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ORIGINAL ARTICLE

Investigation of mutations in early-onset Parkinson's disease through next-generation sequencing analysis

Tuba Sözen Turk[1], Ahmet Acarer[2], Asude Durmaz[3], Burak Durmaz[3], Figen Gokcay[2], Zafer Colakoglu[2], Haluk Akın[3], Ozgur Cogulu[4], Ayca Aykut[3]

[1]Department of Medical Genetics, İzmir City Hospital, Medicine İzmir, Türkiye
[2]Department of Neurology, Ege University Faculty of Medicine, İzmir, Türkiye
[3]Department of Medical Genetics, Ege University Faculty of Medicine İzmir, Türkiye
[4]Department of Pediatric Genetics, Ege University Faculty of Medicine İzmir, Türkiye

ABSTRACT

Objectives: This study aimed to expand our understanding of the genetic basis of Parkinson's disease (PD) by investigating individuals diagnosed with early onset PD (EOPD) or those with a suspected genetic predisposition to PD.

Patients and methods: Thirty patients (18 females, 12 males; mean age: 33.2±6.4 years; range, 15 to 48 years) diagnosed with EOPD between January 2018 and December 2019 were included in the study. A targeted next-generation sequencing analysis was conducted on 10 genes (SNCA, LRRK2, VPS35, PARK2, PINK1, PARK7, ATP13A2, PLA2G6, FBXO7, DNAJC6) known to be associated with PD etiology. Additionally, the MLPA method was used to investigate eight genes (SNCA, PARK7, LRRK2, ATP13A2, PINK1, GCH1, PRKN, and UCHL1) for large deletions and duplications.

Results: Mutations in PD-associated genes were identified in seven out of the 30 patients included in the study. Four patients exhibited mutations in the PRKN gene: three had defined deletion mutations (exon 5 deletion, exon 2 deletion, and exon 3 and 4 deletion), and one had a splice site mutation newly identified in this study (c.1083+1delG). Two patients displayed a point mutation in the PLA2G6 gene (c.1705C>T), and one patients had a point mutation in the PINK1 gene (c.1247C>T). The clinical and genetic characteristics of these patients were analyzed to explore genotype-phenotype correlations.

Conclusion: This study is one of the few in Türkiye to examine the molecular etiology of EOPD. The identified mutations in the PRKN, PLA2G6, and PINK1 genes provide valuable insights into genotype-phenotype correlations in PD cases and contribute to the existing literature.

Keywords: Early-onset Parkinson's disease, next-generation sequencing, novel mutation.

The advent of next-generation sequencing (NGS) has ushered in a new era in genomics research, offering unparalleled speed, cost-effectiveness, and throughput. Understanding molecular genetics is crucial in the context of movement disorders, and NGS serves as a powerful tool for unraveling the complexities associated with these conditions. It significantly enhances diagnostic accuracy by providing a comprehensive genetic profile, minimizing the risk of misdiagnosis. This precision in diagnosis is essential for effective patient management and timely interventions.

Early-onset Parkinson's disease (EOPD) is characterized by the onset of the first motor symptoms before the age of 50, typically occurring between 20 and 50 years, and accounts for approximately 4% of all Parkinson's disease (PD) cases.[1] About 15% of individuals diagnosed with PD have a positive family history.[1] The neurodegenerative process of the disease is believed to be influenced by a combination of genetic and environmental factors, along with aging. Approximately 10 to 15% of cases follow a single-gene inheritance pattern (autosomal recessive [AR], autosomal dominant [AD], and, rarely,

Correspondence: Ayca Aykut, MD. Ege Üniversitesi Tıp Fakültesi, Tıbbi Genetik Anabilim Dalı 35100 Bornova, İzmir, Türkiye.
E-mail: aycaaykut@hotmail.com
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X-linked recessive). Most mutations associated with this single-gene inheritance are linked to either AD (*SNCA, LRRK2, GCH1, UCHL1)* or AR *(PARK2, PINK1, PARK7, ATP13A2)* forms of PD. The age at diagnosis often helps differentiate AD PD (typically after age 50) from AR PD (typically before age 40) in PD patients with the single-gene inheritance pattern.[2] The majority (around 80 to 85%) are sporadic cases. In recent years, the use of NGS methods has become widespread in elucidating the genetic basis of PD.[2]

In our study, we targeted individuals diagnosed with EOPD or those with a suspected genetic predisposition to PD. Next-generation sequencing and multiplex ligation-dependent probe amplification (MLPA) were employed to investigate disease-associated variants, screen at-risk family members, generate community-specific data, identify novel mutations in genes, expand the understanding of the disease's genetic etiology, and contribute to the existing literature.

