CRISPR/Cas9 Mediated-Disruption of *ZNF543* Gene: An Approach Towards Discovering Its Relation to *TRIM28* Gene in Parkinson's Disease

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Abstract

Genetic studies of familial forms of Parkinson's disease (PD) have shown that the *ZNF543* gene is a candidate gene that operates relevant to this disease. However, until now there is no evidence for *ZNF543* gene function in PD, and mechanisms resulting from its mutation had not been elucidated. Given the same genetic location of the *ZNF543* gene with *TRIM28* and effects of both of them on PD pathogenesis, we surmised that *ZNF543* may act as a transcription factor for *TRIM28* gene expression. By knocking out the *ZNF543* gene via the CRISPR/Cas9 editing platform, we assessed the functional effect of loss of expression of this gene on *TRIM28* gene expression. Four sgRNAs with different PAM sequences were designed against two parts of the regulatory region of *ZNF543* gene, and highly efficient disruption of *ZNF543* expression in human neuroblastoma cell line was evaluated by polymerase chain reaction and T7 endonuclease assay. Moreover, evaluation of *TRIM28* gene expression in *ZNF543*-knocked-out cells indicated a significant increase of *TRIM28* gene expression, suggesting that *ZNF543* probably regulates the expression of *TRIM28*. This approach offers a window into pinpointing the mechanism by which *ZNF543* gene mutations mediate PD pathogenicity.

1. Introduction

Parkinson's disease (PD) is the second common neurodegenerative disorder, with a prevalence of 0.3%, affecting approximately 1–2% of individuals over the age of 60 years (De Lau and Breteler 2006; Klemann et al. 2017). Recent studies have shown that familial PD genes act as starting points in the pathogenesis of PD. In addition to *SNCA*, which is the first Parkinson's disease-related gene, several risk loci associated with PD, harboring common causative genes such as *DJ-1*, *DNAJC6*, *EIF4G1*, *LRRK2*, *PARK2*, *PINK1*, *PLA2G6*, and *SYNJ1*, have been identified for PD or other parkinsonism disorders (Bonifati 2014; Sasan et al. 2020). In recent years, genome-wide association approaches such as exome and genome sequencing have investigated various PD candidate variants (Nalls et al. 2019; Jansen et al. 2017; Simon-Sanchez et al. 2009; Satake et al. 2009; Saad et al. 2011). Using large-scale whole-exome sequencing, Jansen et al. identified 27 risk-associated genes with loss-of-function variants in PD individuals. Genetic knockdown of *ZNF543* gene as a candidate disease-causing gene (by siRNA) showed decrement of the mitochondria number per cell, indicating the role of this stop gain variant in PD pathology (Jansen et al. 2017). On the other hand, Rousseaux et al. demonstrated that α -synuclein accumulates within the nucleus through tripartite motif-containing 28 (TRIM28) protein (Rousseaux et al. 2018; Rousseaux et al. 2016). Within the nucleus, α -synuclein binds to the mitochondrial transcription activator peroxisome proliferator-activated receptor gamma-coactivator 1 α (*PGC1 α*) promoter, which leads to inhibition of *PGC1 α* promoter and reduction of *PGC1 α* mRNA and protein, resulting in mitochondrial dysfunction and reduced levels of mitochondrial biogenesis (Siddiqui et al. 2012; Eschbach et al. 2015). Inspired by the similar effects of knocking down the *ZNF543* gene and overexpressing TRIM28 on PD and their genomic co-localization (chromosome 19q13.43), we reasoned that they may be related to each other. *ZNF543* is a member of the Kruppel-associated box (KRAB)-containing zinc finger proteins family, which function as transcription factors. Most of these factors are

associated with KRAB domain, which is an almost transcriptional inhibitor domain (Ecco, Imbeault, and Trono 2017). This fact and pathologically overlapping of ZNF543 and TRIM28 led us to ask whether ZNF543 acts as a transcription factor for *TRIM28* gene expression.

