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Identification & characterization of leucine-rich repeat kinase 2 & parkin RBR E3 ubiquitin protein ligase variants in patients with Parkinson's disease

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Background & objectives*: Parkinson's disease (PD) is a motor disorder that affects movement. More than 24 loci and 28 associated genes have been identified to be associated with this disease. The present study accounts for the contribution of two candidates, leucine-rich repeat kinase 2 (*LRRK2*) and parkin RBR E3 ubiquitin protein ligase (*PRKN*) in the PD patients, and their characterization *in silico* and *in vitro.

***Methods*: A total of 145 sporadic PD cases and 120 ethnically matched healthy controls were enrolled with their informed consent. Mutation screening was performed by direct DNA sequencing of the targeted exons of *LRRK2* and all exons flanking introns of *PRKN.* The effect of the pathogenic *PRKN* variants on a drug (MG-132) induced loss of mitochondrial membrane potential (ΔΨM) was measured by a fluorescent dye tetramethylrhodamine methyl ester (TMRM).**

***Results*: Twelve and 20 genetic variants were identified in *LRRK2* and *PRKN*, respectively. Interestingly, five out of seven exonic *LRRK2* variants were synonymous. Further assessment in controls confirmed the rarity of two such p.Y1527 and p.V1615. Among the pathogenic missense variations (as predicted *in silico*) in *PRKN,* two were selected (p.R42H and p.A82E) for their functional study *in vitro,* which revealed the reduced fluorescence intensity of TMRM as compared to wild type, in case of p.R42H but not the other.**

***Interpretation & conclusions*: About 6.2 per cent of the cases (9/145) in the studied patient cohort were found to carry pathogenic (as predicted *in silico*) missense variations in *PRKN* in heterozygous condition but not in case of *LRRK2* which was rare. The presence of two rare synonymous variants of *LRRK2* (p.Y1527 and p.V1615) may support the phenomenon of codon bias. Functional characterization of selected *PRKN* variations revealed p.R42H to cause disruption of mitochondrial membrane potential (ΔΨM) rendering cells more susceptible to cellular stress.**

Key words: Codon bias - *LRRK2* - mitochondrial membrane potential - Parkinson's disease - *PRKN* - TMRM

Parkinson's disease (PD) is a progressive neurodegenerative motor disorder caused due to loss of dopaminergic neurons in the midbrain. It is a multifactorial complex disorder resulting from interaction between multiple genetic and environmental factors and imposes social and economic burden as the populations undergo ageing. Clinically, PD is characterized by four cardinal motor symptoms: resting tremor, bradykinesia, rigidity and postural instability; associated with some non-motor symptoms such as loss of smell, trouble in sleeping, fainting and constipation. The secondary disease manifestation includes mask-like face, speech changes, sialorrhoea, *etc*. Sometimes, the secondary features appear first, leading to Parkinsonism by years or even decades[1]. A recent genome-wide association study (GWAS) and meta-analysis revealed the identification of more than 24 loci and 28 associated genes causing the disease either in monogenic (by single candidate gene mutation) or compound heterozygous (more than one heterozygous point mutations in a number of candidate genes giving additive effect) form[2,3]. A number of studies have shown the candidate gene mutations in *PRKN* (parkin RBR E3 ubiquitin protein ligase)[4-6], PTEN-induced kinase 1 (*PINK1*)[7,8] and *DJ-1*[9] in different patient cohorts from India but unable to find any pathogenic variant or risk factor in leucine-rich repeat kinase 2 (*LRRK2*)[10,11] except in one report where a single EOPD (early-onset PD) female patient of 748 PD patients was observed to possess G2019S mutation in *LRRK2* in heterozygous condition[12]. The present study was designed for the identification and characterization of DNA variants in *PRKN* and an assessment of DNA variants identified in *LRRK2* in PD patients.

The genetic locus of *PARK8* (cytogenetic location 12q12) was first identified in a series of families from Japan[13]. Later on, the same chromosomal locus was shown to be linked with several families with PD worldwide[14] and a number of mutations were also identified in the gene situated in this locus, *i.e*., *LRRK2*[15]. The mutation frequency in it is high for PD and all the mutations reported so far are inherited dominantly. *LRRK2* encodes a protein with five putative functional domains: an N-terminal LRR domain, a Roc (Ras of complex protein) domain, a COR (C-terminal of Roc) domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) domain and a C-terminal WD40 repeat domain[16]. It encodes a 2527-amino acid protein with a molecular mass of approximately 250 kDa[16]. The protein is primarily cytoplasmic and associated with particulate membrane structures, such as mitochondria, microsomal membranes, endoplasmic reticulum and the Golgi apparatus but not integrated into membranes[16]. Rather it dimerizes on kinase activity and also able to phosphorylate itself[17].

