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Article

Re-evaluation of Genetic Variants in Parkinson's Disease Using Targeted Panel and Next-Generation Sequencing

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Abstract

Parkinson's disease (PD) is a complex disorder with a significant genetic component. Genetic variations associated with PD play a crucial role in the disease's inheritance and prognosis. Currently, 31 genes have been linked to PD in the OMIM database, and the number of genes and genetic variations identified is steadily increasing. To establish a robust correlation between phenotype and genotype, it is essential to compare research findings with existing literature. In this study, we aimed to identify genetic variants associated with PD using a targeted gene panel with next-generation sequencing (NGS) technology. Our objective was also to explore the idea of re-analyzing genetic variants of unknown significance (VUS). We screened 18 genes known to be related to PD using NGS in 43 patients who visited our outpatient clinic between 2018−2019. After 12−24 months, we re-evaluated the detected variants. We found 14 different heterozygous variants classified as pathogenic, likely pathogenic, or VUS in 14 individuals from nonconsanguineous families. We re-evaluated 15 variants and found changes in their interpretation. Targeted gene panel analysis with NGS can help identify genetic variants associated with PD with confidence. Re-analyzing certain variants at specific time intervals can be especially beneficial in selected situations. Our study aims to expand the clinical and genetic understanding of PD and emphasizes the importance of re-analysis.

Keywords: Parkinson's disease; parkinsonism; next generation sequencing; reanalysis; Canakkale population; Parkinson's genetic

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by Lewy bodies in the midbrain and the loss of dopaminergic neuron activity, particularly in the substantia nigra (Rodriguez-Oroz et al., 2009). PD is the second most common neurodegenerative disease worldwide (de Lau & Breteler, 2006; Rodriguez-Oroz et al., 2009; van den Eeden et al., 2003). The increased prevalence in some racial groups might be due to the carrier status of certain genetic variants (Chillag-Talmor et al., 2011). Studies suggest that first-degree family members of a person with PD are at 2·7-fold increased risk. Epidemiology studies have reported that there is a 10−30% positive family history for PD, although penetrance may vary (Marder et al., 2003; Sveinbjornsdottir et al., 2000).

Until 1997, PD was mostly considered a sporadic disorder with certain environmental contributions (Langston et al., 1983). In 1997, an article identified the *SNCA* gene loci for PD (Polymeropoulos et al., 1997). The genetic etiology of PD is estimated to account for 5−15% of cases (Kalinderi et al., 2016). Studies have shown an increasing correlation between early age diagnosis and genetic background (Alcalay et al., 2010; Marder et al., 2010). Increasing amounts of data suggest that specific genetic alleles exhibit a Mendelian inheritance pattern for PD. *SNCA*, *LRRK2*, *VPS35*, *PRKN*, *PINK1*, *GBA*, and *DJ-1* genes are the leading candidates for suggested monogenic PD, although other genes have also been linked to Mendelian forms and sporadic cases of PD (Bandres-Ciga et al., 2020; Yan et al., 2017). For sporadic cases of PD, which comprise a significant proportion of cases, a small number of genetic loci have been attributed to the etiology. Sporadic cases of PD are mostly explained by the combined effects of genetic and nongenetic factors (Kalinderi et al., 2016). In this study, we reanalyzed the genetic data of 43 subjects and found 14 different heterozygous variants, including two novel variants, emphasizing the importance of reanalysis.

Materials and Methods

Ethical Issues

The ethical committee of Canakkale Onsekiz Mart University Faculty of Medicine reviewed the study for the cohort, and permission was obtained (No: 2020-12/September 23-2020). All subjects enrolled in this study were informed, and a signed consent form was obtained from subjects or legal family members. Patient privacy and confidentiality were protected when nonidentifiable cohort data were used.

