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Case report

Copy number analysis from whole-exome sequencing data revealed a novel homozygous deletion in *PARK7* leads to severe early-onset Parkinson's disease

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ABSTRACT

Parkinson's disease (PD), a neurodegenerative disease characterized by both motor neuron and non-motor neuron symptoms, is the most frequent neurodegenerative disease after Alzheimer's disease. Both genetic and environmental factors take part in disease etiology. Most cases are considered complex multifactorial diseases. About 15% of PD appear in the familial form, and about 5% of all cases arise from a single gene mutation. Among Mendelian causes of PD, PARK7 is one of the autosomal recessive forms due to loss-of-function mutations in both gene alleles. Both single nucleotide variants (SNVs) and copy number variations (CNVs) are observed in PARK7. This study presents an Iranian family with familial PD where some relatives had psychiatric disorders. A homozygous 1617 bp deletion in a female with early-onset PD was detected through copy-number analysis from whole-exome sequencing (WES) data in this consanguineous family. Further investigation by surveying microhomology revealed that the actual size of the deletion is 3,625 bp. This novel CNV that was in the PARK7gene is supposed to co-relation with early-onset PD and infertility in this family.

1. Introduction

Parkinson's disease, the second most frequent neurodegenerative disease, is characterized by motor neuron features besides non-

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motor findings [1]. The four main motor neuron symptoms are rest tremor, rigidity, and bradykinesia often accompanied by postural instability [2]. Insomnia, depression, constipation, and hyposmia are common associated non-motor findings [3]. The onset of PD is

Nomenclature

PD	Parkinson's disease
WES	Whole-exome sequencing
CNV	Copy number variation
SNV	Single nucleotide variation
WGS	Whole-genome sequencing
Ct	Cycle of threshold
NAHR	Non-allelic homologous recombination

around 60 years in most cases but can be variable. Regarding the age of onset, PD could classify into three subsets: 1) juvenile-onset if it is in <20 years, early-onset adult Parkinson's disease if it is in 20–50 years, and late-onset adult Parkinson's disease if the age of onset is > 50 years [1]. PD is a complex disease that arises from the interplay of non-genetic and genetic factors. This interplay leads to death in dopaminergic neurons and in following PD features. PD usually occurs in sporadic form, but about 15% of cases have a history of this disease. In about >5% of all cases, pathogenic variant(s) in a single gene is the cause of this disease. Monogenic PD can inherit in an autosomal (dominant or recessive) or rarely an X-linked manner. Among monogenic causes of PD, loss-of-function mutations in PARK7 (DJ1) is a rare cause of PD in adults (indeed in early-onset adults with the average age of the onset 32 years) that inherit in an autosomal recessive manner. Patients with mutations in PARK7 occasionally have an intellectual disability with or without seizures besides Parkinsonian features. Slow progression, lower-limb dystonia, dyskinesia, and hyperreflexia are other attributes of PD patients with mutations in PARK7 [4]. Heterozygous for PARK7 mutations has a high PD risk, so genetic diagnosis is crucial for risk assessment in family and genetic counseling. Homozygous or compound heterozygous loss-of-function mutations in PARK7 have complete penetrance for early-onset PD [5].

PARK7 islocated on 1p36.23 and consists of 7 exons and encodes a protein with 189 amino acids. PARK7 acts as a positive regulator of androgen receptor-dependent transcription; may also act as a chaperone responding to oxidative stress; apparently, proper functions of this gene protect neurons against oxidative stress and subsequent cell death [6].