Another gene assessed in the present study is *PRKN*, is an important component of the mitochondrial quality control pathway[16]. It is an E3 ubiquitin ligase situated on *PARK2* locus (cytogenetic location 6q25.2-q27)[16]. It was first identified to be deleted in one Japanese patient with juvenile PD[18]. Since then, a number of studies reported homozygous or compound heterozygous mutation in *PRKN* responsible for causing autosomal recessive juvenile PD. LRRK2 protein, by its COR domain, interacts with the C-terminal R2 RING finger domain of PARKIN[19].

Material & Methods

A total of 145 patients from Uttar Pradesh, Bihar and part of Madhya Pradesh participated in the present study after their clinical diagnosis from departments of Neurosurgery and Neurology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, and their written informed consent. The study protocol was approved by the Institutional Human Ethics Committee (Ref. no. F. Sc. /Ethics Committee/2015-16/1). Sampling was started in the year 2013; enrolment of studied number of cases was completed in 2016. The experiments were conducted over the years 2015-2017.

All the patients were apparently sporadic in nature as reported by the proband and one or two close relatives, including 37 early-onset (≤50 yr) PD (EOPD) cases. All the registered cases had at least two of the four classical symptoms of PD. The cases with Parkinsonism and traumatic brain injury carrying the symptoms of PD were excluded from this study. The control group comprised 120 unrelated ethnically matched healthy volunteers with no positive family history for PD or any other neurological disorders. Approximately 6 ml of peripheral blood sample was collected each in heparinized syringes from patients and controls.

Mutation screening: Genomic DNA was extracted from whole blood as per standard protocol[20] and purified using phenol-chloroform-isoamyl alcohol method[21]. For *LRRK2*, exons 30-41 covering ROC, COR and kinase domain and flanking introns were selected for mutational screening. For *PRKN*, all the exons and exon-intron boundaries were sequenced in

patients[16]. Primers were designed using Primer3web v4.0.0 software. Polymerase chain reaction (PCR) was carried out using 25-50 ng of genomic DNA in ABI verity 96-well thermal cycler (Applied Biosystem, USA). Leftover primers and dNTPs were removed by exonuclease I and recombinant shrimp alkaline phosphatase (rSAP) (USB Affymetrix, USA). Purified PCR products were labelled with ABI Big Dye Terminator V3.1 cycle sequencing reagent (Applied Biosystem, USA) and sequenced in ABI 3130 Genetic Analyzer. Resulting sequences were compared with the available National Center for Biotechnology Information (NCBI) GenBank database using NCBI-Basic Local Alignment Search Tool (BLAST). The status of the variants, if reported anywhere, was obtained using MutationTaster[22], dbSNP and PDmutDB (*http://www.molgen.ua.ac.be/PDmutDB*). Some of the *LRRK2* variants were assessed among controls using either restriction fragment length polymorphism (RFLP) or allele-specific PCR (Table I). For PCR-RFLP 2U of *HpyCH4IV* (New England Biolabs Inc, USA) was used per reaction.

In silico analysis of the variants: After determining the status of the variants, the pathogenic variants were sorted. Such variations with no amino acid change were studied at the messenger RNA (mRNA) level using the Mfold server (*http://unafold.rna.albany.edu/?q=mfold/RNAFolding-Form*)[23]. On the other hand, alteration in protein secondary structure for the missense variations was observed using *PSI-PRED*. For analyzing protein tertiary structure, first, protein models were generated based on the templates searched by the software SWISS-MODELLER (*https://swissmodel.expasy.org/)* on homology basis; second, the three-dimensional (3D) protein structures (.pdb files) were viewed and compared by the superimposition of wild and mutant proteins using CHIMERA (*https://www.rbvi.ucsf.edu/chimera/*).

Site-directed mutagenesis: Two changes in *PRKN*, predicted to be pathogenic, were incorporated in the mammalian expression vector of PARKIN (pRK5-Myc-Parkin, Addgene, USA)[24] using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, USA). Mutagenic primers were designed as instructed in the guideline.

Cell culture: COS7 fibroblast cell line and HeLa cervical cancer cells were maintained in DMEM (Sigma, USA) supplemented with 10 per cent foetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) (HiMedia, Mumbai) and grown in five per cent (v/v) CO_2 in a humidified incubator for studying *PRKN* mutants *in vitro*.