Study Population

This retrospective study examined the genetic data of 43 patients who were referred to our clinic between 2018 and 2019 with indications of clinically diagnosed PD (based on the International Parkinson and Movement Disorder Society criteria,) parkinsonism

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Table 1. Custom designed NGS gene panel

Genes	Ref Seq	OMIM number	Genes	Ref Seq	OMIM number
ATP13A2 (ATPase, Type 13A2)	NM_022089.3	610513	*MAPT* (Microtubule-Associated Protein TAU)	NM_001123066.3	157140
SNCA (Synuclein, Alpha)	NM_001146055.2	163890	*PLA2G6* (Phospholipase A2, Group VI)	NM_001349867.1	603604
DNAJC13 (DNAJ/HSP40 Homolog, Subfamily C, Member 13)	NM_015268.3	614334	*PRKN (PARK2)* (Parkin)	NM_004562.2	602544
GIGYF2 (GRB10-Interacting Gyf Protein 2)	NM_001103146.1	612003	*PARK7* (Parkinson Disease 7)	NM_007262.4	602533
HTRA2 (Htra Serine Peptidase 2)	NM_181575.4	606441	*PINK1* (Pten-Induced Putative Kinase 1)	NM_032409.2	608309
LRRK2 (Leucine-Rich Repeat Kinase 2)	NM_198578.3	609007	*VPS35* (Vacuolar Protein Sorting 35)	NM_018206.5	601501
SYNJ1 (Synaptojanin 1)	NM_003895.3	604297	*EIF4G1* (Eukaryotic Translation Initiation Factor 4-Gamma, 1)	NM_001194947.1	600495
CHCHD2 (Coiled-Coil-Helix Domain-Containing Protein 2)	NM_016139.3	616244	*FBXO7* (F-Box Only Protein 7)	NM_012179.3	605648
FGF20 (Fibroblast Growth Factor 20)	NM_019851.2	605558	*UCHL1* (Ubiquitin Carboxyl-Terminal Esterase L1)	NM_004181.4	191342

Note: OMIM, Online Mendelian Inheritance in Man (catalog); Ref Seq, Reference Sequence (database).

without a clear diagnosis (at least one cardinal symptom of PD for more than one year), family history, and incidental genetic findings. The data were collected and re-evaluated in late 2020. Clinical history, pedigree analysis, age, sex, history of drug use, and exposure to pesticides were obtained during clinical observation. Phone calls were made to gather missing data due to the COVID-19 pandemic. The patients exhibited a wide range of symptoms, including rigidity, tremor, dyskinesia, postural instability, dysphagia, axial deformities, sleep disturbances, memory deficits, reduced cognitive function, dementia, hallucinations, mood disorders, autonomic dysfunctions (mostly orthostatic hypotension, urogenital dysfunction, constipation, excessive sweating, etc.), sensory symptoms (decreased olfactory senses), and pain disorders. Any imaging studies involving probands were also noted.

Genetic Analyses

We conducted genetic analyses on 43 patients in this study using a custom-designed gene panel containing 18 genes associated with PD. The 18 genes, their transcript numbers, and related clinical OMIM numbers are summarized in Table 1. The gene panel was specifically designed to detect any single nucleotide variation and copy number variation changes in exons, exon-intron junctions, and splicing regions (+-10bp). DNA was extracted from peripheral blood using the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Germany), and genes were sequenced on the Ion S5TM System (Thermo Fisher Scientific, USA) after library preparation. The sequence reading results were aligned to the reference human genome (hg19/GRCh37.p13) via Torrent Suite™ Software to obtain the Binary Alignment/Map (BAM)-Variant Calling Format (VCF). The Franklin Genoox database was used for annotation in the reanalysis process. All variants were classified and reclassified according to American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants as pathogenic (P), likely pathogenic (LP), variants of unknown significance (VUS), likely benign (LB), or benign (B). The ClinVar database was used for annotation of variants in addition to variant frequency in the gnomAD database. Clinically significant variants were then reported in three categories based on their pathogenicity: pathogenic variants, likely pathogenic variants, and VUS. We compared the first and second interpretations of the variants, and confirmation of variants was achieved using the IGV genome browser 2.9.4.