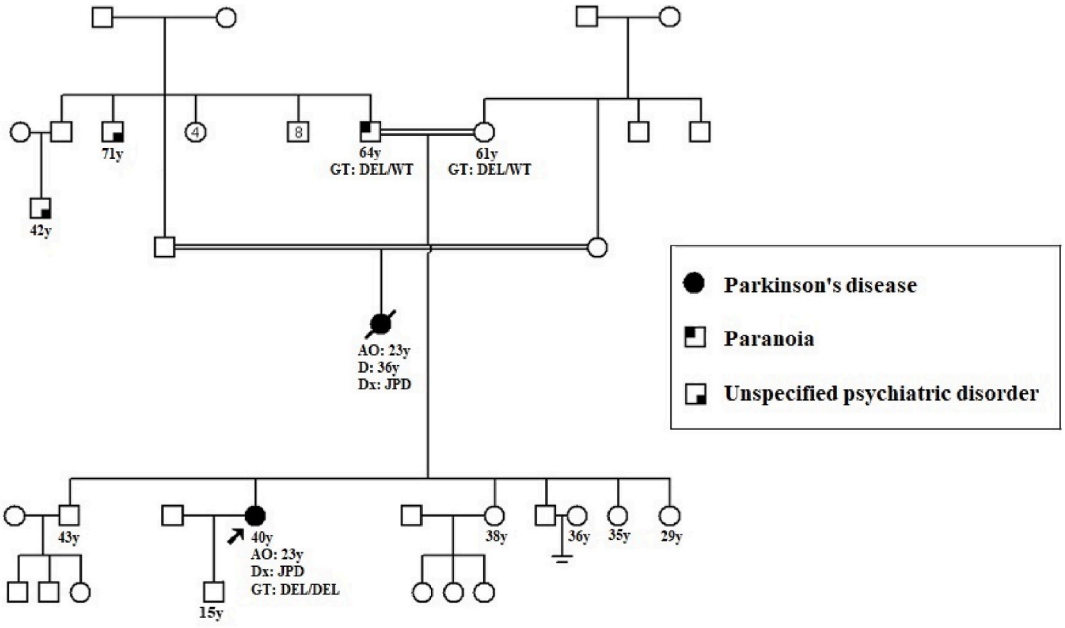


Fig. 1. Pedegree of the patient. Pedigree of the patients (had drawn by Cyrillic 2). Pedigree indicates familial PD. Some of her relatives manifested psychological problems. Her cousin had a similar condition and died at 36 years old because of apnea. Proposita's kid is a 15 years healthy boy, and her parents are 5th-degree relatives that are not shown here for simplicity. The brother of the case was infertile. All people in the pedigree were asked about the non-motor symptoms of Parkinson's disease (such as anosmia, constipation, REM sleep behavior disorder, etc.), who did not show any of the symptoms till the last survey. Only the case and her parents were genotyped; others were not genotyped due to unwillingness or unavailability. (#y represents age, D represents age at death, and AO represents age of onset; other acronyms include Dx: age of diagnosis, JPD: juvenile Parkinson's disease GT: genotype, DEL: deletion in *PARK7*, WT: wild type for *PARK7*).

Copy Number Variations (CNVs) are known by losing or gaining larger than 50 bp. According to the ACMG classification, the impact of CNVs could vary from benign to pathogenic [7]. Analysis CNVs are more challenging compared with SNVs because, first, fewer population studies are performed for analysis and categorizing these variants, second, fewer prediction tools are developed to predict their effects, and last, evolutionary conservation is less practical in these types of variants.

Among the many methods for detecting CNVs, there is a bioinformatical method, ExomeDepth, which extracts CNVs from WES data. The logic of ExomeDepth is to compare the read depth of exomes with the optimized set of exons i.e. similar in some properties