Transfection and stress induction: 1×10^6 cells/well were seeded for transfection in a 6 well plate format. The wild-type and mutant constructs of *PRKN* were prepared using Plasmid Midi Kit (Qiagen, Germany). Transfection was performed next day (after 24-30 h) at 85-90 per cent cell confluences using lipofectamine 2000 (Invitrogen, USA). Cells were kept at 37°C CO_2 incubator for two days. For stress study, cells were divided into two groups: one group was treated with 15 µM of a proteasomal inhibitor MG-132

Table I. Leucine-rich repeat kinase 2 (*LRRK2*) variants assessed in controls

Variant (technique)	Primer sequence (5'-3')	Annealing (°C)	RE	Wild (bp)	Mutant (bp)
p.Tyr1527Tyr (RFLP)	F: GTTAGCACTGAATTTGCCAACC	52	*HpyCH4IV*	394	270,124
	R: GAACCGTATGGATATTCTCTCAAC				
p.Val1615Val (ARMS)	F: GAGAAATTAGGTACTGTGTTGCAC	58	NA	468,179	468,179
	R: GTCAGTAGGAGGTTTACACTAGAAGC				
	W: GGTGTTTTGGACAACCTTCC				
	M: GGTGTTTTGGACAACCTTCT				
p.Ser1647Thr (ARMS)	F: GAGAAATTAGGTACTGTGTTGCAC	58	NA	468,277	468,277
	R: GTCAGTAGGAGGTTTACACTAGAAGC				
	W: TTCTAGGAGCTTAAAATACTGTGA				
	M: TTCTAGGAGCTTAAAATACTGTGT				

RE, restriction endonuclease; F, forward; R, reverse; W, wild type allele; M, mutant allele; NA, not available

(Sigma, USA) and the rest were treated with vehicle control, *i.e*., 15 µM of dimethyl sulfoxide. Cells were incubated for 24 h.

Protein isolation and Western blot analysis: Following transfection, one more group of cells were taken for protein isolation from the same set. For this, cells were trypsinized and collected by centrifugation followed by cell lysis using radioimmunoprecipitation assay (RIPA) buffer. Bradford assay was performed for quantification of isolated proteins. Qualitative Western blot analysis was performed to check the normal protein formation in all PARKIN mutants as compared to wild type. For this, mouse-anti-c-Myc monoclonal antibody (Sigma, USA) was used as primary antibody since the construct had an upstream tag of c-Myc. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken for positive control, which was detected using monoclonal mouse-anti-GAPDH antibody (Sigma, USA). The secondary antibody used was the goat-anti-mouse antibody (B-Genei, Bengaluru) tagged with HRP. Proteins were detected following application of 3,3'-Diaminobenzidine.

Mitochondrial membrane potential (ΔΨM) assay using fluorescence-activated cell shorting (FACS): After 24 h of drug (MG-132) treatment cells were harvested with trypsin, pelleted and resuspended in phosphate-buffered saline containing 200 nM of mitochondrial membrane potential (ΔΨM) sensitive fluorescence dye tetramethylrhodamine methyl ester (TMRM) (Thermo Fisher Scientific, USA) on ice. Cells were incubated for 15-20 min and analyzed immediately in the fluorescence-activated cell shorting (FACS) caliber BD flow cytometer (Biosciences, USA) with 488 nm argon laser using FL-2 channel. For each sample, 10,000 cells (events) were analyzed in triplicate. Data were acquired on a logarithmic scale using cell quest pro software (Biosciences, USA). Fluorescence shift of cell population was obtained by overlying the histograms of wild type and each of the *PRKN* mutants. The experiment was repeated twice.

Results

Identification of genetic variants in (LRRK2): Twelve DNA variants were identified (Table II), including seven exonic and five intronic. Interestingly, more than 70 per cent of the exonic variants were synonymous and more interestingly, two of them (p.Tyr1527Tyr and p.Val1615Val), situated on the COR domain of LRRK2 protein were predicted to be pathogenic by MutationTaster (*http://www.mutationtaster.org/*). Neither of these two variants was found in dbSNP or ClinVar. The allele frequency of both the above-mentioned variants in our studied patient cohort was only 0.34 per cent. Neither of these two variants was found in 240 ethnically matched control chromosomes.

Application of Mfold for prediction of alteration in mRNA folding caused by these two synonymous changes at minimum free energy (δG) revealed no significant change in case of c.4581 T>C (p.Tyr1527Tyr) (Fig. 1A and B), but several points of structural alterations (Fig. 2B) were observed in case of c. 4845 G>A (p.Val1615Val) (Fig. 2A and B). The calculated δδG (δGMutant- δGWild-type) was +2.3 kcal/mol. It appeared that more the positive value of δδG, lesser the stability of mutant RNA as compared to wild type. The nucleotide length for this *in silico* analysis was restricted to 150 bp with the variant of interest positioned in the middle (shown by asterisks in Figs 1 and 2). Structural alteration was observed in the case of c.4581 T>C (p.Tyr1527Tyr) too when the nucleotide length was increased to 300 bp. However, those changes were not considered because the number of possible structures grows exponentially with the increasing length of the sequence[22].

The allele frequency of one missense variant (c.4939 T>A, leading to p.Ser1647Thr) was also determined in the control group. The minor allele frequency of this variant was 40.5 per cent in controls, as compared to 29.54 per cent in patient group (odds ratio: 0.585, % confidence interval: 0.2883-1.1872, P=0.1376). Statistical significance was set at P<0.05. Hence, this variant was not associated with PD in the studied cohort. Two out of five intronic changes, IVS36+30 del A and IVS39+4A>T, those identified in this study are reported for the first time.

