(3)P, $PI(3,5)P_2$, and PI(5)P [41]. Low levels of $PI(3,5)P_2$ have already been reported in mice exhibiting central and peripheral nervous system neurodegeneration due to Vac14 deficiency and in mice and patients with pathogenic *FIG4* mutations [34, 42]. Moreover, both patients and mice with *VAC14* mutations have been reported to exhibit vacuolation in both cultured fibroblasts and

affected neurons that are thought to arise from defects in the intracellular membrane trafficking, particularly in the retrograde transport from late endosomes to the trans-golgi network (TGN) [34, 40]. Taken together, the *VAC14* p.Ala562Val mutation might also result in vacuolation and impaired retrograde transport from late endosomes to the TGN, likely supporting its pathogenicity.

We concluded that *Parkin* is the most common mutated gene in our population, being found mutated in 71.42% (n = 40) of our examined patients with mutations in known genes (n = 56). Despite the observed, variable phenotypic expressivity in our patients with *Parkin* or *PINK1* mutations, the phenotype observed in the majority of *Parkin* and *PINK1* mutation carriers was indistinguishable from one another. It was mainly characterized by an early-onset presentation, resting tremor of the limbs as an onset symptom, and slow disease progression. Given the presence of seizures in three different families with *SYNJ1* mutations, we propose that seizures should be considered in prospective subjects with *SYNJ1* mutations and parkinsonism. Lastly, given the progressive dystonic phenotype observed in patients with *VAC14* mutations, we suggest nominating *VAC14* as *DYT27* gene and categorizing its phenotype as hereditary progressive dystonia with dystonic gait as a symptom of onset, followed by parkinsonian symptoms.

The finding of *VAC14* as a novel gene for hereditary progressive dystonia is very interesting as it sums up to the large list of parkinsonism-related proteins, including alpha-synuclein, Lrrk2, VPS35, parkin, auxilin, and Synaptojanin 1, that act as important regulators of synaptic vesicle endocytosis and trafficking pathways at synapses. This together suggests the polyphosphoinositide signaling pathway as a relevant therapeutic target for neurodegenerative diseases such PD, parkinsonism, and now dystonia.

Acknowledgements We are deeply grateful to all patients and relatives for their generous contribution to this study.

Compliance with Ethical Standards The local ethics committees at each participating medical center approved this study, and informed consent, according to the Declaration of Helsinki, was obtained from all participants.

Funding This work was in part supported by the Shahid Beheshti University of Medical Sciences, the American Parkinson Disease Association (APDA; CPR), and the National Institute of Neurological Disorders and Stroke of the National Institutes of Health (R01NS079388; CPR).

Conflict of Interest The authors declare that they have no competing interests.

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