

Screening of Genetic Mutations in Early Onset Parkinsonism Patients: A Family Based Study in Tamil Nadu Population

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ABSTRACT Parkinson's disease (PD) is a progressive motor system disorder which distress several parts of the brain, in particular substantia nigral area that controls balance and movement. The intend of the study was to identify the polymorphism in SNCA (α -Synuclein) and Parkin genes using PCR-RFLP and chromosomal analysis by GTG banding in 23 early onset PD patients who are below the age of 50. The results were analyzed and SNCA polymorphism was observed as missense mutation A53T with G–A transition whereas Parkin is also observed with G–A transition and change in amino-acid S167N. The chromosomal abnormality resulted with 22q11.2 deletion which is an increased risk factor in early-onset PD. Therefore, the resulted polymorphism is the foremost report in Tamil Nadu population and the researchers assure that this would be a unique study from the previous researches in India.

INTRODUCTION

Parkinson's disease (PD) is a type of movement disorder, which occurs due to the loss of the dopamine and it is the second most common neurodegenerative disorder following Alzheimer's disease. It affects substantia nigral area that controls balance and movement. The presence of intraneural inclusions, lewy bodies and loss of neurons in the substantia nigra are the distinguished pathological features (Fearnley et al. 1991). Usually, PD before the age of 50 is early-onset whereas after 50 named as late-onset disease. PD symptoms are subtle and occur gradually (Javier et al. 2015). The prevalence rate

in North America and West European countries is about 150–200 per 100,000 (Zhang and Roman 1993) and lower in China, Japan and Africa (45–80 per 100,000). In India its occurrence is around 14–27 per 100,000 individuals (Singhal et al. 2003). It can be rarely inherited in families either as an autosomal dominant, autosomal recessive trait (Biswasa et al. 2006) or as sporadic cases. Hereditary and environmental factors increase the chance of PD and are likely to inflict a major socio-economic burden on aging populations.

Till now more than 16 PD related loci has been identified after the discovery of α -synuclein (SNCA) (Corti et al. 2011). The following five major genes have shown to cause PD: PARK2 (Parkin), PINK1, PARK7 seen in autosomal recessive PD whereas SNCA and LRRK2 genes seen in autosomal dominant form (Thomas and Beal 2007). PARKIN, DJ-1 and PINK 1 mutations have been detected in autosomal recessive juvenile Parkinsonism (Kitada et al. 1998; Funayama et al. 2002; Valente et al. 2004). Parkin and DJ-1 mutations are significant in causing familial PD (Healy et al. 2004; Hedrich et al. 2004).

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SNCA is α -synuclein protein seen on chromosome 4q is the major fibrillar component which constitutes the pathological hallmark of Parkinson's disease (Dawson and Dawson 2003). Among various genetic abnormalities, point mutation was the first to be identified, carrying an autosomal dominant inherited PD with an altered primary structure of alpha-synuclein (Polymeropoulos et al. 1997). It is the only gene that produces PD even when it is expressed in multiple copies (Uchiyama et al. 2008; Pihlstrom and Toft 2011; Moura et al. 2012). The function of SNCA is considered to be involved in the regulation of dopamine release and transport, induces fibrillization of microtubule associated with protein tau (Ferese et al. 2015). It exerts neuroprotective phenotype in non-dopaminergic neurons by inhibiting p53 expression and transactivation of proapoptotic genes leading to decreased caspase-3 activation (Hashimoto and Masliah 1999; da Costa et al. 2002; Tang et al. 2008).

Recently, autosomal recessive juvenile Parkinsonism is the other prevalent form resulted with mutations in the parkin gene (*PARK2*) (Kitada et al. 1998). *Parkin*, encoding the E3 ubiquitin ligase parkin, is one of the largest genes in human and it is located on chromosome 6q25.2-q27 (McAvoy et al. 2008). *PARK2* gene mutations were first identified in Japanese patients (Kitada et al. 1998) and were also found in patients from various ethnic groups (Leroy et al. 1998; Lucking et al. 1998; Abbas et al. 1999; Nisipeanu et al. 1999). Apart from these multiple gene involvement in PD, previous case studies of Parkinsonism and a hemizygous deletion of chromosome 22q11.2 also known as DiGeorge or Velocardio-facial syndrome may be an increased risk of early-onset PD (Zaleski et al. 2009; Booij et al. 2010). The effect of this deletion is less clear on aging and neurodegenerative processes (Butcher et al. 2013). The midbrain dopaminergic cell loss and the presence of Lewy bodies (Braak et al. 2003; Milber et al. 2012) are necessary in establishing a 22q11.2 deletion which is a risk factor for PD.