PATIENTS AND METHODS

In this prospective study, index cases diagnosed with EOPD at the Ege University Faculty of Medicine, Department of Neurology between January 2018 and December 2019 and referred to the Medical Genetics Department for molecular genetic investigation were examined. Thirty patients (18 females, 12 males; mean age: 33.2±6.4 years; range, 15 to 48 years), all without familial relationships with each other, were included in the study. Data from medical records and clinical examinations were recorded, including personal and family history information. A pedigree analysis covering at least three generations was constructed for each case. The study protocol was approved by the Ege University Medical Research Ethics Committee (Date: 04.03.2020, No: 20-3T/27). Written informed consent was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients clinically diagnosed with EOPD and followed up at the Department of Neurology were examined for mutations in 10 genes *(SNCA, LRRK2, VPS35, PARK2, PINK1, PARK7, ATP13A2, PLA2G6, FBXO7, and DNAJC6)* associated with EOPD using targeted NGS. The MLPA method was applied to assess eight genes *(SNCA, PARK7, LRRK2, ATP13A2, PINK1, GCH1, PRKN, and UCHL1)* for significant deletions and duplications.

DNA isolation

The DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).[3] The extracted DNA quantity was measured with the Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, San Francisco, CA, USA), following the manufacturer's instructions.[3]

Next-generation sequencing analysis

Library preparation was performed using the Ion Chef System (Thermo Fisher Scientific), following the manufacturer's protocols. Ion Reporter software was used for mutation analysis, and variants were individually assessed based on clinical phenotype, minor allele frequency (MAF) score, and pathogenicity scores calculated by prediction programs.[4] Variants were filtered to retain nonsynonymous changes with a MAF score <0.01 using combined datasets from the 1000 Genomes Project, the Exome Variant Server project, the Genome Aggregation Database (gnomAD) Barcoded libraries were generated from 10 ng of DNA per sample using the Ion AmpliSeq Chef Solutions and the Custom Ion AmpliSeq Panel (Thermo Fisher Scientific).[5] This panel consisted of 884 amplicons covering 242.96 kb, including coding regions, 10 base pair exon-intron boundary regions, and other regions with defined mutations (e.g., deep intron and UTR), achieving a coverage rate of 98.72%.

The resulting libraries were clonally amplified onto Ion Sphere Particles (ISPs) using emulsion polymerase chain reaction (PCR) within the Ion Chef System, following the manufacturer's guidelines. Enriched ISPs were then loaded onto 530 chips, allowing for 16 samples per sequencing run. Sequencing was conducted using an Ion S5 Sequencer with an Ion 530 Chip and an Ion 530 Kit-Chef Kit (all from Thermo Fisher Scientific).[5]

The sequences were aligned to the reference genome hg19, and base calling was performed using the Torrent Suite software (Thermo Fisher Scientific, Massachusetts, USA). The annotated variant calling file was filtered in the Ion Reporter Software to display only the variants relevant to the 10 PD-related genes *(SNCA, LRRK2, VPS35, PARK2, PINK1, PARK7, ATP13A2, PLA2G6, FBXO7, and DNAJC6).* According to the American College of Medical Genetics and Genomics guidelines, variables were classified. Varsome and Franklin databases were used as in silico analysis programs to evaluate the pathogenicity of the variants.

Multiplex ligation-dependent probe amplification analysis

The probe mixtures within the SALSA MLPA Parkinson Kit (MRC Holland, Amsterdam, the Netherlands), specifically P051-D2-0618 and P052-D2-0618, were used for deletion and duplication analyses. The genes included in these probe mixtures were *SNCA*, LRRK2, PINK1, PARK2 (PRKN), DJ1, ATP13A2, GCH1, and UCHL1.