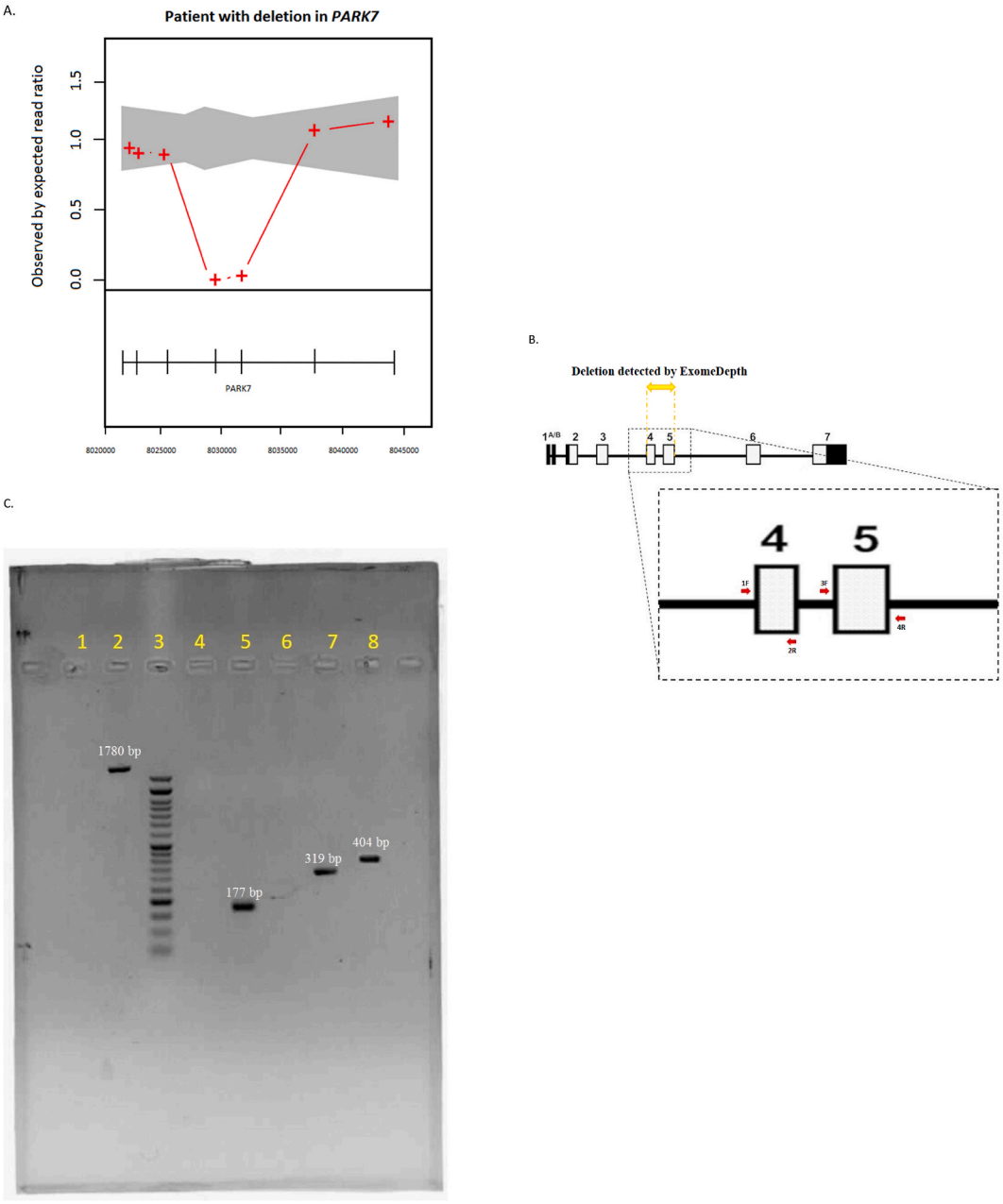


Fig. 2. First line for surveying the deletion. A. The result of ExomeDepth shows 8020000–8045000 nucleotides of chromosome 1 (GRCh37/hg19); the red crosses show the relative presence of exons in the PARK7 gene. Exons 4 and 5 were not present in the exons library. The gray zone shows the area of 90% confidential. *Note that according to the results of PCR gel electrophoresis, the deletion size must be larger than the ExomeDepth prediction. B. Two-headed yellow arrow shows the deletion site according to ExomeDepth data. Red arrows are the approximate annealing sites of the primers (F1, 2R, 3F, and 4R in order from left to right). PCR product sizes of each pair are presented in Table 1. C. PCR product results. 1) F1-R4 product in the patient, 2) F1-R4 product in control, 3) DNA marker (ladder 50 bp), 4) F1-R2 product in the patient (exon 4), 5) F1-R2 product in control, 6) F3-R4 product in the patient (exon 5), 7) F3-R4 product in control, and 8) patient DNA control (by other primers specific for *LRRK2* gene). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

like GC% content (not to all exons), and measure deviations from the ratio 1 to predict deletion and duplication [8]. For example, if the ratio was 0.5, it should be interpreted as heterozygous deletion, and if it was 1.5, as duplication. Many variations could affect the depth of an exome, for example, GC% content, PCR bias in making library, etc; hence it is necessary to validate the results (even while ExomeDepth uses an optimized set of exone for comparison).

