Indian Journal of Biochemistry & Biophysics
Vol. 57, April 2020, pp. 202-212

Association of SLC6A3 gene polymorphisms with the pharmacokinetics of Levodopa and clinical outcome in patients with Parkinson's disease

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Received 26 September 2019; *revised 04 October 2019*

Levodopa (LD) is the gold standard for the treatment of Parkinson's disease (PD). Genetic polymorphisms in the SLC6A3 gene (Solute carrier family 6 member 3/DAT-Dopamine Transporter gene) are shown to have a functional impact on levodopa therapeutic response, motor complications of PD and adverse events. Hence the present study was carried out to investigate the association of SLC6A3 polymorphisms with the pharmacokinetics of levodopa and clinical response. A total of 150 PD patients were recruited in the study. Plasma levodopa was analysed by HPLC at 0, 1, 2, 3 and 4 h post levodopa administration and AUC was calculated. Genotyping of SLC6A3 40 bp VNTR and SLC6A3 rs393795 (G>T) polymorphisms was done by the PCR-RFLP method. The result shows that AUC of levodopa was significantly higher in patients carrying homozygous10/10 genotype (P =0008) compared to 9/9 genotype of SLC6A3 40 bp VNTR polymorphism. A similar difference was also observed in early-onset Parkinson's disease (EOPD) and late-onset Parkinson's disease (LOPD) groups. SLC6A310/10 genotype was found to be significantly associated with disease severity (P =0.05) compared with the 9/10 genotype in the EOPD group, however, there was no significant association with dyskinesia. To conclude, patients carrying SLC6A3 40VNTR 10/10 genotype were found to have higher levodopa exposure, disease severity and prone to further neurodegeneration.

Keywords: Dyskinesia, Levodopa, Pharmacokinetics, SLC6A3 polymorphisms

Parkinson's disease (PD) is the second most common neurodegenerative disorders and it is caused by the depletion of dopamine (DA) producing neurons in the basal ganglia in the brain[1] resulting in the cardinal motor symptoms such as bradykinesia (slowness of movements), tremor (shaking) and rigidity (stiffness). Clinical diagnosis of PD is based on at least two of the three cardinal motor symptoms with the asymmetrical onset and a positive response to levodopa or DA agonists. Several factors affect the bioavailability of levodopa and its therapeutic response. Levodopa is the standard drug of choice for the treatment of Parkinson's disease (PD) because of its remarkable clinical efficacy. However, various adverse events, including wearing-off phenomenon, the on-off phenomenon, dyskinesia, and psychiatric symptoms are experienced by the majority of the patients. The response to levodopa depends on various factors including the type of levodopa preparation, its absorption/metabolism, the blood-brain barrier, age at onset of disease, concomitant use of anti-parkinsonian drugs and the intrinsic responsiveness of the patients which include pre- and postsynaptic dopamine receptors[2].

When levodopa is administered systemically, whether oral or intravenous, some levodopa in the peripheral system (Fig. 1) undergoes decarboxylation by aromatic amino acid decarboxylase (AADC/DDC) and gets converted to dopamine which does not cross the blood-brain barrier[3,4]. Some of the administered levodopa is metabolised peripherally by catechol-O-methyltransferase (COMT) and converted to 3-O-methyldopa (3-OMD), which does not work as a

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Abbreviations: AUC, Area under curve; DAT, Dopamine transporter; EOPD, Early onset Parkinsons Disease; H&Y Stage, Hoehn and Yahr stage; LD, Levodopa; LOPD, Late onset Parkinsons Disease; MoCA, Montreal Cognitive Assessment; PCR, Polymerase chain reaction; PD, Parkinson's disease; PK, Pharmacokinetics; RFLP, Restriction fragment length polymorphism; S&E score, Schwab and England score; SLC6A3, Solute carrier family 6 member 3; UKPDRS, UK Parkinson's disease society brain bank clinical criteria; UPDRS III, Unified Parkinson's Disease Rating Scale; VNTR, Variable number of tandem repeats

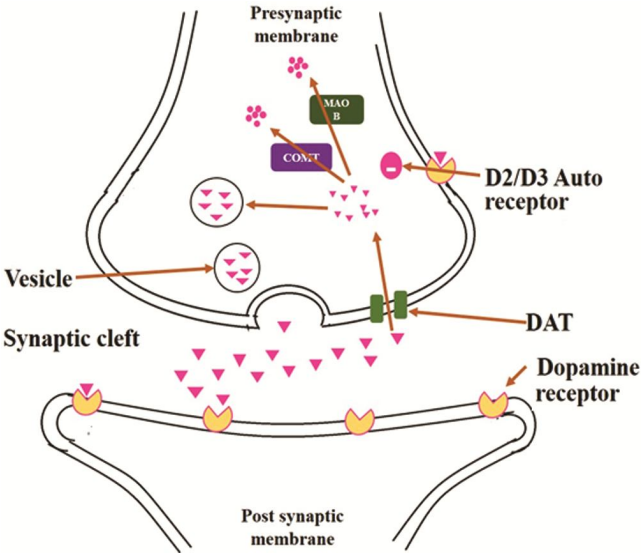

Fig. 1 — Dopamine Metabolism showingreuptake of DA from synaptic cleft into presynaptic neuron. D2/D3 – Dopamine receptor 2/3, DAT – Dopamine transporter (SLC6A3), COMT – Catechol O methyl Transferase, MAO-B – Monoamine oxidase B

dopaminergic neurotransmitter in the striatum[5]. The remaining levodopa crosses the blood-brain barrier, gets converted into dopamine by AADC, stored as dopamine in the synaptic vesicles by vesicular monoamine transporter-2 (VMAT-2), metabolized, released, and reuptake mainly by dopaminergic neurons, the normal striatum[6]. Dopamine transporters (DAT/SLC6A3) are expressed at terminals of dopaminergic neurons and reuptake extracellular dopamine for reuse or metabolization[7-12]. The negative feedback by D2 dopamine receptors and with SLC6A3[12], dopaminergic neurons controls the extracellular dopamine levels.