Samples were denatured at 98°C for 5 min in a thermal cycle device. After denaturing, the device was switched to the waiting phase at 25°C. Then the hybridization program was started in the thermal cycle device. The samples were kept at 95°C for 1 min and then at 60°C for 16 to 20 h. The ligation PCR protocol was started from the thermal cycle device. Afterward, the program was started at 54°C for 15 min and 98°C for 5 min, followed by a waiting phase at 20°C. The PCR mix was prepared. The MLPA PCR protocol was started from the Thermal Cycler device (Thermo Fisher Scientific, Massachusetts, USA). Then the capillary electrophoresis was started. The raw data in the FSA format were analyzed using Coffalyser.net version 131211.0000 (MRC Holland, Amsterdam, the Netherlands). The fragments obtained from capillary electrophoresis were transferred to Coffalyser.net.[6]

Sanger sequencing was performed for segregation analysis and to confirm detected mutations. The MLPA method was also used to conduct segregation analyses for cases with deletions detected.

RESULTS

The mean age at onset was 26.7±6.1 years (range, 14 to 44 years). Thirteen (43.3%) patients had a positive family history of PD. Ten genes associated with the disease *(SNCA, LRRK2, VPS35, PARK2, PINK1, PARK7, ATP13A2, PLA2G6, FBXO7,* and *DNAJC6)* were investigated using targeted NGS analysis. Deletions and duplications in specific genes related to the disease were subsequently examined using the MLPA method.

Mutations were detected in seven (23.3%) out of the 30 patients. Four (57.1%) of them had a family history of PD. Six of these mutations were previously identified, while one was considered a novel mutation (c.1083+1delG). Among these, three were missense mutations, three were large deletions, and one was a splice site mutation. The mutations identified in the cases are presented in Table 1.

TABLE 1
Clinical classification features and disease-causing variants identified in EOPD patients

Patient	Age/Sex	Age on set	Gene	Consanguinity	Inheritance	Zygosity	Transcript	cDNA	Protein	Novel/ previously described	ACMG	Clinical presentation
1	38/F	25	PLA2G6	Yes	AR	Homozygous	NM_001349867.1	c.1705C>T	p.Arg569Trp	Previously described	Pathogenic	Initiating with dystonia in the lower extremities, an akinetic-rigid form
2	44/F	28	PRKN	Yes	AR	Homozygous	NM_004562.3	Exon 5 deletion		Previously described	Pathogenic	Tremor dominant form
3	38/F	27	PLA2G6	Yes	AR	Homozygous	NM_001349867.1	c.1705C>T	p.Arg569Trp	Previously described	Pathogenic	Initiating with dystonia in the lower extremities, an akinetic-rigid form
4	57/M	40	PRKN	Yes	AR	Homozygous	NM_004562.3	Exon 2 deletion		Previously described	Pathogenic	Akinetic-rigid form
5	34/M	14	PINK1	Yes	AR	Homozygous	NM_032409.2	c.1247C>T	p.Pro416Leu	Previously described	Pathogenic	Initiating with dystonia in the lower extremities, an akinetic- form
6	36/M	25	PRKN	Yes	AR	Homozygous	NM_004562.3	Exon 3-4 deletion		Previously described	Pathogenic	Initiating with dystonia in the lower extremities, an akinetic- form
7	28/F	15	PRKN	No	AR	Homozygous	NM_004562.3	c.1083+1delG		Novel	Pathogenic	Tremor dominant form

EOPD: Early onset Parkinson's disease; cDNA: Complementary deoxyribonucleic acid; ACMG American College of Medical Genetics; AR: Autosomal recessive.

All the mutated genes *(PLA2G6, PRKN, and PINK1)* had AR inheritance. In the segregation analysis, the parents of the patients were studied by using sanger sequencing or MLPA methods, and it was shown that both parents were heterozygous carriers of the relevant alteration. Since only the fathers of Case 1 and Case 4 were deceased, their fathers could not be included in the segregation analysis. The mother of Case 4 and the parents of Case 5 were not included in the segregation study because they did not want to give consent for testing.