2. Case presentation

The case was a 40-years-old Iranian woman with early-onset adult Parkinson's disease with familial history of PD and some psychological disorders (Fig. 1). When she was 23 years old, the first sign of PD manifested, the disease started with a tremor in her left hand. Her parents were 5th-degree relatives, and her cousin had a similar problem (age of onset was same as proposita) and died in 36 years because of obstructive sleep apnea. Her Father was paranoid, and her uncle and one of her cousins had an unconfirmed psychological disorder.

The case's main complaint was a seizure, dysphagia, constipation, tremor, bradykinesia, falling, and illusion. Examinations reveal ataxic gait and standing, rigidity, hypertonia, and eye movement "catch-up" saccades with the slowness of eye movements in the vertical plane in cranial nerve examination. An MRI brain scan reveals almost no abnormality. The score for Montreal Cognitive Assessment (MoCA [[18]]) was far below the defined normal range (6/30), and the score for Frontal Assessment Battery (FAB [10]) was zero. Beck Depression Inventory (BDI [11]) revealed some signs of depression. Unified Parkinson's Disease Rating Scale (UPDRS [12])overall score was 152/199, indicating the high severity of PD.

Medications prescriptions included Levodopa, Isicom, PARKIN C, NORSTOR, and Levoparkin for ameliorating Parkinsonism features, and some other medications including sodium valproate, Depakine, EPILIM, Divalproex, ORLEPT, ORFIRIL, Clozapine, LEPONEX, and clonazepam for seizure, psychiatric features, and sleep problems.

No environmental factors with indications of PD were mentioned. In addition, positive family history along with a consanguinity marriage implied a high probability of a genetic factor in causing the disease.

3. Material and methods, and results

As meant for genetics analysis, whole-exome sequencing was done. Under Helsinki's declaration [13], 5 cc of whole blood of patients was venipuncture, and lymphocyte's DNA was extracted by salting out protocol [9]. WES performed at Helmholtz Center Munich (Munich, Germany). Paired-end 100 bp library prepared, and exons captured using SureSelect Human All Exon v6 kit (Agilent Technologies, CA, USA), and run on a Hiseq4000 (Illumina, CA, USA), targeted sequences covered at least 20-fold. Data processing is done with a validated in-house pipeline that implements Burrows-Wheeler-Aligner for sequence mapping (GRCh37/hg19) and SAMtools, GATK, ExomeDepth (CNV detection), and custom scripts for variant calling and annotation.

Data analysis uncovered a 1617 bp homozygous deletion (chr1:8029405-8031022) inPARK7 (Fig. 2 (A,B)), 4th and 5th exons located at this site. This variant is not observed in DECIPHER and ClinVar databases. For confirmation, four primers were designed (in Table 1). The location of primers, respectively, is the first primer at the 3' end of intron 3, the second at exon 4, the third at intron 4, and the fourth at the intron 5. Primers were designed by assisting primer3 and some manual change; the secondary structure of primer was checked by oligo analyzer software, and the specificity of primers was checked by NCBI primer blast. PCR reactions were performed on a 20μl final volume with 10μl of PCR MasterMix Parstos (2x) 100ng genomic DNA and 1pmol from each primer. Primers 1F and 2R were used to survey exon 4 presence, and primers 3F and 4R were utilized for exon 5. These two reactions run with program 1. For surveying deletion size, 1F and 4R primers are used; this reaction ran with a long-range specific PCR program (program2). The PCR results are displayed in Fig. 2 (C), and PCR programs are presented in Table 2.