The functional polymorphisms of genes involving in the above process may have an impact on the therapeutic response of levodopa. In a recent study, it was observed that chronic high-dose (1 mg/kg/day) methylphenidate (an inhibitor of SLC6A3) administration reduced the severity of gait disorders in patients with advanced Parkinson's disease[13] which indicates that pharmacogenetic factors may be partly responsible for the heterogeneity of methylphenidate therapeutic response. In another study, SLC6A3 was found to be the most powerful determinant of dopamine neurotransmission[14]. The genetic studies further reveal that the 9- and 10-repeat alleles are the most frequent in patients with PD[15]. In this study, we therefore, investigated the association of genetic polymorphisms in SLC6A3 with levodopa pharmacokinetics and a functional impact on levodopa therapeutic response and adverse events in patients with PD.

Materials and Methods

Study subjects

We recruited 150 Parkinson's disease patients visiting the Department of Neurology at Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. Patients who had secondary Parkinson's disease (drug-induced), atypical Parkinsonian syndromes and other neurological disorders were excluded from the study. All the patients were recruited based on UK Parkinson's disease society brain bank clinical criteria (exclusion criteria of familial PD was not considered) for the diagnosis of PD. Baseline characteristics such as gender, age, height, weight, body mass index, disease duration, age at onset of all of the patients were obtained using a self-designed questionnaire and medical records. The data of levodopa (represents combination of levodopa and carbidopa in 1:4 ratio) response by Unified Parkinson's Disease Rating Scale part III (UPDRS – III) in OFF (12 h without dopaminergic medication)–ON scores (best response after levodopa challenge *i.e.*, approx 1 h after 250 mg levodopa), cognition by MoCA (Montreal Cognitive Assessment), disease severity by modified Hoehn and Yahr scale (H & Y) Scoring, and activity of daily living by Schwab and England (S & E) were collected using standard questionnaire. This study was approved by the Institutional Ethics Committee (NIEC) of Nizam's Institute of Medical Sciences (EC/NIMS/1895(a)/2017) Hyderabad, India. The informed consent was obtained from all the subjects and from the patient's guardians those who unable to give the consent

Sample collection

For the estimation of plasma levodopa, 3 mL blood sample was collected in the fasting condition after overnight withdrawal of L-DOPA and 24 h of withdrawal of dopaminergic agonists and at 0, 1, 2, 3 and 4th h after the levodopa (250 mg) administration. Blood was subjected to centrifugation at 3000 rpm for 10 min for the separation of plasma at 4°C. These plasma samples were aliquoted and stored at −20°C after adding 50 µL of 10% sodium metabisulphite (for the stability of levodopa) per 1 mL of plasma. Genomic DNA was extracted from whole blood using the standard phenol–chloroform extraction protocol.

Pharmacokinetic (PK) analysis of Levodopa

Chemicals and reagents

L-DOPA and Methyl-L-DOPA were purchased from Sigma Aldrich. Acetonitrile and methanol used were HPLC grade and obtained from Fisher Scientific. All other chemicals used were analytical grade.

Levodopa estimation by HPLC

The Shimadzu HPLC with Fluorescence Detector (Prominence series) was used for the estimation of plasma levodopa concentrations. The chromatographic analysis was performed using the KINETEX column (250×4.6 mm) with particle size of 5 µM analytical column, isocratic mobile phase consisted of 10 mM Potassium Hydrogen Phosphate (KH_2PO_4) (pH 3.5) and methanol (HPLC grade) (95:5, *v/v*) at flow rate of 0.4 mL/min for 25 min. The absorbance of L-Dopa and Me-Dopa was monitored using fluorescence detector at excitation 270 nm and emission 320 nm and retention time (RT) at 8 min and 12 min, respectively[16] (Fig. 2). The C_{max} and time to reach C_{max} i.e., T_{max} were obtained directly from the individual plasma–concentration–time data for levodopa. The area under concentration–time curve from time zero to 4 h (AUC0–4 h) was measured using linear trapezoidal summation with extrapolation[17].

Genomic DNA isolation

Genomic DNA was extracted from whole blood using the standard phenol–chloroform extraction protocol.

Genotyping of SLC6A3 40 bp repeat polymorphism IN 3′-UTR

Genotyping of 40 bp-VNTR polymorphism in 3′-UTR region was determined by using standard PCR protocol and the primers used are (i) SLC6A3 forward primer: 5′-TGTGGTGTAGGGAACGGCCTGAGA-′3 and (ii) SLC6A3 reverse primer: 5′- TGTTGG TCTGCAGGCTGCCTGCAT-′3 . Ten microliter volume was used for each PCR reaction, containing 2 pico mole/µL of each primer, 5 µL of Takara Emerald Amp GT polymerase chain reaction (PCR) master mix, together with 3–4 µL of 50 ng/µL genomic DNA using Eppendorf master cycler. PCR conditions for the amplification were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of 30 sec at 94°C, 15 s at 61.5°C and 45 sec at 72°C for an extension, and a final extension of 10 min at 72°C. The amplified PCR fragments were run on 3% agarose gel and were visualized using an ultraviolet transilluminator for genotyping. The low–quality PCR products were subjected to reanalysis. The ten copies of 40 bp repeats (10R) generating a 476 bp band, nine copies of 40 bp repeats (9R) generating a 436 bp band, eight copies of 40 bp repeats (8R) generating a 396 bp band, seven copies of 40 bp repeats (7R) generating a 356 bp band, six copies of 40 bp repeats (6R) generating a 316 bp band, and five copies of 40 bp repeats (5R) generating a 276 bp band (Fig. 3A) [18].