The *PRKN* gene had the highest mutation rate, identified in four (57.1%) out of seven cases. The second most frequently mutated gene was PLA2G6, with mutations found in two (28.5%) out of seven cases. The third gene with mutations was PINK1, with one (14.2%) out of seven cases. Consanguinity between parents was observed in the pedigrees of cases with identified mutations as in the pedigree of Case 7 with homozygous novel mutation (c.1083+1delG) (Figure 1). The phenotypic-genotypic characteristics of the cases are also detailed in Table 1.

DISCUSSION

To date, pedigree and cohort studies have identified several genes responsible for PD, including *SNCA, UCHL1, LRRK2, PRKN, PINK1, DJ1,* and *ATP13A2*.[7] While point mutations in these genes are the most extensively investigated variations in PD cases, larger genomic rearrangements and copy number variations have also been identified as significant mutations contributing to PD.

Approximately 10% of patients exhibit mutations in the *LRRK2, SNCA, PRKN,* and *PINK1* genes.[8-10] These genetic factors influence clinical features such as age of disease onset, symptom manifestation, prognosis, and cognitive function.[8,11-13] The frequency of gene mutations in EOPD has been investigated in various studies.[14,15] Interestingly, mutations in certain genes, such as *LRRK2*, have also been found in a substantial number of non-familial late-onset PD patients.[16]

In our cohort, akinesia/rigidity was more prominent, which aligns with a study that examined the different clinical features of PD based on age of onset, showing that early-onset cases predominantly presented with akinesia/rigidity, whereas late-onset cases more often started with resting tremors.[17]

In a study by Lohmann et al.[18] involving 86 patients diagnosed with AR PD in the Turkish population, alterations in the *PRKN, PINK1*, and *DJ1* genes were investigated. PRKN mutations were detected in 16.9% of cases, while PINK1 mutations were found in 2.6%. The high mutation rate in these AR genes within our population may be attributed to the high prevalence of consanguineous marriages (22%).[19] In our

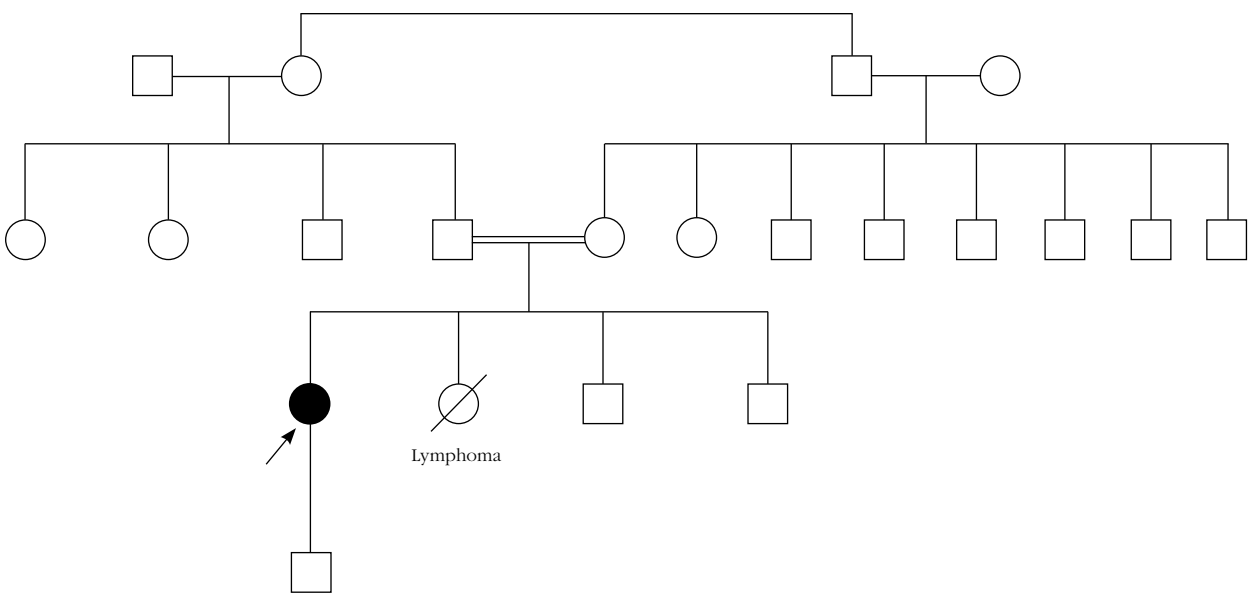

Figure 1. The pedigree of the Case 7 with novel mutation.
• Parkinson disease.

study group, the consanguinity rate among the parents of the seven cases with mutations was 100%. Notably, the three genes with mutations identified in our study *(PRKN, PLA2G6, and PINK1)* follow the AR inheritance pattern.