Identification of DNA variants in PRKN: A total of twenty DNA variants (Table II) were identified in *PRKN* among 145 PD patients. Six variants were predicted to be pathogenic, including four missense (p.Q34R, p.R42H, p.A82E and p.G359D), one synonymous (p.L272L) and the rest intronic (IVS2+35G>A). A total of nine cases (6.2%) were found to carry pathogenic missense variations in *PRKN* in heterozygous condition. The clinical features of these cases are enlisted in Table III. *In silico* study using Mfold revealed the identification of several points of structural alterations in case of the synonymous variant (δδG=−0.5 kCal/Mol) (Fig. 3A and B). The missense variants were studied *in silico*

Table II. Variants identified in leucine-rich repeat kinase 2 (*LRRK2*) and parkin RBR E3 ubiquitin protein ligase (*PRKN*) in Parkinson's disease (PD) patients

Nucleotide change	Location	Amino acid change	Type of change	Allele frequency (%)	Status
Variants identified in *LRRK2*					
c. 4581 T>C	Exon-32	p.Y1527Y	Synonymous	0.34	Reported in ExAC
c. 4624 C>T	Exon-32	p.P1542S	Non-synonymous	1.66	rs33958906
IVS33-28 T>C	Intron-33	NA	NA	27.69	rs1896252
c. 4845 G>A	Exon-34	p.V1615V	Synonymous	0.34	Reported in ExAC
c. 4872 C>A	Exon-34	p.G1624G	Synonymous	34.61	Polymorphism
c. 4911 G>A	Exon-34	p.K1637K	Synonymous	30.67	Polymorphism
c. 4939 T>A	Exon-34	p.S1647T	Non-synonymous	29.54	rs11564148
IVS36+20 C>T	Intron-36	NA	NA	37.5	rs7137665
IVS36+30 del A	Intron-36	NA	NA	37.5	NOVEL
c. 5457 T>C	Exon-37	p.G1819G	Synonymous	67.37	Polymorphism
IVS39+32G>A	Intron-38	NA	NA	33.34	rs17484342
IVS39+4A>T	Intron-39	NA	NA	54.78	NOVEL
Variants identified in *PRKN*					
c. 101A>G	Exon-2	p.Q34R	Non-synonymous	1.72	rs148851677
c. 125G>A	Exon-2	p.R42H	Non-synonymous	0.34	rs368134308
IVS2+25T>C	Intron-2	NA	NA	17.93	rs2075923
IVS2+35G>A	Intron-2	NA	NA	0.68	rs79752410
c. 245C>A	Exon-3	p.A82E	Non-synonymous	0.34	rs55774500
IVS3-9G>C	Intron-3	NA	NA	4.13	Polymorphism
IVS3-15T>A	Intron-3	NA	NA	5.17	Polymorphism
IVS3-35_delA	Intron-3	NA	NA	25.51	Polymorphism
c. 500G>C	Exon-4	p.S167T	Non-synonymous	4.13	Polymorphism
c. 500G>A	Exon-4	p.S167N	Non-synonymous	11.03	rs1801474
c. 707T>A	Exon-6	p.I236N	Non-synonymous	2.75	Polymorphism
c. 816C>T	Exon-7	p.L272L	Synonymous	1.72	rs143902760
c. 822T>G	Exon-7	p.D274E	Non-synonymous	10.68	Polymorphism
c. 838G>A	Exon-7	p.D280N	Non-synonymous	15.51	Polymorphism
IVS7-35G>A	Intron-7	NA	NA	7.58	rs3765474
IVS8-16_20	delCTGCINTRON-8	NA	NA	1.03	Polymorphism
c. 1000C>T	Exon-9	p.R334C	Non-synonymous	1.37	rs199657839
c. 1033C>G	Exon-9	p.P345A	Non-synonymous	15.51	Polymorphism
c. 1076G>A	Exon-9	p.G359D	Non-synonymous	0.68	rs201300874
c. 1138G>C	Exon-10	p.V380L	Non-synonymous	22.06	rs1801582

for the prediction of alterations in protein secondary and tertiary structures. *PSIPRED* analysis (*http://bioinf.cs.ucl.ac.uk/psipred/*) resulted in alteration in protein secondary structure not at the point of variation but other regions of the proteins in cases of p.A82E and p.G359D. No significant alterations were found in the protein secondary structure for the other two missense variations. Tertiary protein conformational changes were observed by superimposition of the wild-type and mutant proteins using SWISS-MODELLER and Chimera for all the four missense variants (Fig. 4A-D). Among these, two pathogenic missense