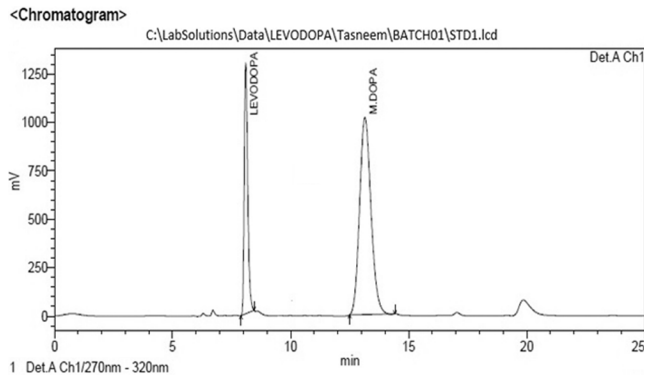

Fig 2 — Estimation of plasma levadopa by reverse-phase HPLC Chromatogram depicts Levodopa and internal standard, methyl DOPA. The retention time (RT) of Levadopa is at 8 min and methyl DOPA at 12 min, respectively

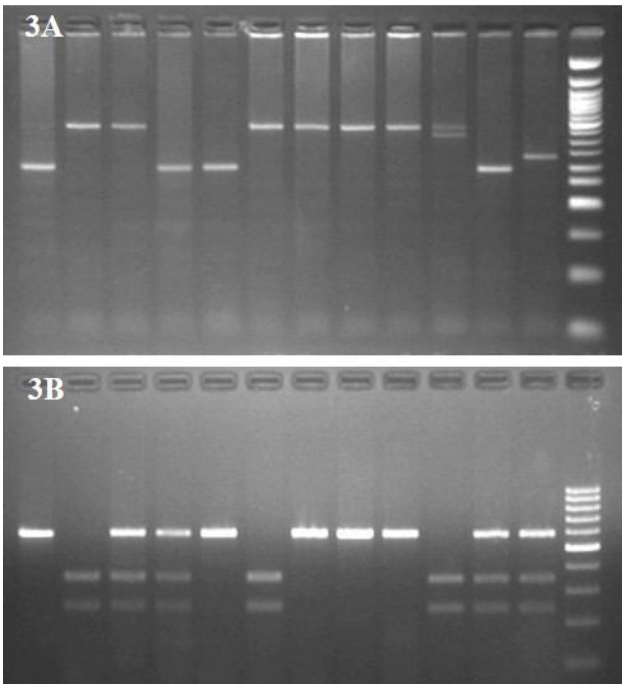

Fig. 3 —(A) Representative gel image of SLC6A3 polymorphism (40 bp VNTR) PCR Product checked on Gel electrophoresis. Lanes 1, 4, 5, 11- 5/5(276 bp, Lanes 2, 3, 6, 7, 8, 9 – 10/10 (476 bp), Lane 10 – 9/10 (436 bp), Lane 12 – 7/7 (356 bp), Lane 13- 50 bp DNA ladder; and (B) Representative gel picture of SLC6A3rs393795 polymorphism, PCR-RFLP Product checked on Gel electrophoresis, Enzyme used Bts I. Lane 1 – Undigested PCR product, Lanes 2,6,10 - Homozygous wild (G/G - 348 bp, 237 bp), Lanes-3, 4, 11, 12 - Heterozygous (G/T - 585 bp, 348 bp, 237 bp), Lanes- 5, 7, 8, 9 - homozygous mutants (T/T - 585 bp)

Genotyping of intronic variant SLC 6A3 G/T (rs393795)

The genotyping of intronic variant G/T (rs393795) polymorphism was done using the PCR RFLP method using the following primers for PCR amplification. (i) Forward: 5′-GCATGTGGAACATTCACCAG -′3. (ii) Reverse: 5′- CAGCCAGCCTTCCTGCAT-′3. The PCR reaction mixture was setup at 95°C initial denaturation for 5 min, followed by 38 cycles at 95°C for 30 sec, 60.2°C for 30 sec, and 72°C for 30 sec, and then a final extension at 72°C for 10 min. The PCR amplification results in 585 bp length amplicons which is subjected to restriction digestion with Bts I enzyme (New England Biolabs, MA, USA) at 55°C for 60 min. Fragments of 348 and 237 bp correspond to for the wild G/G genotype, whereas, 585 bp undigested product corresponds for mutant T/T genotype and 348, 237, 585 fragments correspond for the G/T genotype. These were separated by 2% agarose gel electrophoresis and were visualized using an ultraviolet transilluminator for genotyping (Fig. 3B).

Clinical assessment

MoCA: Montreal Cognitive Assessment (Range: 0-30, ≥26 normal, <26 impaired cognition). H & Y stage: PD severity was determined by modified H & Y staging (0-5, 5 is severe). S & E: Schwab and England activities of daily living scale (100-0% disability).UPDRS III: Unified Parkinson's disease rating scale III clinician-scored monitored motor evaluation. aLR: absolute levodopa response measured as change in UPDRS III (OFF-ON). PDQ 39: Parkinson's disease questionnaire (higher the score, worse will be the quality of life).

Statistical analysis

Genotype frequencies were tested for the deviation from Hardy–Weinberg equilibrium using chi −square ($\chi 2$) test (SLC6A3 40 bp VNTR - P =0.69; SLC6A3 rs393795 - P =0.59). Simple gene counting was performed to find out the allele and genotype frequency distributions. GraphPad Prism Software 5.0 (San Diego, USA) was used for generating bar graphs. Statistical significance for all the tests were considered if the P value is <0.05.