Developing technologies for precise and scalable loss of function gene mutation creates powerful tools for investigating the genes' function in proteins signaling networks, disease mechanisms, and the developmental pathway activation (Joung and Sander 2013; Gaj, Gersbach, and Barbas III 2013). In this regard, genetic screens can play an important role in unbiased discovery and phenotype characterization of specific genetic elements. Genome editing technologies based on the clustered, regularly interspaced, palindromic repeats (CRISPR) system as an RNA-guided platform, have generated a tremendous motivation in the manipulation of genomic information contents moving towards disease-gene association and individualized treatment decision making (Mohammadinejad, Biagioni, et al. 2020; Mohammadinejad, Sassan, et al. 2020; Samare Gholami et al. 2019; Riordan et al. 2015). The type II CRISPR system from *Streptococcus pyogenes* consists of a duplex of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), which provides a potential platform for guiding Cas9 (CRISPR-associated endonuclease)-mediated cleavage. This system has been compatible with a variety of eukaryote cell types and whole organisms to induce targeted mutations derived from DNA break repair of non-homologous end joining (NHEJ) or homologous directed repair (HDR). NHEJ can lead to insertions or deletions (indels) at the targeted site, resulting in a frameshift in the target sequence and thereby disruption of the gene (Jiang and Doudna 2017; Ran et al. 2013).

Herein, hypothesizing that ZNF543 may act as a transcription factor for *TRIM28* gene expression, by using the CRISPR/Cas9 editing platform we assessed the functional effect of loss of the expression of ZNF543 on *TRIM28* gene expression. Four sgRNA with different PAM sequences were designed against two parts of the regulatory region (5'-untranslated region (5'-UTR)) and the core promoter of *ZNF543* gene, and their knock-out efficiency for *ZNF543* gene was evaluated. We achieved a highly efficient disruption of *ZNF543* expression in the human neuroblastoma cell line. Moreover, evaluation of *TRIM28* expression in *ZNF543*-knocked-out cells indicated the higher levels of *TRIM28* mRNA.

2. Materials And Methods

2.1. Bioinformatics analysis

In order to design the synthetic sgRNAs, the sequence of *ZNF543* gene regulatory region include a part of promoter and 5'-UTR was obtained from the eukaryotic promoter database (<https://epd.epfl.ch>) and the Ensembl database (<https://www.ensembl.org>). Four CRISPR RNAs (crRNAs) targeting the *ZNF543* regulatory region named g1-g4 were designed according to the results of the online CRISPR design tool (<http://crispr.mit.edu>) and were synthesized by Bioneer Co. (South Korea). The sense and antisense sequences of each crRNA were listed in Table S1.

2.2. Generation of sgRNAs and Cas9 plasmids

The pSpCas9 (BB)-2A-puro (pX459) expression vector was a gift from Feng Zhang (Addgene plasmid # 48139) which has a crRNA:tracrRNA (sgRNA), Cas9 and puromycin resistance genes. Synthetic sense and antisense strands were annealed to form the double strand crRNA. crRNAs targeting the *ZNF543* gene regulatory region was cloned into pX459 using BbsI enzyme (Thermo Fisher Scientific (USA)), according to the previously published protocol (Ran et al. 2013). The sgRNA cassette insertion into the expression vector was verified by colony PCR and Sanger sequencing by primer listed in Table S2 and the schematic map of primers used in colony PCRs was shown in Figure S1.

2.3. Cell preparation and transfection experiments

The human neuroblastoma SH-SY5Y cells were provided by the Pasteur Institute of Iran. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin–streptomycin antibiotics (Gibco, USA) at 37°C in a 5% CO₂-air humidified atmosphere. The cells were separately seeded in six-well plates and were transfected with Px459 vectors containing the desired sgRNA and Cas9 sequences, using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA). For inducing a deletion mutation, all plasmids (containing g1-g4) were co-transfected in another well of cells. After 48 hours, puromycin (1 µg/mL) was added to the wells and cells were selected for the Cas9/gRNA vector expression after another 48 hours.

2.4. T7 endonuclease assay

In order to confirm the efficiency of the designed gRNAs for disruption of the regulatory region upstream of *ZNF543* gene, T7 endonuclease cleavage assay was performed. To evaluate the mutation efficiency, each of the gRNAs (g1-g4) was transfected to the separate well of the plate, and total genomic DNA was isolated from 10⁶ transfected cells using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The regulatory region of *ZNF543* gene were first amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs) with primers amplifying an 828 bp fragment of *ZNF543* regulatory region containing the target sites of gRNAs (Table S2). PCR amplicons were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific). For T7 endonuclease assays, 400 ng of the purified PCR product was denatured and reannealed in 1X NEB buffer (New England Biolabs) in a total volume of 20 µL, after which 10 U of T7 endonuclease (New England Biolabs) was added to the solution and incubated for 45 min at 37°C and then the reaction stopped with 1 µL of 0.5 M EDTA. The products were then run on a 2.5 % agarose gel. The mutation efficiency of gRNAs with different protospacer adjacent motifs (PAMs) was evaluated by quantifying DNA signals using ImageJ software (<http://rsb.info.nih.gov/ij/>).