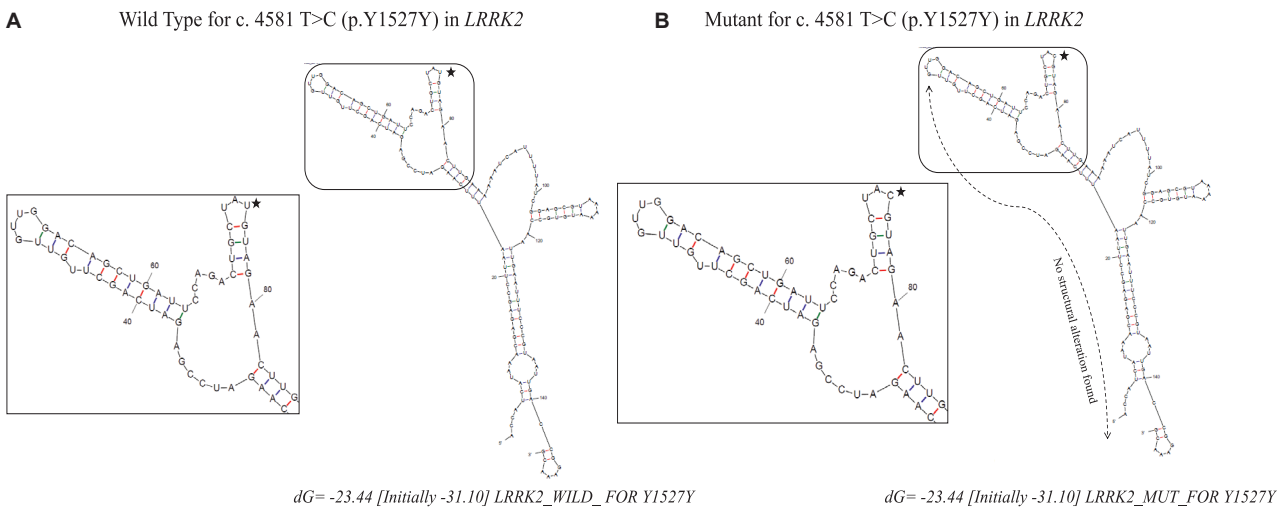

Fig. 1. *In silico* analysis of mRNA structure and stability of c. 4581 T>C (p.Y1527Y) in leucine-rich repeat kinase 2 (*LRRK2*) using Mfold. The nucleotide position has been indicated by a small asterisk . The mRNA secondary structure is stabilized by hydrogen bonds, represented by three different colours; AU blue, CG red and GU green. Inset showing part of image in enlarged form. (**A**) Wild type and (**B**) Mutant (no changes were found in this mutant).

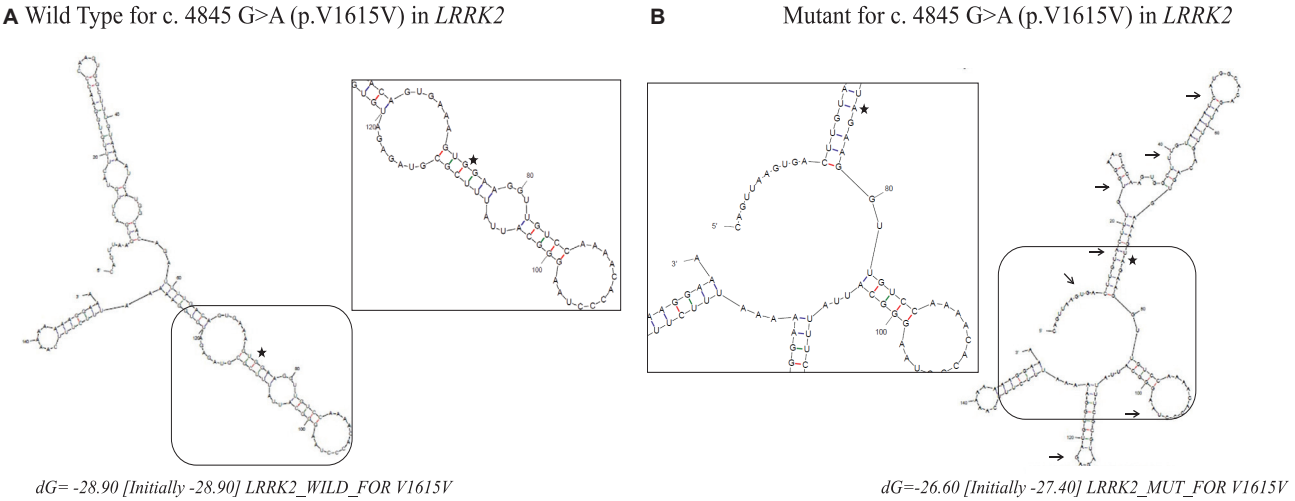

Fig. 2. *In silico* analysis of messenger RNA structure and stability of c. 4845 G>A (p.V1615V) in *LRRK2* using Mfold. The mRNA secondary structure is stabilized by hydrogen bonds, represented by three different colours; AU, blue, CG, red and GU, green. The nucleotide position has been indicated by a small asterisk and the regions altered in mutant are indicated by arrows. Inset showing part of image in enlarged form. (**A**) Wild type and (**B**) Mutant.

variants (p.R42H and p.A82E) with the least allele frequency (0.34%) were selected for their functional characterization. One of which was already reported in the Indian population (p.R42H)[4-6] along with the present study and another one (p.A82E) was previously reported in other than the Indian population[25] but for the first time in the Indian population in our study.