To identify the exact deleted region, two primers surrounding both sides of the deletion are needed. Considering that the cause of many microdeletions is Alu-Mediated NAHR, to prioritize the design of primers, Alu sequences upstream of the fourth exon and downstream of the fifth exon were sought using the UCSC database (https://genome.ucsc.edu/) (Fig. 3 (A)). Then, each Alu family sequence upstream of the fourth exon (5 Alu sequences) with each Alu sequence downstream of the fifth exon (8 Alu sequences; two Aluy were identical) was aligned one by one (35 modes) using NCBI nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Among them, Alusg4 upstream of the fourth exon had significant similarity with Alusq2, another Alusq2 (non-identical), and two Aluy downstream of the fifth exon. So one forward primer and four reverse primers were designed. Using primers PARK7-5F and PARK7-6R (Table 1) and PCR program3 (Table 2), which surrounded Alusg4 and proximal (to deletion) Alusq2, a 580 bp product obtained (Fig. 3

Table 1
Designed primes sequence (left) and size of expected bands (right). Because 2R and 3F are located in deletion, no PCR products are expected by 1F-2R and 3F-4R primer pairs for the patient. PCR product size for 5F-6R in the case is calculated after sanger sequencing and blasting it to genome reference.

row	Primer name	Sequence (5'>3')	Annealing site	Product size (bp)
1	PARK7-1F	TAGGCTATCTCCTGTACTTCCCAC	Intron 3	1F-2R: 177 (in control)
2	PARK7-2R	ACCTCAGATAAATTCTGTGCG	Exon 4	3F-4R: 319 (in control)
3	PARK7-3F	TGAGAAATGCCTTGCTTGG	Intron 4	1F-4R: 1780 (in control)
4	PARK7-4R	CCCACCCACCAATGACGC	Intron 5	1F-4R: 163 (in case)
5	PARK7-5F	GGTGACAGAGGTGAGGGCTTC	Intron 3	5F-6R: 4205 (in control)
6	PARK7-6R	CCAGGACCTCTGGCCCATAAC	Intron 5	5F-6R: 580 (in case)

Table 2
PCR programs.

	Program 1			Program 2		Program 3		
Cycle(#)	Temperature (°C)	Time (second)	Cycle(#)	Temperature (°C)	Time (second)	Cycle(#)	Temperature (°C)	Time (second)
1	94	240	1	93	300	1	94	240
	94	20		93	30		94	30
35	58	20	30	55	15	35	62	30
	72	20		66	60		72	30
1	72	600	1	72	600	1	72	600

(B)); As a result, the actual deletion size is 3625 bp. Alusg4 and proximal Alusq2 are 302 and 312 nucleotides long, respectively. Alusg4 and (proximal) Alusq2 have 83% similarity in 99% overlap. No other similarity was found.

Sanger sequencing of the product and alignment of the sequence with Alusg4 and alusq2 reveals that the recombination occurred somewhere between nucleotides 173 and 202 if Counting is done on the Alusg4 sequence (Fig. 3 (C)). Because complete similarity between these two nucleotides, the template switching site is obscured.

Identification of carriers required a quantitative method; for this purpose, Real-Time-PCR technique was used. After observing the desired size band on PCR gel electrophoresis, Real-Time-PCR was performed with a LightCycler 96 instrument (Roche Diagnostics GmbH, Germany). The case, her parents, and one healthy control were assayed by this technique. Each sample was performed triplicate using 10 μl 2X SYBR green master mix (Biofact, Daejeon, South Korea), plus 2μl of DNA (100ng genomic DNA) samples, 0.5 μl of each forward and reverse primers (10 pmol), and 7μl of Distilled water. Sequences of primers are presented in Table 3. The PCR conditions involved an initial step of 15 min at 95 °C for enzyme activation, followed by 40 cycles, including a denaturation step for 30 s at 95 °C and an annealing step for 20 s at 59 °C, and an expansion step for 15 s in 72 °C. The Albumin (Alb) gene was used as a normalizer to compare the number of copies of exons 4 and 5 of PARK7. Δ_{Ct} between *ALB* and *PARK7* exons curves was 1.6 for the mother and 1.4 for the father. These results (are presented in Fig. 4 (A - D)) indicate that both parents are carriers of the deletion mutation. The difference between the threshold cycle of normalization (*ALB*) and exons 4 and 5 of PARK7 in the parents is more than one; due to the inefficiency of the PCR reaction (efficiency less than 100%) and unavoidable deviations in DNA concentrations.

