PARK8 FORM OF PARKINSON'S DISEASE: INDUCED PLURIPOTENT STEM CELLS-BASED MODEL AND GENOME EDITING OF *LRRK2* MUTATION

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Parkinson's disease (PD) is one of the commonest neurodegenerative disorders worldwide. This age-dependent condition usually occurs in 1–2% of people over 65 years, but sometimes first symptoms can manifest before 50 years of age. PD is a multifactorial disorder for which many causative genes have been identified [9]. At present, the most frequent molecular defect leading to PD are mutations in the *LRRK2* (*leucine-rich repeat kinase 2*) gene on chromosome 12p, and genetic form of the disease was designated as PARK8 [2, 3].

Patients with PARK8 are thought to have abnormal kinase activity of respective mutated protein (called "dardarin"), as it was shown for the most prevalent mutation G2019S located in the protein kinase domain – the finding suggesting "gain-of-function" hypothesis [4, 5, 12]. Mutation G2019S (6055G>A) was found in patients from different ethnic groups having both familial and sporadic PD [7, 13]. However, exact molecular mechanisms underlying this molecular form remain to be elucidated, and no cure for *LRRK2*-G2019S carriers has been proposed so far.

One of the most attractive model for studying pathogenesis of PD on cellular level are induced pluripotent stem cells (iPSC). They represent cells obtained from differentiated somatic cells (for instance, from fibroblasts) and capable of generating all types and populations of human cells [10]. iPCS-based models of neurodegenerative disorders, apart from their use in the search for new neuroprotective compounds, may help to overcome difficulties in studying mechanisms of neurodegenerative process that are connected, in part, with limited access to human neurons. On neurons obtained by iPSC technologies from PD patients carrying *LRRK2*-G2019S mutation Liu et al. (2012) observed defects of neuronal membranes and nuclear architecture, that ultimately leads to neuronal damage and loss of dopaminergic potential [6]. These abnormalities may be reversed either by inhibiting dardarin pathological kinase activity or by targeted correction of the mutant gene. Last years it has been

shown that *LRRK2* may participate in immune response. Moreover, kinase dysfunction resulting from *LRRK2* mutations in PD patients may influence on such cellular processes as apoptosis, autophagia, and cytoskeletal dynamics [9].

A growing role in modern neurobiology is attributed to targeted genome editing with the use of artificial nuclease systems (CRISP/Cas9, etc), a "hot" technology that makes it possible to finely correct genetic defects cell cultures [8]. Taking into account the development of new ways in neurotransplantation, genome editing seems especially promising for the application on specialized neurons and iPCS derived by genetic reprogramming from patients with hereditary forms of neurodegeneration [11].

In this work, we performed correction of mutation G2019S in the *LRRK2* gene on iPSC culture obtained from fibroblasts of a patients with autosomal dominant PD.

Material and methods

Construction of plasmids expressing components of CRISP/Cas9 system. To increase specificity of our CRISP/Cas9 system, we used the following bioinformatic resources and programs: www.gemome-engineering.org, www.dna20.com/ecommerse/cas9/input , and www.e-crisp.org. Selected two-strand DNA spacers were cloned into vector pgRNA-dCas(D10A)-Neo using restriction at the Bbs I site as decribed [1]. *E. coli* cells were then transformed by 10 μl ligase mix with the standard protocol. On further analysis, grown colonies (10 for each two-chain spacers) were checked for the presence and the size of insertions using PCR of bacterial colonies, without extraction of plasmid DNA. The following primers were used:

1) U6 Forw: GAGGGCCTATTTCCCATGATTCC and Scaffold Rev: GCAC-CGACTCGGTGCCACT;

2) U6 Forw: GAGGGCCTATTTCCCATGATTCC and one of the internal primers for each guide, respectively (primers are available on request).

PCR products were analyzed on 1.5% agarose gel. As the result, 34 colonies (from 50 clones analyzed) contained insertion of necessary length.

For the correction of mutation in *LRRK2* we used 120 bp single-strand oligonucleotides (Evrogen, Russia, available on request).