2.5. Detection of deletion mutation in *ZNF543* and DNA sequencing

To generate deletion mutations in the regulatory region of *ZNF543* gene, all plasmids (pX459- g1-g4) were co-transfected into the SH-SY5Y cells. As aforementioned, total DNA was extracted and target regions were amplified using Phusion High-Fidelity DNA Polymerase and primers listed in Table S2. The amplification products were run on a 1.5 % agarose gel and then purified using a column-based

precipitation kit (Bioneer). The purified PCR fragments related to control and transfected cells were submitted for Sanger sequencing in order to investigating the deletion mutation (Bioneer, South Korea).

2.6. Relative quantification of *ZNF543* and *TRIM28* gene expression

To evaluate the efficiency of designed gRNAs (g1-g4) for creating the deletion mutation in *ZNF543*, reverse transcriptase (RT)-PCR was performed. Total RNA was extracted from control and transfected cells using Trizol reagent (Zaver Zist Azma, Iran). cDNA was synthesized from 2 µg of the total RNA by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The cDNA products equivalent to 200 ng of total RNA were used as the template in a 25 µL PCR system. Moreover, the relative mRNA expression of *ZNF543* and *TRIM28* genes was evaluated by quantitative real-time PCR (qRT-PCR) using SYBR Green PCR Master Mix (SMObio, Taiwan). Expression levels of genes of interest in cells transfected by different gRNAs were normalized against *GAPDH* mRNA as an endogenous control. Primer sequences used in these assays were listed in Table S2.

2.7. Statistical analysis

The real-time PCR results were shown as mean $\pm$ SD and P values of < 0.05 were considered as the criterion for the statistical significance. Gene expression data were conducted by one-way ANOVA followed by Tukey's post hoc test using the SPSS software (version 24).

3. Results

3.1. Generation of gRNAs expressing plasmids

To selectively disrupt the *ZNF543* gene expression in human neuroblastoma SH-SY5Y cells, four sgRNAs (g1-g4) targeting a part of the promoter and the 5'-UTR of *ZNF543* gene (Position: chr19:57,831,623 – 57,832,069 = $\Delta 447$ bp), were designed and selected based on the prediction of high on-target and low off-target effects (Fig. 1a). Synthetic sense and antisense strands were annealed to form a double stranded DNA with sticky-ends (Fig. 1b), and ligated into pSpCas9 (BB)-2A-puro (pX459) expression vector linearized with BbsI restriction enzyme. Colony PCR and Sanger sequencing results using CRISPR-Cas9 specific primers confirmed the correct insertion of g1-g4 into the pX459 expression vectors (Fig. 1c and 1d).

3.2. Detection of the deletion mutation in the regulatory region of *ZNF543* gene

Gene editing efficiency of the pSpCas9 (BB)-2A-puro-g1-g4 (pX459-g1-g4) system was evaluated in the SH-SY5Y cells. To generate deletion mutations in the regulatory region of *ZNF543* gene, all plasmids (pX459- g1-g4) were co-transfected into SH-SY5Y cells. Un-transfected cells were used as the control. After 48 hours, puromycin (1 µg/mL) was added to the cells, and cells were assayed for the Cas9/gRNA

vector expression after another 48 hours. In order to detection of the deletion mutations, the genomic DNA of un-transfected cells, puromycin-untreated transfected cells and puromycin-treated transfected cells were used as the template for PCR reactions by primers listed in Table S2. As displayed in Fig. 2a, Lane 1, un-transfected cells showed an 828 bp band on the agarose gel corresponding to the wild-type *ZNF543* gene target site. Puromycin-untreated transfected cells represented both bands of 828 bp and 381 bp corresponding to the wild-type and mutated *ZNF543* gene target site, respectively (Fig. 2a, lane 2). Transfected cells treated with puromycin exhibited a characteristic 381 bp band on the agarose gel which is representative of the deletion mutation of the regulatory region of *ZNF543* gene in these cells (Fig. 2a, lane 3). These results imply that the pX459-g1-g4 system has high efficiency for generating deletion mutations in a part of the regulatory region of *ZNF543* gene in SH-SY5Y cells. The sequencing results of PCR products were also completely consistent with our prediction for occurring the deletion mutations in *ZNF543* gene target site. As shown in Fig. 2b, the deletion mutation extends from – 235 to + 212 of the *ZNF543* gene, including a GC-box and a CCAAT-box positioned in the – 30 and – 79 regions of the target site, respectively