Results

Demographic characteristics of PD patients

The demographic characteristics of PD patients are shown in (Table 1). There were 106 male and 44 were female patients. The age of the male patients and female patients were 58.52 ± 9.80 years and 57.00 ± 10.24 years, respectively. The age of late– onset PD (>50 years) (LOPD) patients is 64.32 ± 0.66 years, which was significantly high (P =<0.0001) compared to early– onset PD (≤50 years) (EOPD) patients *i.e.*, 49.89 ± 0.99 years. The disease duration was 6.18 ± 3.40 years. The disease duration was significantly higher in EOPD cases (7.18 ± 0.51 years) compared to LOPD cases (5.40 ± 0.29 years) (P =0.002). The disease duration was higher in male patients as compared to females (Male: 6.51 ± 2.53 years, female: 5.40 ± 3.29 years, P =0.07). The clinical diagnostic parameters such as the mean disease severity by modified H & Y stage, cognitive assessment by MoCA, a motor performance by UPDRS III OFF and ON scores were recorded at the baseline. The mean of disease severity *i.e.,* modified H & Y stage was 2.50 ± 0.77, which was not different between the EOPD-LOPD groups, male and female groups. The mean cognitive assessment score MoCA was 28.06 ± 3.40, there is no significant difference between male and female EOPD and LOPD groups. The mean motor performance - UPDRS III 'OFF' score was 50.49 ± 11.82 and 'ON' score was 17.42 ± 7.20, and the side effect of levodopa therapy *i.e.*, dyskinesia score was found significantly higher in male patients 1.60 ± 1.16 (P =0.02) compared to female patients 1.13 ± 1.01. The pharmacokinetic parameter *i.e.,* AUC of levodopa was found significantly higher in females 1810.92 ± 1390.77 ng/mL/h (P =0.04) compared to males *i.e.*, 1418.43 ± 958.60 ng/mL/h, whereas it could not reach significance with EOPD and LOPD groups (Table 1).

Genotype and allele distribution of SLC6A3polymorphisms

The genotype distribution of SLC6A3 40 bp VNTR polymorphism shows a higher frequency of 10/10 repeat genotype with 42.6% genotypic distribution, followed by 9/10 repeats with 28% genotype distribution, and 9/9 repeat with 24%. The frequency of 10 repeat-allele was 56%, 9 repeat-allele frequency was 38% (Table 2). For the SLC6A3 intronic variant rs393795 polymorphism, the frequencies of GG, GT, and TT genotype were 38%, 49% and 19%, respectively. The allelic frequency of G-allele was 63% and T-allele was 37% (Table 2).

Association of Pharmacokinetics of Levodopa with genotypes

As shown in (Table 3), AUC of levodopa was significantly higher in patients with SLC6A3 40 bp VNTR homozygous 10 repeat (10/10: 1744 ± 147.50

Table 1 — Demographic characteristics of Parkinson's disease patients

	Parameter	Mean ± SD	*P* value
1.	Age (years) (n= 150)	58 ± 10	
	Male (n=106)	58.5 ± 9.8	0.39
	Female (n=44)	57 ± 10.2	
	EOPD (N=65)	49.8 ± 0.9	<0.0001*
	LOPD (N=85)	64.3 ±0.6	
2.	Age at onset (Years)	51.9 ± 10.1	
	Male	52 ± 10.2	0.80
	Female	51.6 ± 9.6	
	EOPD (n=65)	42.7 ±0.8	<0.0001*
	LOPD(n=85)	58.8 ±0.6	
3.	PDD (Years)	6.1 ± 3.4	
	Male	6.5 ± 2.5	00.07
	Female	5.4 ± 3.2	
	EOPD (n=65)	7.1 ± 0.5	0.002*
	LOPD (n=85)	5.4 ± 0.2	
4.	aLR (n= 150)	33.5 ± 10.9	
	Male	33.1 ± 10.5	0.50
	Female	31.8 ± 11.6	
	EOPD	35.6 ± 1.3	0.02*
	LOPD	31.6 ± 1.7	
5.	LEDD (mg)	968.2 ± 462.3	
6.	AUC (ng/mL/h)	1535 ± 1111.4	0.04*
	Male	1418 ± 958.6	
	Female	1810 ± 1390.7	
	EOPD	1448 ± 127.9	0.40
	LOPD	1602 ± 128.3	
7.	S & E (n= 150)	73.6 ± 1.5	
	Male	73.4 ± 1.2	0.82
	Female	74 ± 1.7	
	EOPD	73.4 ± 1.8	0.89
	LOPD	73.7 ± 1.2	
8.	MoCA (n= 150)	28 ±3.4	
	Male	28.3 ±3.8	0.05*
	Female	27.2 ±2.1	
	EOPD	28.5 ±0.6	0.16
	LOPD	27.7± 0.2	
9.	Dyskinesia	1.4 ± 1.1	
	Male	1.6 ± 1.1	0.02*
	Female	1.1 ± 1.01	
	EOPD	1.7 ± 0.1	0.009*
	LOPD	1.2 ± 0.1	
10.	H & Y (n= 150)	2.5 ± 0.7	
	Male	2.5 ± 0.8	0.62
	Female	2.5 ± 0.6	
	EOPD	2.5 ± 0.1	0.89
	LOPD	2.5 ± 0.07	
11.	UPDRS III OFF (n= 150)	50.4 ± 11.8	