4. Discussion

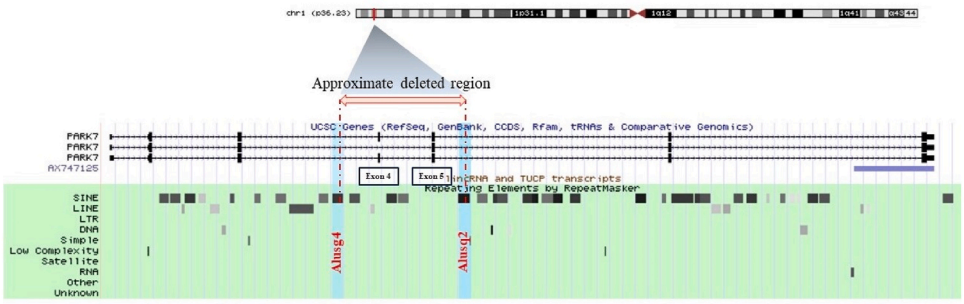
PARK7is an established cause of early-onset PD. Hypomorph or null morph mutations in this gene, via the loss-of-function mechanism, made neurons susceptible to oxidative stress and cell death. Death of dopaminergic neurons in substantia nigra leads to parkinsonism features. Copy number variations comprise a remarkable share of PARK7 mutations [[7,14],]. The mutations of this class are usually surveyed by a specific MLPA kit or aCGH, but in the near future, whole-genome sequencing (WGS) will supersede all. Alternatively, in this project, the ExomeDepth approach was replaced MLPA and aCGH techniques, and led to the detection of a deletion in PARK7. WES data is worthful data, by different strategies other than single nucleotide variants (SNVs), CNVs, and some structural variants are also identified. But WES is unable to detect variants in introns (SNVs, CNVs, or structural variants with a breakpoint in an intron); as we expect to see a 163 bp band in the PCR using primer 1 and 4 (instead of a 1780 bp band in the control DNA), but did not observe any band. It means that the deletion is extended to introns, which is undetectable by the ExomeDepth approach. In this case, the deleted region proposed by ExomeDepth is precisely from the beginning of exon 4 to the end of exon 5. This fact is crucial in survey heterozygous deletion or carrier detection by this method. NGS-based detection of CNVs has a higher resolution in comparison with aCGH. Analysis of CNVs in WES data could increase the positive detection rate of WES. By descending massively parallel sequencing costs, and developing reliable long-read methods with high, whole-genome sequencing could detect any types of mutations and become the first line of genetic diagnosis.

Howbeit, it is debated how many CNVs are provoked by Alu repeats misalignment; here, to cut costs of Precise identification of the deletion boundary, surveying for microhomology was done. Though, Such a finding might be serendipitous, depending on the target sequence (length, complexity, and the number of established micro-homologies) may be worth trying. However, WGS is still preferred if available. Randomly breakage of DNA is another origin of indels where the mentioned method is completely useless, while WGS does not have such limitations.

A range of loss-of-function mutations has been identified in thePARK7 gene. Among the mutation spectrum, in addition to nonsense mutations, frameshift mutations, and deletions, some missense mutations such as p.L166P also have been found, which causes protein instability [15]. Missense mutations cause misfolding or defects in dimerization (like in the case of p.L166P); the misfolded proteins are leaded to the proteasome. As reported by the Human Gene Mutation Database (HGMD®) to data 36 mutations in *PARK7* have been reported, including 19 missense/nonsense mutations, 1 regulatory, 2 splicing-site, 8 gross deletion, and 6 small indels. This deletion removes exons 4 and 5 in the *PARK7* gene, and as exon 5 is an asymmetrical exon (with an incorrect multiple of 3 nucleotides), leading to a frameshift that reaches a stop codon (TGA) after translating nine amino acids in exon 6. Most probably, mRNA will destroy by non-sense mediated decay, and even if cells could ignore this stop codon, protein could not be functional. This variant is compatible with the mechanism of loss-of-function, previously observed in mutations in PARK7 leading to PD.