Cell culturing and transfection. iPSCs from a patient with PARK8 form of PD were cultured on a medium for feeder-free culturing of embryonic stem cells mTESRI (Stem Cell Technologies, Canada). Cells were passed every 5–7 days using 1 mg/ml dispase (Invitrogen, USA). Lipophilic transfection with Turbofect (Fermentas) was used for introducing genetic constructs (plasmids and single-strand donor DNA molecules) into iPSC.

PCR of target regions of LRRK2 and Sanger sequencing. Genomic DNA was extracted with Wizard genomic DNA purification kit (Promega) or K-Sorb-100 (Syntol, Russia). PCR and real-time PCR reactions were carried out on genetic amplifier Verity (Applied Biosystems, USA). Analysis of nucleotide sequence in the *LRRK2* target regions and of RNA guide spacers was carried out on automated Genetic Analyzer 3130 (Applied Biosystems, США). Below are provided primers and probes used in this work.

LRRK2 Forw: GATTTCCTGTGCATTTTCTGGCAG ;

LRRK2 Reverse: CTCACATCTGAGGTCAGTGGTTAT C;

wild WT: 5'-(FAM)-TTGCTGACTACGGCATTGCT-(BHQ1)-3';

mutant: 5'-(R6G)-CTGACTACAGCATTGCTCAGTAC-(BHQ2)-3'.

Results and discussion

We obtained culture of iPSC, IPSPDL2.15L, from a patient with PARK8 form of PD carrying mutation *LRRK2*-G2019S. The presence of mutation was confirmed by direct sequencing (fig. 1). For the correction of this mutations, the IPSPDL2.15L cells were transfected with different variants of combinations of plasmid pgRNA-dCas(D10A)-Ne and single-strand probe as a donor (fig. 2).

In 48 hours after transfection, selective antibiotics G-418 was added to the cells in working concentration 75 μg/ml. Selection was carried out during 10 days, and then the survived clones were grown up on Petri plates. Genomic DNA was extracted from a part of the cells, and PCR-amplified targets were directly sequenced. The analysis of a part of *LRRK2* nucleotide sequence showed that A to G change at site 6055 restoring normal genotype was achieved with the use of guide pairs # 18/17 and 18/23, with simultaneous addition of a single-strand probe acting as a wild-type donor fragment for homologous region of exon; however, the populations of cells were heterogeneous (fig. 3). On the other side, correction of mutation with the use of guides # 5/21 turned out to be completely non-effective.

To select clones with corrected mutation, we dispersed the cell edited with guide pairs # 18/17 and 18/23 on a 24-hole plate and let grow during one week. The grown clones were then reseeded on a second (doubling) 24-hole plate. Ranked cells from the first plate were removed with the use of trypsin and washed with PBS, and genomic DNA was extracted on a K-Sorb-100 column. With the use of high-affinity Pfu DNA polymerase (Fermentas) and respective pairs of primers and probes we analyzed target genome-edited regions on real-time PCR regimen. We found one clone (out of 21 clones analyzed) carrying wild

(normal) nucleotide sequence, while other clones were heterogeneous and contained both mutated and normal sequences. Sanger sequencing of PCR fragment of the above-mentioned clone confirmed effective correction of the G2019S mutation (fig. 4).

To conclude, we successfully edited genomic mutation on iPSCs obtained from fibroblasts of a patient with *LRRK2*-associated PD. On the next step of our studies, "normalized" iPSCs will be differentiated into dopaminergic neurons, with detailed comparisons of morpho-functional characteristics of neuronal cultures carrying mutant and wild genotypes. Such comparisons will allow clarifying fine molecular mechanisms of parkinsonism associated with common mutation G2019S. Targeted correction of genotype with getting mutation-free dopaminergic neurons is of great importance for the development of neurotransplantation in PD. The iPSC-based approach expands possibilities of personalized neurology and improves outcomes of treatment in patients with genetic forms of PD.