EOPD –Early onset parkinsons disease, LOPD- Late onset parkinsons disease, PDD – Parkinsons disease duration, LED – Levodopa equivalent daily dosage, AUC-Area under curve, S&E – Schwab & England activity of daily living, H&Y-Hoehn and Yahr staging, MoCA-Montreal Cognitive Assessment scale; P value < 0.05 considered as significant

ng/mL/h, *P* =0.008) compared with homozygous 9 repeats (9/9: 1002 ± 10980 ng/mL/h) (Table 3). The AUC of levodopa in heterozygous genotype 9/10 (9/10: 1726 ± 187.10 ng/mL/h, *P* =0.002) was also significantly higher compared with homozygous 9 repeat (9/9: 1002 ± 10980 ng/mL/h) of SLC6A3 40 bp VNTR polymorphism (Table 3). Further the data was

segregated into EOPD and LOPD groups based onage at onset of PD. As shown in (Table 3), AUC of levodopa was significantly higher in EOPD patients with SLC6A3 40 bp VNTR homozygous 10 repeat (10/10: 1616 ± 207.9 ng/mL/h, *P* =0.002) as compared to EOPD patients carrying homozygous 9 repeat (9/9: 796.30 ± 113.60 ng/mL/h, *P* =0.01). Similarly, LOPD patients carrying 10/10 (1825.00 ± 203.00 ng/mL/h, *P* =0.001) had higher AUC as compared to LOPD patients carrying homozygous 9 repeat (9/9: 1121.00± 157.60 ng/mL/h, *P* =0.02). A similar observation was seen with heterozygous 9/10 repeats as compared to 9/9 homozygous repeats in patients with EOPD or LOPD. However, there was no significant difference in AUC of levodopa withwild and variant genotypes was observed with SLC6A3 rs393795 (G/T) (Table 3). The mean AUC values, when studied within disease duration groups (<10 years and > 10 years – as disease progression occurs with the duration as well as levodopa absorption in to brain), the homozygous 10 repeat, heterozygous 9/10 repeat showed significantly higher AUC of levodopa when compared to homozygous 9 repeat in <10 years disease duration group. However, there were no significant differences in AUC values of levodopa of 10/10 (1749 ± 157.3), 9/9 (1018 ± 118.5), and 9/10 (1762 ± 202.2) genotypes in patients with > 10 years disease duration (Table 3).

Table 2 — Genotype and allele frequencies of SLC6A3 polymorphisms

SNP	Genotype	PD (n=150)	Allele	
SLC6A3 40 bp VNTR All the cases (n=150)	10/10	64 (42.6 %)	10 Allele	170 (56.6 %)
	9/10	42 (28 %)	9 Allele	114 (38 %)
	9/9	36 (24 %)	7 Allele	10 (3.3 %)
	7/7	05 (3.3 %)	5 Allele	06 (2 %)
	5/5	03 (2 %)		
SLC6A3 40 bp VNTR In EOPD group (n=65)	10/10	25 (38.4 %)	10 Allele	75 (57.6 %)
	9/10	25 (38.4 %)	9 Allele	49 (37.6 %)
	9/9	12 (18.4 %)	7 Allele	04 (3 %)
	7/7	02 (3 %)	5 Allele	02 (1.5 %)
	5/5	01 (1.5 %)		
SLC6A3 40 bp VNTR In LOPD group (n=86)	10/10	39 (45.3 %)	10 Allele	96 (55.8 %)
	9/10	18 (20.9 %)	9 Allele	66 (38.3 %)
	9/9	24 (27.9 %)	7 Allele	06 (3.4 %)
	7/7	03(3.4 %)	5 Allele	04 (2.3 %)
	5/5	02 (2.3 %)		
SLC6A3 r393795 (G/T) In all the cases (n=150)	G/G	58 (38.6 %)	G Allele	189 (63 %)
	G/T	73 (48.6 %)	T Allele	111 (37 %)
	T/T	19 (12.6 %)		
SLC6A3 r393795 (G/T) In EOPD group(n=65)	G/G	24 (36.9 %)	G Allele	77 (59.2 %)
	G/T	29 (44.6 %)	T Allele	53 (40.7 %)
	T/T	12 (18.4 %)		
SLC6A3 r393795 (G/T) In LOPD group (n=86)	G/G	34 (39.5 %)	G Allele	113 (65.6 %)
	G/T	45 (52.3 %)	T Allele	59 (34.3 %)
	T/T	07 (8.13 %)		

EOPD –Early onset Parkinson's disease, LOPD –Late onset Parkinson's disease, VNTR –Variable number of tandem repeats.

Table 3 — Association of AUC of Levodopa with SLC6A3 polymorphisms

SNP	Genotypes	AUC ng/mL/h	P value
SLC6A3 40 bp VNTR	10/10	1744 ± 147.5	0.0008*
	9/9	1002 ± 109.8	
	9/10	1726 ± 187.1	0.002*
	9/9	1002 ± 109.8	
	EOPD 10/10	1616 ± 207.9	0.01*
	EOPD 9/9	796.3 ± 113.6	
	LOPD 10/10	1825 ± 203	0.01*
	LOPD 9/9	1121 ± 157.6	
	EOPD 9/10	1574 ± 225	0.02*
	EOPD 9/9	796.3 ± 113.6	
	LOPD 9/10	1937 ± 320.8	0.01*
	LOPD 9/9	1121 ± 157.6	
	<10 Years 10/10	1749 ± 157.3	0.001*
	<10 Years 9/9	1018 ± 118.5	
	>10 Years 9/10	1762 ± 202.2	0.003*
	>10 Years 9/9	1018 ± 118.5	
SLC6A3 r393795 (G/T)	G/G	1624 ± 143.8	0.35
	G/T	1443 ± 130.8	
	G/G	1624 ± 143.8	0.75
	T/T	1532 ± 265.9	
	G/T	1443 ± 130.8	0.75
	T/T	1532 ± 265.9	
	EOPD G/G	1637 ± 149.8	0.68
	EOPD T/T	1502 ± 326.3	
	LOPD G/G	1489 ± 567.4	0.83
	LOPD T/T	1646 ± 381.4	
	<10 Years G/G	1493 ± 137.7	0.41
	<10 Years T/T	1017 ± 228.5	
	>10 Years G/G	1489 ± 567.4	0.83
	>10 Years T/T	1646 ± 381.9	

P value < 0.05 considered as Significant, * indicates significant value,EOPD –Early onset Parkinson's disease, LOPD –Late onset Parkinsons Disease.