Results

A total of 43 patients from 39 families were enrolled in this study, consisting of 22 females and 21 males, with a median age of 53.4 years. Only three cases showed consanguinity in the pedigree analysis. We identified 14 different clinically significant reportable variants (classified as pathogenic, likely pathogenic, or VUS) in 14 individuals from nonconsanguineous families. Eight (57%) of the probands were considered late onset (50 years of age or after), and 4 (28%) of the probands were considered early onset (before 50 years of age) when considering probands with a clinical PD diagnosis or having at least one long-term cardinal parkinsonism symptom. Probands who had a family member diagnosed with PD or showed at least one long-term parkinsonism symptom constituted 20 (46%) of the cohort. Of this subgroup, 8 (40%) had clinically significant variants. Only one proband had an incidental genetic test result regarding PD-related genes. None of the three juvenile onset probands (20 years of age and before) had any clinically significant variant. Table 2 summarizes the clinical and demographic characteristics of the patients with clinically significant variants. Out of the 14 (32%) clinically significant variants, 3 (7%) were classified as P or LP, while 11 (26%) were VUS. Two variants were not reported in databases and were defined as novel variants. There was no homozygosity or compound heterozygosity. We have summarized the details in Table 3.

Reanalysis Findings

In the reanalysis process, 14 variants were found to be benign or likely benign, despite being classified as VUS in the first

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Table 2. Clinical and demographical characteristics of probands with clinically significant variant

Clinical characteristics	Laboratory ID													
	P-02	P-10	P-12	P-13	P-14	P-23	P-25	P-26	P-28	P-31	P-33	P-37	P-38	P-39
Age	2	29	75	97	77	40	53	57	64	66	70	54	54	70
Gender	M	F	M	F	F	M	M	F	F	F	M	F	F	M
Symptom onset	–	25	53	80	75	37	N/A	54	60	50	65	50	–	65
Motor symptoms	–	+	+	+	+	+	+	+	+	+	+	+	–	+
Sensory symptoms	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Dementia	–	–	+	–	–	–	–	–	+	–	–	–	–	–
Autonomic dysfunction	–	–	–	–	–	–	–	+	–	+	–	–	–	–
Compatible imaging studies	N/A	–	+	+	+	+	+	+	+	–	N/A	N/A	N/A	+
Family history	–	+	+	–	–	–	+	+	+	+	–	+	+	+

Table 3. Laboratory IDs of probands, reference number, codon, mutation type, allele frequency and clinical significance according to different databases of variants detected in the cohort

Lab. ID	Gene	Transcript	Variant	Protein	Effect	Allel Freq. (gnomAD)	ACMG	ClinVar	İnterpretation
P-2	*FBXO7*	NM_012179.4	c.1546G>C	p.Asp516His	Missense	0.00002841	VUS	Benign (1);VUS (3)	VUS
P-10/ P-25	*FGF20*	(NM_019851.3)	c.499G>C	p.Val167Leu	Missense	–	VUS	–	VUS
P-28/ P-25	*PRKN*	(NM_004562.3)	c.245C>A	p.Ala82Glu	Missense	0.003484	Benign	Benign (1);VUS (2)	VUS
P-12	*SYNJ1*	(NM_003895.3)	c.700G>A	p.Ala234Thr	Missense	0.00005656	Likely benign	VUS	VUS
P-13	*EIF4G1*	(NM_001194947.1)	c.3142A>G	p.Ser1048Gly	Missense	0.00002387	Likely pathogenic	–	Likely pathogenic
P-14	*SYNJ1*	(NM_003895.3)	c.3863C>T	p.Pro1288Leu	Missense	0.0003041	Likely benign	VUS	VUS
P-23	*VPS35*	(NM_018206.6)	c.506+6T>C	–	Splicing	0.000007991	VUS	–	VUS
P-26	*PRKN*	(NM_004562.3)	c.136G>A	p.Ala46Thr	Missense	0.000003978	VUS	Benign	VUS
P-31	*DNAJC13*	(NM_015268.4)	c.3872A>G	p.Glu1291Gly	Missense	0.004565	Benign	Likely benign (2);VUS (1)	VUS
P-33	*ATP13A2*	(NM_022089.4)	c.2859G>A	p.Thr953=	Splicing	0.000007960	Likely pathogenic	Benign (1); Likely benign (1); VUS (4)	Likely pathogenic
P-38	*ATP13A2*	(NM_022089.4)	c.2816T>C	p.Leu939Pro	Missense	–	VUS	–	VUS
P-39	*LRRK2*	(NM_198578.4)	c.2915A>G	p.Asp972Gly	Missense	0.00006385	VUS	VUS	VUS
P-39	*EIF4G1*	(NM_198241.3)	c.1403C>A	p.Ala468Glu	Missense	–	VUS	–	VUS
P-37	*LRRK2*	(NM_198578.4)	c.4915delA	p.Arg1639GlyfsTer15	Frameshift	–	Pathogenic	–	Pathogenic