Functional characterization of pathogenic PRKN variants: Since *PINK1* is known to be stabilized and activated upon mitochondrial membrane depolarization and stimulates its downstream E3 ubiquitin ligase, *PRKN*[26]; experiments were performed under cellular stressed condition. A potent cell-permeable proteasomal inhibitor MG-132 which induces the loss of ΔΨM, increases intracellular ROS and renders cells to be eliminated by apoptosis was employed[27]. This stress-induced alterations in ΔΨM were assessed by lipophilic TMRM which senses the negatively charged mitochondria by its delocalized positive charge and accumulates inside healthy, non-apoptotic cells in an inner membrane potential-dependent manner[27]. After

Table III. Clinical data for the patients carrying pathogenic variations as predicted *in silico*

Variation	Patient ID	Age, yr (sex)	Age at onset (yr)	Family history	Clinical features	Response to L-DOPA
c. 101A>G (p.Q34R)	PD-41	52 (males)	48	No	Tremor on left side of the body, rigidity, masked face, postural instability	Yes
	PD-48	45 (females)	43	No	Tremor in both hands, severe headache, bradykinesia	Yes
	PD-111	35 (males)	33	No	Tremor in whole body, rigidity, postural instability, depression	No
	PD-139	65 (male)	60	No	Tremor started on left hand, then spread over whole body, facial tremor, speech disability, postural instability, dementia	Yes
	PD-141	47 (females)	45	No	Tremor in both hands and legs, rigidity, bradykinesia, postural instability	No
c. 125G>A (p.R42H)	PD-75	55 (female)	53	No	Tremor in whole body, bradykinesia, rigidity, postural instability, nausea and vomiting	Yes
c. 245C>A (p.A82E)	PD-76	55 (males)	54	No	Tremor in both hands and legs, bradykinesia, postural instability	Yes
c. 1076G>A (p.G359D)	PD-112	53 (male)	51	No	Tremor on the left side of the body, speech disability, problem in swallowing food, bradykinesia	No
	PD-116	60 (females)	57	No	Tremor in both hands, bradykinesia, postural instability	Yes

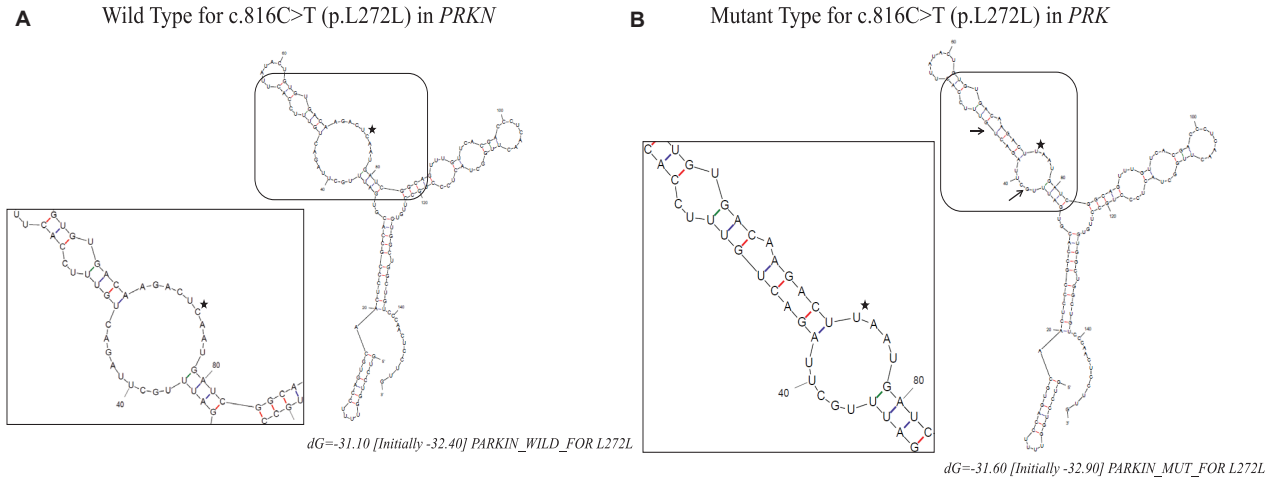

Fig. 3. *In silico* analysis of messenger RNA structure and stability of c.816C>T (p.L272L) in parkin RBR E3 ubiquitin protein ligase (PRKN) using Mfold. The mRNAsecondary structure is stabilized by hydrogen bonds, represented by three different colours;AU blue, CG red and GU green. The nucleotide position has been indicated by a small asterisk and the regions altered in mutant are indicated by arrows. Inset showing part of image in enlarged form. (**A**) Wild type and (**B**) Mutant.

performing FACS assay using TMRM, fluorescent shift was observed in the case of one mutant (p.R42H) but not in the other mutant (p.A82E) of PARKIN, as compared to that of wild type (Fig. 5A and B).