Mutations in the *LRRK2* and *SNCA* genes are well-known causes of AD PD. In a study involving 91 patients with a family history consistent with AD inheritance in the Turkish population, a mutation rate of 3.3% was detected. Specifically, one (1.1%) patient had an *LRRK2* gene c.6055G>A (p.Gly2019Ser) mutation and two (2.2%) patients had an *SNCA* gene duplication.[20] In our series, no mutations were detected in genes associated with AD PD. The low number of cases with mutations in the examined AD genes might explain this absence, and the relatively high consanguinity rates in Türkiye make AR genes more noteworthy than AD genes.[21]

The *PRKN* gene is the most common cause of AR PD and accounts for approximately 50% of parkinsonism cases with onset before age 40. Mutations in this gene are highly diverse, including missense, nonsense, frameshift, exon deletions, or duplications, as noted in genomic rearrangements.[21-24] This rate is consistent with our findings (57.1%).

The onset age for individuals with mutations in the *PRKN* gene is typically around 30 but can range from childhood to around 50.[2,25] In our study, the onset ages for individuals with *PRKN* mutations were 28, 40, 25, and 15 years, with a mean onset age of 26.7±6.1, which aligns with the literature.

The *PRKN* gene comprises 12 exons, and approximately half of the mutations involve genomic rearrangements, including large deletions and duplications.[26] According to the 2019[4] release of the Human Gene Mutation Database (HGMD), 378 mutations associated with this gene have been reported, with 161 (42.5%) being large deletions. In our study, among the four patients with PRKN mutations, three (75%) had large deletions, and one (25%) had a splicing mutation. The rate of large deletions in our series was higher than that reported in the literature.

In the HGMD, 16 (4.2%) mutations involving splicing in the *PRKN* gene are reported, and the rate of splicing mutations in our PRKN gene series was also higher than that found in the literature. While mutations in this gene can be detected by sequencing analysis at a rate below 50%, they can be identified at a rate above 50% with MLPA. Therefore, the simultaneous use of both methods is beneficial. In our study, three out of the four mutations were large deletions, which were also detected in sequencing analysis, supporting this approach.

Hedrich et al.[27] screened 379 carriers of PRKN mutations, and a higher incidence of deletions and duplications was observed in exons 2 and 9. Similarly, a 2019 study by Pandey et al.[28] reported that 32% of variants in the *PRKN* gene were located in exon 2. One of the *PRKN* mutations in our series (25%) was found in exon 2, although no mutations were detected in exon 9. Bouhouche et al.[29] identified exons 2 through 8 as a mutation hotspot region. The deletion mutations (exon 2, exon 3-4, and exon 5) identified in three cases in our study were consistent with this hotspot mutation region.

The homozygous splicing mutation (c.1083+1delG) detected in our series has not been previously reported in the literature. This mutation results from the deletion of the first nucleotide guanine in intron 9. Segregation analysis showed that the parents of the individual with this mutation were heterozygous carriers. This particular case (Case 7) had an onset age of 15 and was followed as juvenile parkinsonism. Despite the parents being first-degree cousins, the clinical presentation was tremor-dominant parkinsonism. Constipation accompanied the clinical symptoms of this case, and clinical follow-up revealed an early requirement for levodopa, impulsivity, and frontal dysfunction. In a 2011 study by Ghazavi et al.,[30] a homozygous missense mutation (c.1083+1G>A) in the same region of intron 9 was reported. The onset age of the male case was 18, and the clinical presentation included classic parkinsonism symptoms such as tremors, bradykinesia, rigidity, postural instability, a good response to levodopa, voice changes, and alterations in handwriting. The parents of this case were known to be relatives. These two cases, with different types of mutations in the same region, exhibited very similar clinical presentations.