Association of clinical factors with SLC6A3 polymorphisms

The disease severity score of PD assessed by utilising modified H & Y scale (scale 1-5, a score of 5 is severe), revealed significantly more severe disease in patients with homozygous 10/10 genotype of SLC6A3 polymorphism with EOPD group (10/10: 2.82+0.19) compared to heterozygous 9/10 EOPD group (9/10: 2.34 ± 0.14) indicating the disease progression and severity with the EOPD 10/10 genotype of SLC6A3 40 bp VNTR polymorphism (Table 4). Significant higher disease severity with disease duration (comparing less than 10 years *vs.* more than 10 years) was observed with homozygous 9/9 genotype of 40 bp VNTR and polymorphism rs393795(G>T) G/T genotype (Table4).

Correlation of Clinical Factors with Levodopa response

Absolute levodopa response (aLR) is a measure of levodopa response obtained by subtracting the ON score of UPDRS III with the OFF UPDRS III score. Absolute levodopa response was correlated with clinical factors such as age at onset of disease (EOPD and LOPD), disease duration and AUC of levodopa. The age at onset of PD patients was correlated with aLR by genotypes of the SLC6A340 bp VNTR polymorphism, where we found a significant negative correlation with homozygous 10 repeat genotype (r = −0.2757, P =0.02) and homozygous 9/9 repeat genotype (r = −0.3598, P =0.03) of SLC6A340 bp VNTR polymorphism (Table 6). Another clinical parameter, disease duration when correlated with aLR by the genotypes, we could find a significant positive correlation with homozygous 10 repeat (r =0.27, P =0.02), heterozygous 9/10 repeat (r =0.59, P =<0.0001) and homozygous 9/9 repeats (r =0.3, P =0.02) (Table 6) of SLC6A3 40 bp VNTR polymorphism. The motor performance was examined by UPDRS III score, which when correlated with aLR, shows a significant positive correlation with 10/10 repeat (r =0.27, P =0.02), 9/10 repeat (r =0.59, P =<0.0001) and 9/9 repeats (r =0.3, P =0.02) of SLC6A3 40 bp VNTR polymorphism (Table 6). Further aLR was correlated with AUC of levodopa and found no correlation with 10/10 repeat and heterozygous 9/10 repeats of SLC6A3 40 bp VNTR polymorphism (10/10: r = −0.14, P =0.26; 9/10: r = −0.16, P =0.28) while homozygous 9/9 repeat showed positive correlation (r =0.31, P =0.06) with AUC of levodopa (Table 6).

Table 4 — Association of SLC6A3 polymorphisms with disease severity

SNP	Genotypes	H & Y Stage	*P* value
SLC6A3 40 bp VNTR	10/10	2.66 ± 0.10	0.14
	9/10	2.43 ± 0.11	
	10/10	2.66 ± 0.10	0.23
	9/9	2.47 ± 0.10	
	9/10	2.43 ± 0.11	0.79
	9/9	2.47 ± 0.10	
	10/10 EOPD	2.82 ± 0.19	0.05*
	9/10 EOPD	2.34 ± 0.14	
	10/10EOPD	2.82 ± 0.19	0.38
	9/9EOPD	2.54 ± 0.20	
	10/10 LOPD	2.56 ± 0.11	0.96
	9/10 LOPD	2.55 ± 0.19	
	10/10 LOPD	2.56 ± 0.11	0.48
	9/9 LOPD	2.43 ± 0.12	
	<10Y DD 10/10	2.61 ± 0.10	0.13
	<10Y DD 9/10	2.37 ± 0.12	
	<10Y DD 10/10	2.61 ± 0.10	0.17
	<10Y DD 9/9	2.39 ± 0.10	
	<10Y DD 9/10	2.37 ± 0.12	0.89
	<10Y DD 9/9	2.39 ± 0.10	
	>10Y DD 10/10	3.16 ± 0.47	0.81
	>10Y DD 9/10	3.00 ± 0.40	
	>10Y DD 10/10	3.16 ± 0.47	0.82
	>10Y DD 9/9	3.33 ± 0.33	
	>10Y DD 9/10	3.00 ± 0.40	0.57
	>10Y DD 9/9	3.33 ± 0.33	
	<10Y DD9/9	2.39 ± 0.10	0.01*
	>10Y DD9/9	3.33 ± 0.33	
SLC6A3 r393795 (G/T)	G/G	2.75 ± 0.11	0.008*
	G/T	2.38 ± 0.08	
	G/G	2.75 ± 0.11	0.002*
	T/T	2.65 ± 0.15	
	G/T	2.38 ± 0.08	0.13
	T/T	2.65 ± 0.15	
	<10Y DDG/T	2.34 ± 0.07	0.01*
	>10 Y DD G/T	3.25 ± 0.47	

DD, Disease Duration; EOPD, Early onset PD; LOPD, Late onset PD;Disease severity assessed by modified H & Y stage (0-5, 5 is severe disease).