However, Western blot analysis reveals normal protein production for both the mutants *in vitro*. Both the wild and mutants produced proteins of ~ 52 kDa (as determined by the molecular protein marker, pg-

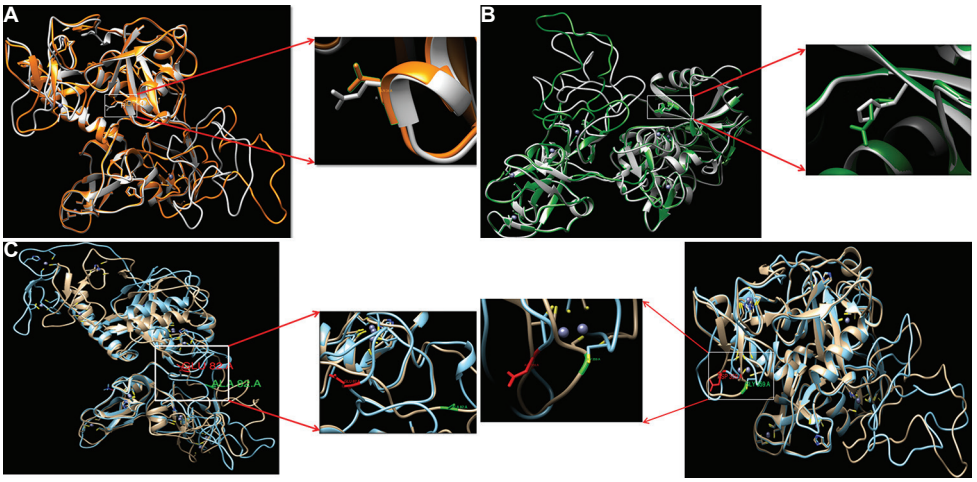

Fig. 4. *In silico* analysis of protein tertiary structure of wild type and mutant protein. Models were generated based on the templates searched in the software (SWISS-MODELLER) on homology basis. The 3D protein structures (.pdb files) were viewed and compared by superimposition of wild and mutant proteins using CHIMERA. (**A**) PARKIN wild versus p.Q34R, (**B**) PARKIN wild v p.R42H, (**C**) PARKIN wild versus p.A82E and (**D**) PARKIN wild versus p.G359D.

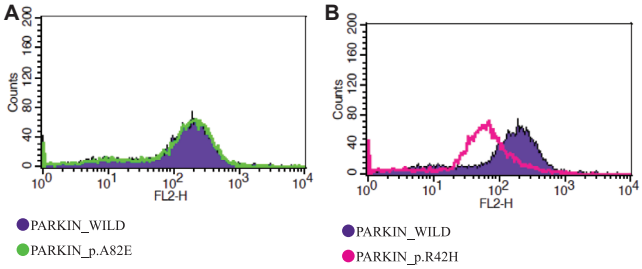

Fig. 5. Overlaid histogram plots derived by cell quest pro software after performing FACS for TMRM labelled cells under stressed condition; (**A**) *PARKIN* wild versus p.A82E (**B**) *PARKIN* wild versus p.R42H.

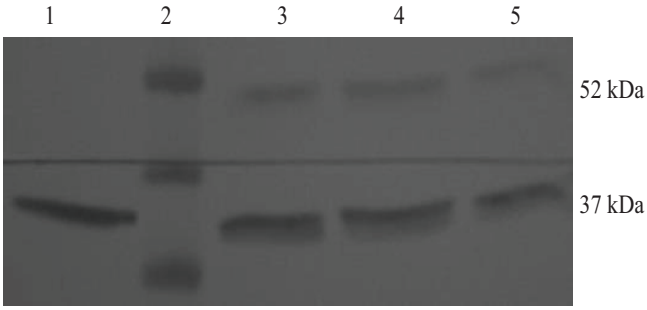

Fig. 6. Western blot analysis to detect wild type and mutant *PARKIN* proteins tagged with c-Myc: 1. Empty vector, 2. Protein marker, 3. *PARKIN*_WILD, 4. *PARKIN*_P. A82E, 5. *PARKIN*_ p.R42H.

pmt2922) which matches with the expected size of it (Fig. 6).