In our study, the second most common mutation was found in the *PLA2G6* gene, with a prevalence of 28.5% (two out of seven cases). This gene, typically associated with an atypical clinical phenotype, is often linked to conditions such as dementia, pyramidal symptoms, ataxia, psychiatric symptoms, ocular disorders, and early motor complications. Sheerin et al.[31] reported that *PRKN, PINK1*, and *DJ1* were responsible for PH with typical parkinsonism symptoms, while

ATP13A2, FBXO7, DNAJC6, SYNJ1, and *PLA2G6* were associated with more complex phenotypes presenting atypical symptoms. Various mutations in *PLA2G6* have been reported in association with atypical AR PD[32-34] and sporadic EOPD.[35,36]

Although the clinical symptoms in most cases begin in childhood or early adulthood (8 to 36 years), they exhibit considerable heterogeneity. Distinct dystonia, ataxia, cognitive decline, a good response to levodopa, and drug-induced dyskinesia are characteristic features.[37] In our study, the onset ages of the two cases with mutations in the *PLA2G6* gene were 25 and 30 years, which are consistent with the information reported in the literature.

PLA2G6 is a 17-exon gene. According to the HGMD, 141 mutations have been reported in association with this gene, with the majority (102 [72.3%] out of 141) being missense/nonsense mutations. Small deletions and splicing mutations are the next most common types. The mutation in *PLA2G6* identified in two cases in our study was the same and was of the missense type, consistent with the mutation types most commonly reported in the literature.

The mutation detection rate for the gene using sequence analysis is 85%, while the detection rate for deletions/duplications with MLPA is ≤12.5%.[38] Therefore, sequence analysis should be the primary method for examining this gene. As expected, the point mutation identified in both of our cases was detected through sequence analysis.

In our series, the third most frequent mutation (one [14.2%] out of seven cases) was identified in the *PINK1* gene. *PINK1*-related parkinsonism is the second most common cause of AR PD after *PRKN*.[39-42] However, since *PLA2G6* was not evaluated in these studies, a direct comparison of the mutation detection ranking in our study with theirs is not possible.

Valente et al.[41] and Hatano et al.[43] reported that mutations in the *PINK1* gene were found in 1 to 7% of Caucasian patients with EOPD. In Japanese patients, this rate was approximately 9%.[39] The rate identified in our series was slightly higher than the rates reported in the literature.

The *PINK1* gene comprises eight exons. According to the HGMD, 134 mutations are associated with this gene, and 102 (76%) are missense/nonsense mutations, followed by large deletion mutations (11%). The *PINK1* mutation identified in our series is also of the missense type, aligning with the most commonly reported mutation group.

The mutation detection rate for this gene using sequence analysis is over 88%, while the rate for deletions/duplications detection with MLPA is less than 12%.[44] Therefore, sequence analysis should be the primary method for examining this gene. As expected, the *PINK1* mutation in our series was detected using sequence analysis.

The *PINK1* mutation c.1247C>T (p.Pro416Leu) identified in our series was previously reported by Lohmann et al.[18] in 2012. This mutation results in the substitution of proline with leucine at position 416 in exon 6, a universally conserved amino acid position crucial for protein function. A mutation very close to this, p.Pro416Arg, was previously identified as a homozygous mutation in a Jordanian family with a very early onset of symptoms (23 and 25 years old).

In the study by Lohmann et al.,[18] the homozygous p.Pro416Leu mutation was identified in a case with symptom onset at the age of 52, and the symptoms regressed significantly after levodopa treatment. No phenotypic differences were observed between family members who were heterozygous or homozygous for the mutation, suggesting that heterozygous mutations are also relevant for the *PINK1* gene.

In our study, Case 5, who carried the *PINK1* mutation, was diagnosed with juvenile parkinsonism with dystonia in the lower extremities at the age of 14. This case had a family history of PD in both the father and a male sibling. The male sibling's disease onset was at age 43. Clinical follow-up indicated the progression from dystonia to akinetic PD and highlighted the presence of impulse control disorder. Segregation analysis could not be performed as the patient had no contact with other family members. The onset age of Case 5 is considered quite young compared to other cases with the same mutation reported in the literature.

Our study identified no mutations in the *LRRK2, SNCA, VPS35, ATP13A2, DJ1, FBXO7, DNAJC6, GCH1,* and *UCHL1* genes. This limitation could be attributed to the relatively small sample size.