Regarding genotype-phenotype correlation, the patient has classical features of early-onset PD and psychiatric agreeing with-PARK7 mutation. She responds well to levodopa. PARK7 has a role in male fertility, so infertility in proposita's brother (have no child after six years of marriage) may cause by this mutation [16]. No specific complications have been reported for the carriers of PARK7

A.



B.

C.

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Identities:257/309(83%),  Gaps:9/309(2%), Strand: Plus/Plus

Query  1    GGCCGGCCACAGTGGTTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGATGGGTGTA  60
            ||||||  | ||||||  ||||||||||||||||||||||||||||||  |||  |||   |
Sbjct  1    GGCCGGGCGCAGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCTGAGGCGGGCAGA  60

Query  61   TCAC--GAGGTCAGGAGTTTGAGACCAGCCTGGTCAAGAGGGTGAAACCCCATCTTTACT  118
            ||||  ||||||||||||||  ||||||||||||  ||  | ||||||||||||||| ||||
Sbjct  61   TCACCTGAGGTCAGGAGTTCAAGACCAGCCTGGCCACCATGGTGAAACCCCATCTCTACT  120

Query  119  AAAAATACACAGATTAGCCGGGCACAGTGGTGGGTACCTGTAATCCCAGTTGCTTGGGAG  178
            |||||||||  | |||||||  |||    ||||  |||  ||||||||||||||  | ||  |||||
Sbjct  121  AAAAATACAGAAATTAGCCAGGCGTGGTGGCGGGCGCCTGTAATCCCAGCTACTCGGGAG  180

Query  179  GCTGAGGCAGGAGAATCGCTTGAATCCGGGAGACGGAAGTTGCAGTGAGCTGAGATCA--  236
            ||||||||||||||||||||||||  |||||||  ||||  ||||||||||||  |||||
Sbjct  181  GCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCAGTGAGCCGAGATTGTG  240

Query  237  C---TGCACTCTAACCTGGGTGACA-GAGCAAGACTCTGTCTCaaaaaaaaaaaaaaaag  292
            |   |||||||  | ||||||  ||||  ||||  |||||   ||||||||||||||| ||||
Sbjct  241  CCGTTGCACTCCAGCCTGGGGGACACGAGCGAGACTTCGTCTCAAAAAAAAAATAAAAT-  299

Query  293  aaaagaaaa  301
            ||||  ||||
Sbjct  300  AAAATAAAA  308
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(caption on next page)

Fig. 3. Confirmation of deleted location and size of the deletion. A. The results of surveying interspersed genome repeats (surroundingPARK7) using the UCSC database are shown in the figure. The two highlighted SINE (Alusg4 and Alusq2 with the same orientation) are proposed to provoke the NAHR. B. The result of PCR in the case using primers 5F and 6R. The left side is DNA ladder 50 bp, and the right side is the PCR product in the case. No clear single band was observed in carriers and healthy control.
C. The alignment result of Query (Alusg4) and subject (Alusq2) in the NCBI nucleotide blast is presented here. The yellowish highlighted sequence is where same as the sanger sequence obtained in the case. Recombination must have occurred in the bluish area, but because the sequences of Query and subject in this region are similar, the exact nucleotide where switching took place can not be determined.