Discussion

LRRK2 has been implicated in both familial and sporadic[28] forms of PD. A number of studies have identified ethnic-specific variant alleles in *LRRK2* for disease susceptibility and pathogenic mutations as well. For example, R1628P and G2385R are two Asian-specific risk factors[29]. The large size of *LRRK2* (51 exons) limits its mutational screening to exons underlying functional domains only. In the present study, mutational screening of *LRRK2* was conducted only for exons covering ROC, COR and kinase domains. No novel or reported mutation was found in this region neither was any association of SNP p.Ser1647Thr (rs11564148) (P=0.1376) was found with disease. In a previous association study[30] performed among populations grouped as Afrikaner Caucasian, non-Afrikaner Caucasian, Black African and mixed ancestry resulted in association of rs11564148 with PD in the Black African population only. The minor allele frequency (MAF %) was 0 per cent in PD and 13.4 per cent in ethnically matched controls (P=0.004). Association of synonymous variants in *LRRK2* with PD was not reported so far, however, in the present study, two synonymous variants situated in COR domain of LRRK2 in two unrelated EOPD patients were identified in heterozygous form. These are c. 4581 T>C (p.Tyr1527Tyr) and c.4845 G>A (p.Val1615Val), identified in a female and a male patient, respectively. Both these variants were absent in the rest of the patients and controls. It is apparent that these are rare variants and further validation is needed to know their intracellular effects. Since there are many upcoming theories of codon biasness and role of synonymous codon position in affecting mRNA secondary structure, stability, rate of translation, posttranslational

modification, *etc*.; the occurrence of such synonymous variants should not be overlooked. However, studying these two variants using Mfold resulted several points of alterations in mRNA secondary structure affecting its stability in case of c.4845 G>A (p.Val1615Val) (Fig. 2A and B) indicating its potential to cause disease.

All the exonic variants (p.Q34R, p.R42H, p.A82E, p.G359D and p.L272L) identified in *PRKN* in our study predicted to be pathogenic, were reported previously from different patient cohorts (south and east) from India[4-6] except one (p.A82E). This variant was, however, not reported in the Indian population[29]. Interestingly, four out of five cases carrying p.Q34R were of young-onset (Table III). However, no other significant correlation in clinical features was found among the nine cases carrying pathogenic missense variations. Computational analysis reveals that the secondary structure though not affected (except in p.A82E and p.G359D), tertiary conformation of the protein was affected by all the pathogenic missense variants (Fig. 4). The synonymous variant, p.L272L was found to cause alteration of mRNA secondary structure *in silico* (Fig. 3A and B).

In PD mitochondrial biogenesis to maintain the healthy mitochondrial pool is greatly compromised. PINK1 and PARKIN are two important components involved in these pathways. In healthy cellular conditions, PINK1 is degraded inside mitochondria with the help of PARL. Upon mitochondrial membrane depolarization, the import of PINK1 is blocked. PINK1 being stabilized on mitochondria recruits PARKIN and activates it by phosphorylation. The E3 ubiquitin ligase PARKIN ubiquitinate the downstream molecules and targets for degradation[25]. Thus, PARKIN, in PINK1-dependent manner prevents impaired mitochondria from being fused and eliminates it by mitophagy. Hence, augmenting mitophagy by activating the PINK1/Parkin pathway is an attractive target for studying the mutation in PINK1 and PARKIN. In the present study, PINK1 was stabilized by using the protease inhibitor MG-132. Characterization of *PRKN* revealed the function of it in recovering cellular stress which gets compromised in case of one mutant (p.R42H).This variant, situated in the UBL (ubiquitin like) domain of the protein is exclusively reported in the Indian population. The decreased fluorescence intensity of TMRM signifies impaired ΔΨM in p.R42H as compared to that of wild type (Fig. 5B). However, another studied variant, p.A82E, situated in between UBL and RING0 domain did not result in such decrease of fluorescence intensity compared to wild type in stressed condition (Fig. 5A) signifying that it is not affecting the ΔΨM in this experimental set up though this variant was found to cause structural alteration both in secondary and tertiary protein structures *in silico*. This observation was similar for both the experiments performed in COS7 as well as HeLa cells. Western blot analysis further confirmed that the mutations did not affect the full-length protein formation (Fig. 6).

This study suggests that the frequency of cases carrying pathogenic missense variation in *PRKN* in the PD patient cohort is 6.2 per cent (9/145) and is rather rare in *LRRK2*. The presence of two rare synonymous variants in two unrelated PD patients was found to be pathogenic *in silico* and may support the phenomenon of codon bias though experimental validation is further needed. Functional characterization of *PRKN* mutations revealed p.R42H for causing ΔΨM disruption, rendering cells more susceptible to cellular stress as observed *in vitro*. Hence, the computational analysis followed by experimental validation is necessary to establish the disease-causing potential of a nucleotide variant.

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