Currently, there is no definitive algorithm for the molecular diagnosis of PD. However, given the need to distinguish overlapping phenotypes and identify submicroscopic structural variations, multigene panels are considered a preferred option, as they are also capable of detecting large exon deletions.

In studies investigating the molecular etiology of PD, the simultaneous use of NGS and MLPA techniques is common. For example, a study conducted in Morocco by Bouhouche et al.[29] with 145 patients with PD employed chromosomal microarray (CMA) and NGS concurrently. In populations with a high rate of consanguinity, such as Morocco, they emphasized the potential of CMA as a first-line diagnostic test and its effectiveness in identifying candidate genes for recessive diseases. The combination of CMA and NGS was highlighted as a significant step in understanding the genetic basis of AR PD.

Given the high consanguinity rate in Türkiye and the fact that all cases with identified mutations in our series were linked to AR PD, this combined approach was deemed valuable.

Targeted NGS-based panel tests enable the detection of higher mutation rates in diseases with a low prevalence of monogenic forms. The advantage of designing specific panels by selecting genes where mutations are relatively more common has proven beneficial, particularly in diseases like PD, where monogenic forms constitute a small percentage of cases.

Although cases showing an AD inheritance pattern were identified in our study, no mutations were detected in genes responsible for AD PD. This may be due to the small sample size and the typical onset age of the disease being generally under 50 years. Additionally, the 23 cases without detected mutations may represent instances where genetic susceptibility plays a significant role, as suggested by family histories and clinical findings. These cases might still harbor mutations in other genes associated with PD, or they could benefit from whole exome sequencing in the future to identify new candidate genes.

The limited number of cases in our study was a constraint, leading to relatively low diversity in the mutated genes identified. Studies with a larger number of participants may reveal mutations across different inheritance patterns and types. Future efforts are planned to reevaluate the clinical features and family histories of cases without detected mutations using NGS and MLPA. Subsequently, whole exome sequencing may be considered to identify potential candidate genes and expand the understanding of the genetic etiology of PD.

In conclusion, this study identified a novel splice site mutation in the *PRKN* gene, previously unreported in the literature, as the cause of EOPD. The mutations identified in the *PRKN, PLA2G6*, and *PINK1* genes contribute to the genotype-phenotype correlation in patients with EOPD, providing valuable insights into cases reported in the literature. In our community, mutations in the *PRKN* gene, particularly large deletions, are relatively common among patients with EOPD. Our study, one of the few investigating the molecular etiology of EOPD phenotypes in the Turkish population, makes a significant contribution to the literature by identifying disease-associated variants, screening other at-risk family members, generating community-specific data, and expanding the genetic understanding of the disease by identifying mutations in new genes. Additionally, this study is among the first in Türkiye to explore the molecular etiology in cases with familial parkinsonism. The identified mutations in the *PRKN, PLA2G6,* and *PINK1* genes support the genotype-phenotype correlation for patients with EOPD, as documented in the literature. Given that mutations in the PRKN gene, particularly large deletions, are quite common in familial PD cases within our population, this study facilitated the screening of other at-risk family members and provided mutation-specific genetic counseling. By contributing to the identification of disease-associated variants, generating data specific to our community, and expanding the genetic etiology of the disease, this study offers a valuable contribution to the scientific literature.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Conceived and designed the study, performed the data analysis, and interpreted the results. She was responsible for the overall data interpretation and statistical analysis: T.S.; Contributed to the patient recruitment, clinical evaluation, and management of patient data. He also participated in the review of clinical findings and provided input on the manuscript: A.A.; Assisted in data collection, contributed to clinical assessments, and was involved in interpreting clinical outcomes: A.D.; Worked on patient recruitment, managed the clinical data, and supported the clinical evaluation process: B.D.; Contributed to patient recruitment, data collection, and clinical assessments: F.G.; Participated in patient recruitment and clinical evaluations: Z.C.; Contributed to clinical evaluations, data interpretation, and review of the manuscript. He also provided guidance in clinical aspects throughout the study: H.A.; Led the clinical evaluation, patient recruitment, and provided expertise on the interpretation of clinical findings: O.C.; Coordinated the data analysis,

contributed to the interpretation of results, and was responsible for statistical analysis. She played a key role in manuscript writing and final revisions: A.A.

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