3.3. T7 endonuclease assay

Using the T7 endonuclease cleavage assay, we analyzed targeted mutagenesis in transfected cells. DNA extracted from un-transfected and transfected cells with pX459-g1-g4 plasmids were subjected to PCR and T7 endonuclease cleavage. T7 endonuclease assay using the PCR products was performed. The assay showed cleavage bands of the *ZNF543* regulatory region in transfected cells with pX459-g3 and pX459-g4 plasmids (Fig. 3a). The assay in transfected cells with pX459-g1 and pX459-g2 plasmids showed that cleavage bands are very close to bands of the control (data not shown). This can be due to the position of the g1 and g2 target sites which is in the beginning of the PCR products. The mutation percentages of each gRNA construct with the different PAM sequences were estimated from band intensities using Image J (Fig. 3b). Investigating signals of DNAs by ImageJ showed GGG and CGG PAM sequences next to the targeted sites by g1 and g3, respectively, are more efficient in the mutation generation compare to TGG and GGG PAM sequences next to the targeted sites by g2 and g4, respectively. These results highlight that the pX459-g1-g4 system can generate the indel mutation in an efficient manner in SH-SY5Y cells.

3.4. Relative quantification of *ZNF543* and *TRIM28* gene expression

To evaluate the efficiency of designed gRNAs (g1-g4) for *ZNF543* gene disruption in SH-SY5Y cells, reverse transcriptase-PCR (RT-PCR) was performed. As displayed in Fig. 4a, RT-PCR products of total RNA extracted from co-transfected cells with pX459-g1-g4 plasmids didn't show any band on the agarose gel compared to untransfected cells. Moreover, the relative mRNA expression of *ZNF543* gene was evaluated by quantitative real-time PCR (qRT-PCR). As shown in Fig. 4b, the expression of gRNAs targeting the regulatory region of *ZNF543* gene in SH-SY5Y cells significantly decreased the *ZNF543* gene expression.

g1 and g2 had a slight effect on decreasing the *ZNF543* expression, while g3 and g4 decreased it by more than 75%. The mRNA levels of *ZNF543* decreased by more than 95% when the regulatory region was targeted with a mix of four gRNAs, indicating a mix of all pX459-g1-g4 plasmids has higher efficiency for *ZNF543* gene disruption. Hence, for evaluating the effect of *ZNF543* gene disruption on *TRIM28* gene expression, g3 and a mix of all four gRNAs were used for the next experiments. Co-transfected cells with pX459-g3 and the mix of all pX459-g1-g4 plasmids represented a significant expression change for *TRIM28* expression compared to untransfected cells, which indicates a probable role of *ZNF543* for *TRIM28* gene expression (Fig. 4c).