Note: VUS, variants of unknown significance; ACMG, American College of Medical Genetics and Genomics standards and guidelines.

interpretation. Only one variant, *PRKN*(NM_004562.3): c.136G>A, was originally classified as benign or likely benign and later reclassified as VUS. Table 4 shows a comparison between the first interpretation and reanalysis results for the variants, which occurred 12–24 months apart.

Discussion

Genotype-Phenotype Correlation

In this study, we aimed to contribute to the literature by making phenotype correlations and emphasizing the importance of reanalysis of VUS by using a targeted gene panel with next-generation sequencing (NGS). We performed genetic testing on 43 patients and procured 14 different variants from 14 different subjects. To date, this study is the widest cohort with the largest gene panel study conducted with the NGS technique in Turkey, where approximately 150,000 patients have been diagnosed with PD, according to the Turkish Neurology Society.

We designed this study with 18 genes that are related to PD. A targeted gene panel with the NGS method gives us the possibility to study multiple genes with high confidence. The percentage of P or LP variants found in the cohort was 7%, which is consistent with the literature. The detected variants were all heterozygous, and the total percentage of clinically significant variants remained

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Table 4. Comparison of the variants according to first evaluation and reanalysis

Gene	Transcript	Variant	ClinVar First	ClinVar Reanalysis	ACMG First	ACMG Reanalysis
PINK1	NM_032409.3	c.1251+43C>T	–	–	VUS	Benign
PINK1	NM_032409.3	c.1252-25T>C	–	–	VUS	Benign
FGF20	NM_019851.3	c.616G>A	–	Benign	VUS	Benign
LRRK2	NM_198578.4	c.1653C>G	Benign(1); VUS(1)	Benign	VUS	Benign
DNAJC13	NM_015268.4	c.4385G>A	–	Benign	VUS	Benign
SYNJ1	NM_003895.3	c.1000A>C	–	Benign	VUS	Likely benign
GIGYF2	NM_001103146.3	c.3524-26T>G	–	–	VUS	Benign
GIGYF2	ENST00000409451.3	c.1311T>C	–	–	VUS	Benign
LRRK2	NM_198578.4	c.4624C>T	Benign (1); VUS (1)	Benign	VUS	Benign
PINK1	NM_032409.3	c.737G>A	–	–	VUS	Likely benign
GIGYF2	NM_001103146.1	c.3104C>G	–	–	VUS	Benign
EIF4G1	NM_001194947.1	c.1424C>A	N/A	–	N/A	VUS
VPS35	NM_018206.6	c.151G>A	VUS	Benign	Likely benign	Benign
PRKN	NM_004562.3	c.-30T>C	VUS	Likely benign	Benign	Likely benign
PRKN	**NM_004562.3**	**c.136G>A**	**Benign**	**Benign**	**Likely benign**	**VUS**

Note: VUS, variants of unknown significance; ACMG, American College of Medical Genetics and Genomics standards and guidelines. Bold type indicates the only variant showing reverse evolution.

consistent with the literature (Blauwendraat et al., 2020; Gasser, 2009; Kalinderi et al., 2016).

The c.3142A>G (p. Ser1048Gly) variant in *EIF4G1* was found in a patient with clinically diagnosed PD in her 70s. A study from France concluded that *EIF4G1* variants are the etiological cause for late-onset PD (Chartier-Harlin et al., 2011). Another study found that the R1205H variant was detected in familial cases but cited one of the relatives who was also carrying the variant even though he/she had no symptoms, which suggests reduced penetrance (Nuytemans et al., 2013). Last, a wide European cohort of PD patients concluded that *EIF4G1* variants cause late-onset PD with low penetrance (Huttenlocher et al., 2015). This patient shows compatibility with these reports.