Acknowledgements

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Figures

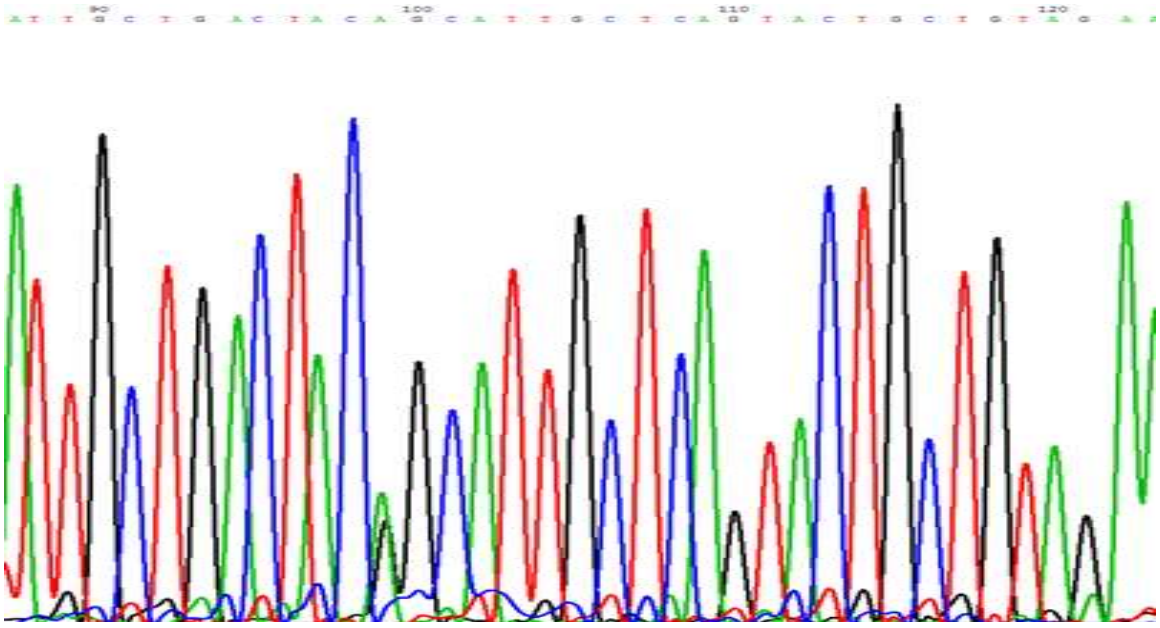


Figure 1. Mutation G2019S in the *LRRK2* gene (arrow) on iPSC line IPSPDL2.15L obtained from fibroblasts of a patient with PARK8.