Association of Pharmacokinetics of Levodopa with adverse events and Side effects of Levodopa therapy

The adverse events following levodopa treatment were recorded by the interview. The most common adverse events observed in 20% of the patients were nausea, vomiting, and headache and 6.6% of the patients were reported leg cramps The most common side effect faced by 78% of the patients was dyskinesia. Following long-term therapy of levodopa,

Table 5 — Association of SLC6A3 polymorphisms with cognition

SNP	Genotypes	MoCA	*P* value
SLC6A3 40 bp VNTR	10/10	27.98 ± 0.24	0.001*
	7/7	24.80 ± 1.77	
	10/10	27.98 ± 0.24	0.03*
	5/5	25.33 ± 2.90	
	9/10	28.21 ± 0.26	0.0009*
	7/7	24.80 ± 1.77	
	9/10	28.21 ± 0.26	0.02*
	5/5	25.33 ± 2.90	
	9/9	27.89 ± 0.42	0.02*
	7/7	24.80 ± 1.77	
	10/10 EOPD	28.12 ± 0.39	0.65
	10/10 LOPD	27.90 ± 0.30	
	9/10 EOPD	28.28 ± 0.26	0.75
	9/10 LOPD	28.11 ± 0.52	
	9/9 EOPD	28.50 ± 0.48	0.39
	9/9 LOPD	27.75 ± 0.55	
SLC6A3 r393795 (G/T)	G/G	27.59 ± 0.35	0.21
	G/T	28.10 ± 0.23	
	G/G	27.59 ± 0.35	0.64
	T/T	27.89 ± 0.43	
	G/T	28.10 ± 0.23	0.69
	T/T	27.89 ± 0.43	
	G/G EOPD	28.25 ± 0.38	0.25
	G/G LOPD	27.33 ± 0.46	
	G/T EOPD	29.00 ± 1.26	0.42
	G/T LOPD	28.19 ± 0.25	
	T/T OPD	28.08 ± 0.55	0.58
	T/T LOPD	27.57 ± 0.71	

P value < 0.05 considered as Significant, * indicates significant value, DD –Disease Duration, EOPD–Early onset PD, LOPD – Late onset PD, Cognition was assessed by modified MoCA Score(Range: 0-30, ≥26 normal, <26 impaired cognition).

patients experienced dyskinesia. The dyskinesia score was recorded using the UPDRS IV questionnaire, and the total score was correlated with AUC for each patient. We could not find a significant association of dyskinesia with AUC of levodopa by 10/10, 9/10, 9/9 genotypes of SLC6A3 40 bp VNTR polymorphisms. Similarly, no serious adverse events were noted in the present study.

Dopamine transporters and the AUC of Levodopa

In the present study, we found a significant association between the SLC6A3 genotype and the AUC of levodopa. SLC6A3 is one of the most powerful components to determine the dopamine metabolism in the striatum[14]. In some of the studies, it has been suggested that higher baseline levels of SLC6A3 expression were observed with patients with SLC6A3 rs28363170 10/10 genotypes than patients with 9/9, 9/10 and 5/5 genotypes and thus lower synaptic dopamine levels (due to greater dopamine reuptake)[19-22]. The situation could be more critical in advanced PD where marked degeneration of the presynaptic neurons within the striatum is reported. Hence, one can postulate that plasma levodopa levels may vary depending on the genotypes of SLC6A3 VNTR polymorphism, where 10/10 genotype may exhibit higher plasma levodopa levels (High SLC6A3 expression with 10/10 genotype leads to higher reuptake from synaptic cleft resulting in less levodopa absorption across blood– brain barrier). To support the above observation, we found a significant higher plasma levodopa concentration in patients with 10/10 genotype, suggesting that the higher expression of SLC6A3 in the striatum with 10/10 genotype and higher reuptake of dopamine may be the mechanism involved.

Dopamine transporters and the clinical observations of PD

We found that SLC6A3 genotypes were not associated with disease severity as assessed by the H & Y stage. When the data were compared between EOPD and LOPD groups, significantly high disease severity was observed with homozygous 10/10 genotype compared to the heterozygous 9/10 genotype of SLC6A3 polymorphism in EOPD group. Further segregation of data into less than 10 years disease duration and more than 10 years disease duration of PD, significantly higher score of H & Y stage was found with the homozygous 9/9 genotype with>10 years disease duration compared to homozygous 9/9 genotype of <10 years disease duration group. A similar finding was noted in patients with heterozygous G/T genotype of SLC6A3 intronic polymorphism. Cognitive assessment in PD patients was not associated with genotypes of SLC6A3 polymorphisms, but when compared in EOPD and LOPD groups, the 10/10 genotype in the LOPD group had significantly poorer cognition compared to heterozygous 9/10 genotype in the same group (Table 5). Significantly poor cognition was seen with 7/7 and 5/5 genotypes, but the number of cases with these genotypes were small (5 and 2, respectively) and hence requires future studies.

Absolute levodopa response and Clinical observations of PD

The motor symptoms response to levodopa is crucial in PD management. Absolute Levodopa Response (aLR) correlates well with clinically

Table 6 — Association of absolute levodopa response (aLR) and clinical factors of PD with SLC6A3 Genotypes