A patient carrying the c.4915del (p. Arg1639GlyfsTer15) variant in *LRRK2* was diagnosed with PD in her early fifties. The patient had a history of multiple relatives diagnosed with PD in her family and two relatives with long-term nonintentional tremor without any particular diagnosis. *LRRK2* is one of the most researched genes linked with PD, and it has been related to dominant Mendelian inheritance as well as risk factors for sporadic PD (Dachsel & Farrer, 2010). Several familial PD studies report that different heterozygous *LRRK2* variants show dominant inheritance (Nichols et al., 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). In addition, different groups of studies concluded that single nucleotide changes in *LRRK2* are a risk factor for sporadic PD (Di Fonzo et al., 2006; Tan et al., 2007). This frameshift mutation causes termination of the reading frame. A history of multiple PD cases and other relatives showing cardinal symptoms of the disease without diagnosis in the family might be consistent with the literature. Unfortunately, we did not have the chance to test the other family members. This variant has not previously been mentioned in the literature before according to our knowledge and is therefore considered a novel variant.

The c.2859G>A (p. Thr953=) variant in *ATP13A2* was found in a patient who had a history of typical PD symptoms with advanced age. One study pointed out that two cases of early-onset PD with Italian descent heterozygous mutations in *ATP13A2* might be related to an increased risk of PD (Di Fonzo et al., 2007). Another study in a Chinese Han population stated that the heterozygous A746T variant was seen more frequently in the early-onset PD group than in controls (Lin et al., 2008). In contrast, Podhajska et al. (2012) reported that a cellular study showed heterozygous variants in *ATP13A2* related to PD in previous literature as risk factors do not alter protein stability or subcellular localization but instead impair the ATPase activity of microsomal ATP13A2, which might not be evaluated as sufficient to link with PD. It is fair to say that the accurate correlation of heterozygous *ATP13A2* mutations and PD seems controversial.

Variants of uncertain significance need more complicated and long processes to assess their real contribution to complex diseases such as PD. According to the ACMG, VUS is a class for which we do not have enough data or evidence to make any certain implications about pathogenicity (Richards et al., 2015). Due to the excessive data we gather from gene panels, we are facing a substantial number of variants with uncertain significance, which mostly leads to obscurity. Studies of cancer genetics, which is the most common indication for sequencing, report 34–41% VUS, and this fact might give us a projection (Esterling et al., 2020; Frey et al., 2015). We often benefit from in silico databases to evaluate these variants and perform segregation testing if possible to conclude any verdict. It would be better to consider that every database shapes and changes our understanding of these variants' contribution to any disease, and by nature, these databases are being updated continuously. It is reasonable to deduce that reanalysis and re-evaluation are vital for making clear curations for these variants; likewise, several studies have concluded the same idea

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(Eccles et al., 2015). Variants that have been interpreted as VUS in reanalysis from our clinical experience are shown in Table 3.

The c.1546G>C p. Asp516His variant in *FBXO7* has been reported in the literature before, and two articles about this variant are not consistent with each other; comments on this particular variant are controversial (Ghani et al., 2015; Gorostidi et al., 2016). This variant was an incidental finding, and the proband was asymptomatic.

The c.499G>C (p. Val167Leu) variant in *FGF20* was found in a female patient with early-onset PD diagnosis, and her father also had parkinsonism without a definitive diagnosis and carried the same variant in addition to the c.245C>A (p. Ala82Glu) variant in *PRKN*. Although the c.245C>A (p. Ala82Glu) variant in *PRKN* was not found in the ClinVar and GnomAD databases, it is classified as VUS by ACMG criteria. In addition to these data, previous articles on this specific variant show no agreement on pathogenicity (Erer et al., 2016; Gorostidi et al., 2016).

The c.700G>A (p. Ala234Thr) variant in *SYNJ1* was found in a patient who had been clinically diagnosed with PD. The patient also had multiple family members with dementia. c.3863C>T (p. Pro1288Leu) in *SYNJ1* was found in a patient who had been suffering from atypical symptoms of unilateral bradykinesia, chorea and hemiparesis for more than two years. The pathogenicity of these *SYNJ1* variants has been evaluated according to ACMG criteria mostly linked with in silico databases.