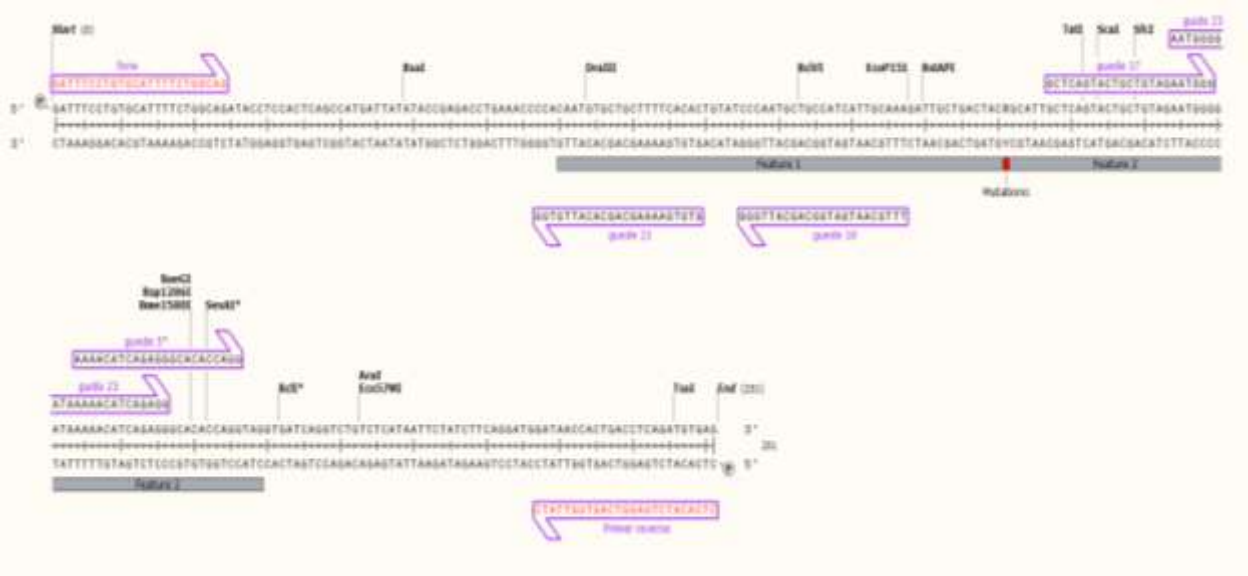
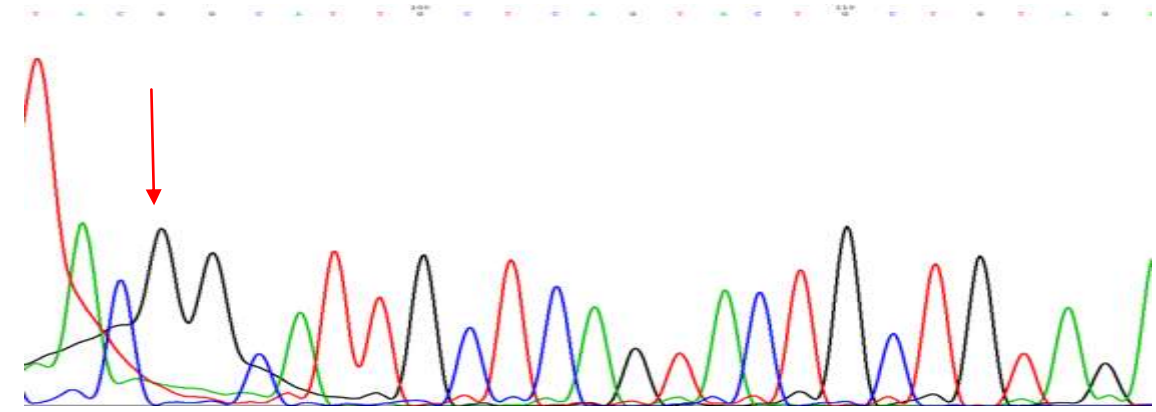
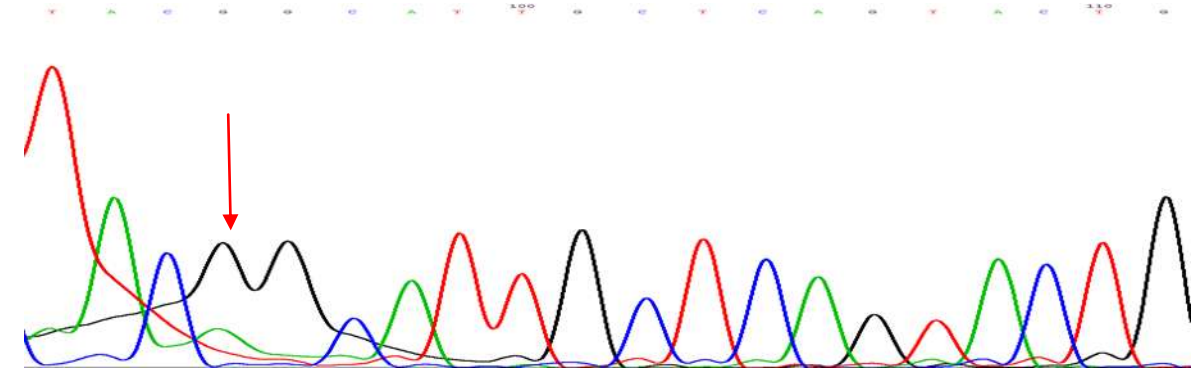


Figure 2. Placing of spacers for RNA guides relative to G2019S mutation on the *LRRK2* sequence.