4. Discussion

Recently, genome-wide association studies have an emphasis on the association of loss of function (LoF) variants with Parkinson's disease (PD). By integrating whole-exome sequencing data and functional evidences, Jansen et al. have suggested that a LoF variant of *ZNF543* gene is a candidate for PD. They showed that knocking down of *ZNF543* gene can decrease the mitochondria number per cell, indicating the role of this variant in PD pathology (Nalls et al. 2019; Jansen et al. 2017). *ZNF543* is a zinc finger protein containing the KRAB domain, which is a transcriptional inhibitory domain (Ecco, Imbeault, and Trono 2017). Up to now, there is no evidence for the *ZNF543* function in PD, and mechanisms resulting from its mutation were not elucidated. Given the role of *TRIM28* in mitochondrial dysfunction and reduced levels of mitochondrial biogenesis in PD, as well as its same location with *ZNF543* gene on chromosome 19 (19q13.43), we surmised that *ZNF543* may act as a transcription factor for *TRIM28* gene expression (Rousseaux et al. 2015) also previous studies have shown that this chromosomal location (19q13) is associated with disorders of the nervous system (Abak et al. 2021). To test whether *ZNF543* itself regulates the levels of *TRIM28* gene expression, we knocked out the *ZNF543* in the human neuroblastoma SH-SY5Y cells via CRISPR/Cas9 technology. For disrupting *ZNF543* gene expression in SH-SY5Y cells, we designed four gRNAs that target a part of the promoter and the 5'-UTR of *ZNF543* gene. The deletion mutations in the regulatory region of *ZNF543* gene were verified by Sanger sequencing, PCR, and T7 endonuclease assay. To evaluate the efficiency of each designed gRNA (g1-g4) for *ZNF543* gene disruption in SH-SY5Y cells, RT-PCR and qRT-PCR were performed. The results showed that the best efficiency for *ZNF543* gene disruption was obtained by g3 and g4, which indicates that the 5'-UTR of the *ZNF543* gene is a critical regulation point for its expression. We proved that a mix of all g1-g4 can create sufficiently high levels of *ZNF543* gene disruption, yielding the output of NHEJ. To address the impact of *ZNF543* disruption on *TRIM28* gene expression, we performed qRT-PCR for *TRIM28* mRNA in the *ZNF543*-knocked out SH-SY5Y cells. We found that the disruption of *ZNF543* gene can significantly increase *TRIM28* gene expression, suggesting that *ZNF543* probably regulates the expression of *TRIM28* gene. The rescue experiment reinforced this hypothesis, and the expression of *ZNF543* cDNA using pcDNA3 plasmid in the cell line model showed a significant decrease in *TRIM28* mRNA expression (data not shown). These results offer a window into understanding the pathogenicity of the stop gain variant of *ZNF543* gene in PD, via the regulation of *TRIM28* gene expression, which is in keeping with previous studies (Rousseaux et al. 2015; Rousseaux et al. 2016). Moreover, this is consistent with other studies

that have shown that reducing *TRIM28* expression can impede a-Syn stabilization in PD (Rousseaux et al. 2018). However, elucidating specific mechanisms resulting from *ZNF543* mutations and its relation with *TRIM28* in PD require to be clarified in future studies.

Declarations

Availability of Data and Material

The data sets generated and analyzed in the current study are available from the corresponding author on reasonable request.

Authors' Contributions

All authors contributed to the study's conception and design. The design and writing of the manuscript were performed by MH and MA. Data collection was performed by MH, HS, and KE, and data analysis was performed by MH, SE, and HR.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Approval

All experiments and procedures were approved by the Animal Research Ethics Committee of the Kerman Neuroscience Research Center, Kerman, Iran (EC/KNRC/86-31).

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Figures

Figure 1

A schematic diagram of the designed sgRNAs (gRNA1-gRNA4) targeting a part of the promoter and the 5'-UTR of ZNF543 gene. b) 2% agarose gel for 1) A sense strand of a designed crRNA 2) An antisense strand of a designed crRNA 3) An annealed double stranded DNA with sticky-ends, M: DNA marker c) Colony PCR products for colonies containing pX459-g1-g4 expression vectors with primers listed in Table S2, 1-3) pX459-g1, 4-6) pX459-g2, 7-9) pX459-g3, 10-12) pX459-g4, M: DNA marker. d) Sequencing results of pX459-g1-g4 expression vectors with specific vector primers listed in Table S2.

a)

b)

TCGACCCTCCGACTTGTGCCCATAGCTGCGCCCGCTTTGTGCGCATTTTT

ZNF543-Deletion mutation

TCGACCCTCCGACTTGTGCCCATAGTAAAGCGGCGGCC

ZNF543-Control

AGCCCCGACGCTGCGCCCGCTTTGTGCGCATTTTT

Figure 2

PCR products of amplifying the regulatory region of ZNF543 gene in un-transfected cells (1), puromycin-untreated transfected cells (2), and puromycin-treated transfected cells (3), M: DNA marker. b) Sequencing results of the regulatory region of ZNF543 gene in un-transfected (control) and transfected cells.

Figure 3

a) T7 endonuclease cleavage products of the regulatory region of ZNF543 gene in un-transfected cells (1), transfected cells with pX459-g3 plasmid (2) and transfected cells with pX459-g4 plasmid (3), M: DNA marker. b) The mutation efficiency of each gRNA construct with the different PAM sequences.

Figure 4

a) 1% agarose gel electrophoresis of RT-PCR product of ZNF543 in control (1) and transfected cells with pX459-g1-g4 plasmids (2), M: DNA marker. b) ZNF543 gene expression in transfected cells (SH-SY5Y) with pX459-g1-g4 plasmids and un-transfected cells (control). c) TRIM28 gene expression in transfected cells with pX459-g3 and a mix of all pX459-g1-g4 plasmids and un-transfected cells (control).

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