The c.506+6T>C variant in *VPS35* has been classified as a VUS because of the variant's low frequency in public genome databases. This variant shows the possibility of affecting splicing. A patient carrying this variant showed early onset unilateral bradykinesia and tremor.

A patient carrying the c.136G>A (p. Ala46Thr) variant in *PRKN* was diagnosed with PD and had multiple family members with PD and dementia separately. This variant has been mentioned in a study enrolled in a Nigerian population and has been found more frequently compared to the control group. A computer-based protein function study stated that there is a small chance that this variant affects protein function (Okubadejo et al., 2008).

c.3872A>G (p. Glu1291Gly) in *DNAJC13* was found in a proband displaying unilateral bradykinesia and tremor who also showed cogwheel rigidity during examination. The proband also had multiple family members with the same symptoms without any clinical diagnosis. A patient carrying c.2816T>C (p. Leu939Pro) in the *ATP13A2* variant was asymptomatic and had a family history of PD and dementia. This variant has not been mentioned in the literature before according to our knowledge and is therefore considered a novel variant.

The c.2915A>G (p. Asp972Gly) variant in *LRRK2* and the c.1403C>A (p. Ala468Glu) variant in *EIF4G1* was found in a patient whose only symptom was unilateral tremor but who had multiple family members with a PD diagnosis. One study from China stated that although the c.1403C>A (p. Ala468Glu) variant in *EIF4G1* was found at a low frequency in public genome databases, it showed no difference from the control group (Ma et al., 2018).

Reanalysis

The main purpose of this study was to emphasize the importance of reanalysis and re-evaluation of variants, particularly VUS. Since the NGS era began, a tremendous amount of genetic data has been generated, and correlating this data with patients' clinical status can often be challenging. Even experienced clinicians have acknowledged significant problems with this issue (Richards et al., 2015; Vears et al., 2017). Reanalysis of variants and patient follow-up by genetic clinics are especially important in these situations. Although there is no international consensus, some societies have published variable opinions about the issue (Carrieri et al., 2017a, 2017b; Dheensa et al., 2017).

One study indicated that reanalysis of variants within 5–6 years has increased clinical benefit from 26% to 47% (Liu et al., 2019). For diseases like PD, which have several candidate genes in addition to candidate variants, reanalysis and clinical follow-up seem inevitable. It has been noted that keeping in touch with patients, scheduling clinical visits at proper intervals, and providing patients with new information about genetic data is vital when making this plan (Carrieri et al., 2017a, 2017b). Various clinical genetic organizations around the Western world have published different statements, but to generalize, the suggestion of reanalysis of VUS and informing the patients was kept favorable (Boycott et al., 2015; Dheensa et al., 2017; Matthijs et al., 2016; Richards et al., 2015). Our clinical experience is shown in Table 4. Fourteen variants had been classified as VUS in the initial evaluation and curated as benign or likely benign in the reanalysis process. We observed an inverse evolution in only one variant, *PRKN*(NM_004562.3): c.136G>A, which had been classified as benign in the initial evaluation and as a VUS in reanalysis. We also added this variant to the final report of the patient. The minimum and maximum re-evaluation times for these data were 12 and 24 months respectively. Similarly, a few reports in the literature have suggested that reanalysis of variants within even a small time gap, such as a 12-month period, provides a clinically significant alteration of data (Ewans et al., 2018; Wenger et al., 2017; Wright et al., 2018).

In conclusion, to our knowledge, this is the first study to use a targeted gene panel with the widest number of genes with NGS method in the largest PD cohort from our country so far. This study has contributed to the genotype-phenotype correlation of PD, demonstrated the benefits of using a targeted gene panel with NGS method for molecular etiology in PD, and, perhaps most importantly, shown that re-evaluating genetic data, particularly VUS, is critical, especially in multifactorial diseases like PD, to arrive at definitive conclusions about variants.

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Authors' contributions. AK, acquisition of data, analysis of the clinical data and design of the clinical experiments, analysis of the sequencing data and writing of the manuscript; AK and FS performed PCR, software, validation; OO, conceptualization, interpretation of data, supervised the study and reviewed the manuscript. All authors read and approved the final manuscript.

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