A



B



C

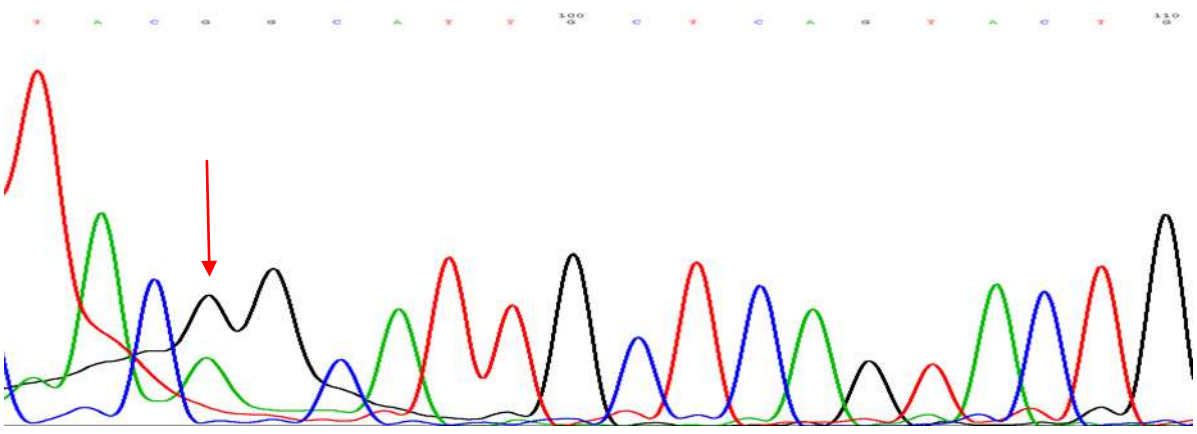


Figure 3. Sequences of different genome-edited clones.
A – Normal donor (fibroblasts). B – iPSC line IPSPDL2.15L after CRISPR/Cas 9 genome editing with the use of RNA guide pair # 18/17. C – iPSC line IPSPDL2.15L after CRISPR/Cas 9 genome editing with the use of RNA guide pair # 18/23. On figures 3B and 3C one can see heterogeneous populations of cells with both mutant and wild-type sequences. The target site is indicated by an arrow.

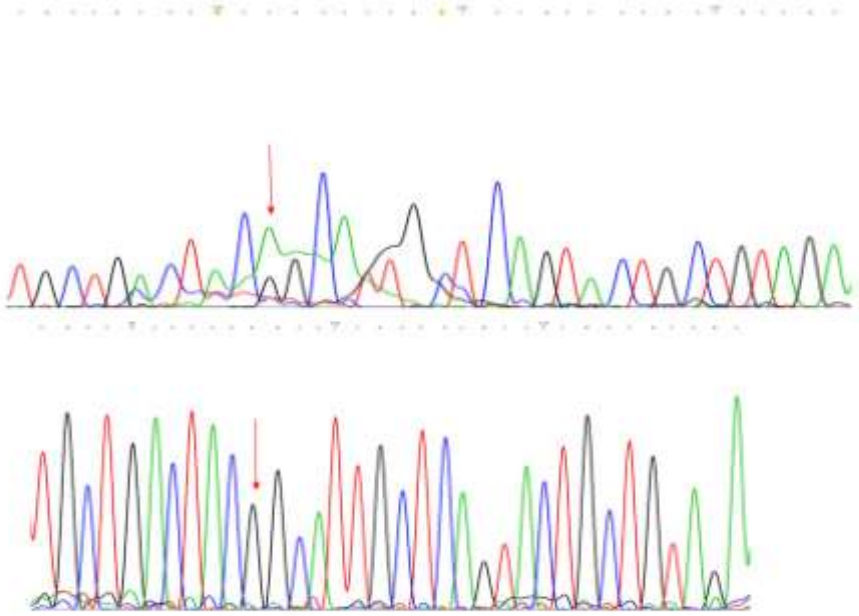


Figure 4. Result of genome editing on a selected clone.
A – Mutant sequence on iPCS line IPSPDL2.15L obtained from a patient with PARK8. B – Normal sequence on the same line after genome editing. The target site is indicated by an arrow.