		Genotype Combinations	r value	*P* Value
Disease Duration		aLR *vs* DD	0.38	<0.0001*
	Overall cases	aLR *vs*DD EOPD	0.37	0.001*
		aLR *vs*DD LOPD	0.37	0.0004*
	SLC6A3 40 bp VNTR	aLR *vs*DD 10/10	0.21	0.08
		aLR *vs* DD 9/10	0.59	<0.0001*
		aLR *vs* DD 9/9	0.36	0.02*
	SLC6A3 r393795 (G/T)	aLR *vs* DD GG	0.40	0.001*
		aLR *vs* DD GT	0.33	0.003*
		aLR *vs* DD TT	0.45	0.05*
UPDRS III OFF Score		aLR *vs* UPDRS IIII OFF	0.78	<0.0001*
	Overall cases	aLR *vs* UPDRS IIII OFF EOPD	0.79	<0.0001*
		aLR *vs* UPDRS IIII OFF LOPD	0.77	<0.0001*
	SLC6A3 40 bp VNTR	aLR *vs* UPDRS IIII OFF 10/10	0.69	<0.0001*
		aLR *vs* UPDRS IIII OFF 9/10	0.81	<0.0001*
		aLR *vs* UPDRS IIII OFF 9/9	0.86	<0.0001*
	SLC6A3 r393795 (G/T)	aLR *vs* UPDRS IIII OFF GG	0.86	<0.0001*
		aLR *vs* UPDRS IIII OFF GT	0.33	0.003*
		aLR *vs* UPDRSS IIII OFF TT	0.87	<0.0001*
Age at Onset	Overall Case	aLR *vs* AGE AT ONSET	-0.23	0.004*
		aLR *vs* AGE AT ONSET EOPD	-0.23	0.08
		aLR *vs* AGE AT ONSET LOPD	-0.15	0.14
	SLC6A3 40 bp VNTR	aLR *vs* AGE AT ONSET 10/10	-0.27	0.02*
		aLR *vs* AGE AT ONSET 9/10	-0.14	0.35
		aLR *vs* AGE AT ONSET 9/9	-0.35	0.03*
	SLC6A3 r393795 (G/T)	aLR *vs* AGE AT ONSET GG	-0.23	0.07
		aLR *vs* AGE AT ONSET GT	-0.25	0.02*
		aLR *vs* AGE AT ONSET TT	-0.23	0.33

P value < 0.05 considered as Significant, * indicates significant value, DD- Disease Duration, aLR – Absolute Levodopa response, EOPD – Early onset Parkinsons disease, LOPD- Late onset Parkinsons Disease.

relevant variables such as motor symptoms, age at onset of PD and disease duration. The present results demonstrated that change in the motor UPDRS-III score and aLR increases with the increase in disease duration. This corroborates results found in the other two studies that aLR increases with disease duration[23,24].

A study by Lees[25], suggests that patients exhibiting higher amplitudes of aLR have a high UPDRS III OFF score. As discussed earlier OFF motor scores are a good representation of substantia Nigra pars compacta degeneration. Patients would then require an increased levodopa dose administration to manage their worsening motor symptoms.

Correlation of aLR by genotypes would allow a more accurate determination of genotype effect on the disease state and clinical factors. Our results revealed higher aLR with a 10/10 genotype and also the AUC was significantly associated with the same genotype.

Late– stage PD is often associated with significant levodopa-induced motor complications such as motor fluctuations and dyskinesia and may be influenced by the AUC of levodopa. Theoretically genotype variations should impact these complications. However, in a recent study, it was shown that there is no difference in dyskinesias among genotypes of SLC6A3 polymorphisms[26]. This is in agreement with our finding that SLC6A340 bp VNTR genotypes were not associated with dyskinesias.

Discussion

This is the first study to demonstrate that the genotypes of the SLC6A3 gene (40 bp VNTR) and intronic variant rs393795 of SLC6A3gene are associated with the inter-individual differences in the therapeutic response to levodopa and clinical observations of PD. As SLC6A3 well known to determine the dopamine metabolism in the striatum[27]and

we observed a significantly higher levodopa exposure in patients with homozygous 10 repeat and heterozygous 9/10 repeat genotypes of SLC6A3 40bp VNTR compared with homozygous 9/9 genotype. A literature search on SLC6A3 genes with the association of levodopa pharmacokinetics did not found any publications on the possible association ofSLC6A3 genotypes with the levodopa of AUC and the association of adverse events in patients with the levodopa treatment. SLC6A3 gene variants was studied to a lesser extent and investigated in PD as the disease risk modifiers for the occurrence of dopaminergic-related complications (such as hallucinations, dyskinesia, and depression)[28]. In the present study, 10/10 genotype of SLC6A3 40 bp VNTR polymorphism showed higher AUC of levodopa, which may contribute to increased risk of dyskinesia as observed in the present study (data not shown). We also found that the patients carrying SLC6A3 40VNTR 10/10 genotype were associated with the disease severity. A possible association with cognitive impairment was also observed in LOPD group with 10/10 genotype.Our results revealed higher aLR with 10/10 genotype and also associated with increased AUC of levodopa, which is in agreement with the previous study that OFF motor scores represents the substantia Nigra pars compacta degeneration and those patients require more and more levodopa administration to manage their worsening symptoms[25].Hence, the knowledge of SLC6A3 genotype could help the physician to predict the disease severity, non-motor symptoms, and risk/benefit balance of therapy and to define the levodopa treatment strategy in patients with PD. We surmise that the genotype may influence the onset of dopaminergic related side effects. In addition to genotypes, peptides like neurotensin, environmental toxins, and other medications are also have role in stability and bioavailability of dopamine in brain[29]. Further to establish the role of genotypes along with other factors.

Conclusion

Our results show that dopamine transporters in particularly, SLC6A3 40VNTR 10/10 genotype significantly influence levodopa concentrations. There was a significant increase in AUC of levodopa in patients carrying SLC6A3 40VNTR 10/10 genotype. This type of observation was also seen in patients in both early-onset and late-onset Parkinson's disease. In addition, SLC6A310/10 genotype was found to be significantly associated with disease severity in patients with early-onset PD. Patients carrying SLC6A3 40VNTR 10/10 genotype was found to have higher levodopa exposure, indicating greater expression of dopamine transporters, greater reuptake of dopamine from the synapse, and thus greater exposure to levodopa contributing to the disease severity and rapid disease progression. However, there was no significant association with dyskinesia was observed with SLC6A3 40VNTR 10/10 genotype.

Conflict of Interest

All authors declare no conflict of interest.

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