Table 3
Primers designed to check the number of copies of exons 4 and 5 of *PARK7* gene using real-time PCR are presented. *ALB* gene has been used as a gene with a specific copy number (2 copies like PARK7) to normalize the data.

row	Primer name	Sequence (5'>3')	Annealing site	Product size (bp)
1	PARK7-E4-F	TAGGCTATCTCCTGTACTTCCCAC	Intron 3	Exon 4: 177
2	PARK7-E4-R	ACCTCAGATAAATTCTGTGCG	Exon 4	Exon 5: 196
3	PARK7-E5-F	TAGTCTGCTGCTGTGAAGGA	Exon 5	*ALB*: 200
4	PARK7-E5-R	TACACCAACACAGATGCCCT	intron 5	
5	ABL-F	GCAGCCAATGAAATACAAAGATG	Intron 1	
6	ABL-R	ACGAAACACACCCCTGGAAT	Exon 2	

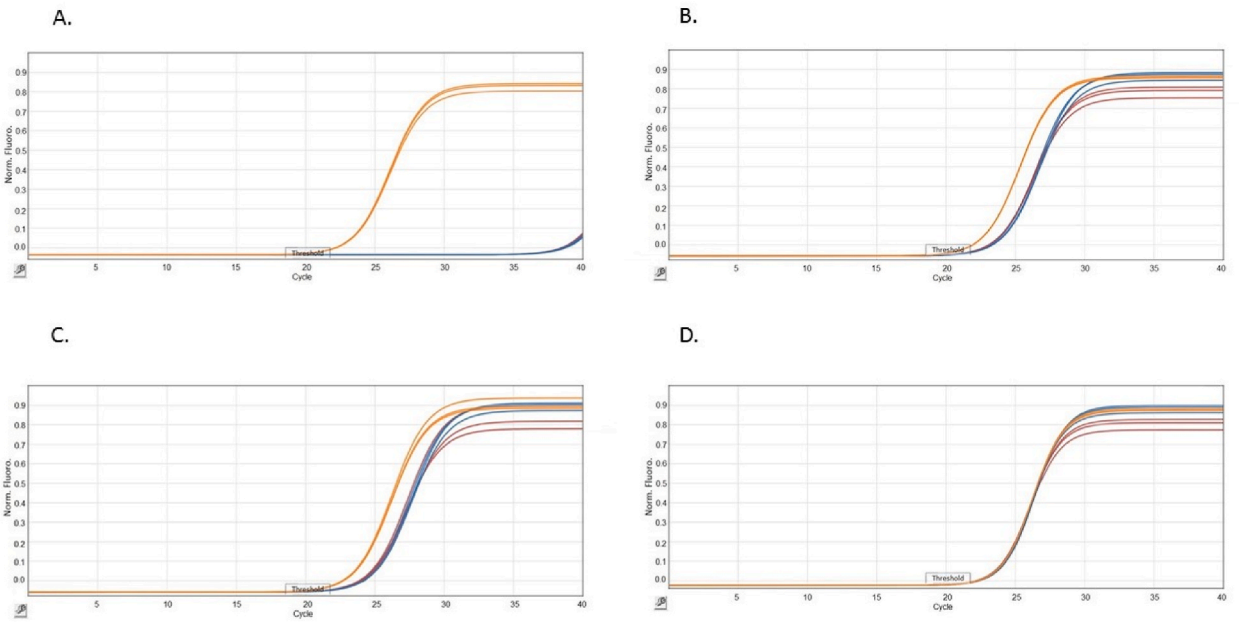

Fig. 4. Real-Time-PCR data. This figure shows the data obtained from the examination of A. In the case, B. her father, C. her mother, and D. a healthy control by Real-Time-PCR. The average Ct in the father's Albumin gene curves was 21.9, and for exonsPARK7 exons, it was equal to 23.3 ($\Delta_{Ct} = 1.4$). These values for the mother were 22.7 and 24.3, respectively ($\Delta_{Ct} = 1.6$), which indicated that the number of copies in these two individuals was half, and as a result, they were carriers. *The yellow curve represents Albumin amplification, and the blue and red curves depict exons 4 and 5 *PARK7*, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutations, and in this family, the parents who were obligated carriers have no striking problems. This variant may also play a role in the psychiatric phenotype in indicated relatives (Fig. 1), but whether a heterozygous mutation in PARK7 increases the risk of developing PD is under debate [17].

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All authors listed have significantly contributed to the investigation, development and writing of this article.

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Data availability statement

The authors do not have permission to share data.

Declaration of interest's statement

The authors declare no competing interests.

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