Objective

In the present investigation, an attempt was made to understand the involvement of SNCA and Parkin gene polymorphism using PCR-RFLP and chromosomal abnormalities by Trypsin

banding in early-onset PD population. Relevantly, in the researchers' study the importance of genetic counseling and genetic testing based on the early onset of PD was discussed. In addition, this is the first kind of report which deals SNCA and Parkin gene in PD patients in Tamil Nadu population.

MATERIAL AND METHODS

Subject Recruitment

The blood sample of 10 mL was collected from 23 PD patients in various hospitals of Tamil Nadu during 2013 to 2016. Equal number of healthy controls was selected. The study was an early-onset form of PD, the subjects were recruited below the age group of 50. Detailed questionnaire on clinical symptoms and history of PD were obtained as well as from review of the patient's hospital records of both control and PD patients.

Chromosomal Analysis

Using 0.5 ml of heparinised peripheral blood samples, karyotyping was conducted by analyzing G-banding. From G-banding, metaphase spreads were performed using phytohaemagglutinin (PHA) since it is one of the standard cytogenetic technique (Moorehead et al. 1960). Lymphocytes were cultured in RPMI 1640 media of 5 ml containing antibiotics and serum of 1.5ml. PHA was included as mitotic stimulant and the samples were incubated for 72 hours at 37°C. At 71st hour, 150 μ l of 0.1 percent colchicine was added to arrest the cells at metaphase stage and incubated again.

Harvesting and Slide Preparation

The cultures were harvested after 72 hours, and centrifuged at 1000 rpm for 7-10 minutes, later on the supernatant was discarded. The cultures are treated with 5ml of hypotonic solution (KCl) and centrifuged at 1000 rpm for 10 minutes, later, the cells were fixed with fixative (3:1, methanol: acetic acid). Fixative washes were performed until a clear suspension was obtained. The prepared slides were then stained using trypsin and Giemsa (GTG). The next step is to prepare the slides were the slides are washed in

running tap water and were kept at 4°C in distilled water. Using a pasteur pipette, few drops of the cell suspension were placed on the slide and dried at 37°C on a slide warmer.

GTG Banding

Slides with cell suspension were treated with 0.5 percent trypsin solution for about 5–20 seconds. The slides were then rinsed in PBS and stained with 10 percent Giemsa (Sigma, USA) solution for 10 minutes. The slides were finally rinsed in distilled water, dried and viewed under microscope. Chromosomal analysis was performed and overall 50 metaphase spreads were screened.

Genotyping

Blood DNA was isolated using the standard procedure and the primer sequences of exon 4 for PCR amplification of *SNCA* gene were previously used in (Polymeropoulos et al. 1997) and for *Parkin* genes it was earlier described by Wang et al. (1999). Thermal cycling parameters for amplification followed initial denaturation for 5 min at 94°C, later 15 sec at 94°C for denaturation, 1 min at 60°C for amplification and extension for 1 min at 72°C. The final extension is for 10 min at 72°C. For the detection of polymorphism in *SNCA* and *Parkin* genes, the restriction enzymes *AlwNI* and *Tsp45I* were used respectively.

Statistical Analysis

The statistical analysis was performed using SPSS software. The statistical significance of the difference in the frequencies of PD (*SNCA* and *Parkin*) genotypes was calculated by t test. All the analysis was performed with IBM-SPSS software 20.0 version. Odds ratios (OR) and confidence intervals were calculated to estimate the strength of the association of polymorphism genotype alleles in patients. Mean and standard deviation were calculated to assess the difference between the patients and controls and the level of significance was calculated by ANOVA.

RESULTS

In this current study total 23 subjects were recruited who are under the age of 50. Table 1

shows the total subject recruited in the study. The average mean $\pm$ SD of the age was calculated for male and female which showed 37.33 ± 5.96 and 40.8 ± 5.58 respectively. Furthermore, the clinical features were analyzed and tabulated in Table 2, where maximum number of patients were in familial form of PD in which three cases revealed with dementia in sporadic form of PD.

Table 1: Total subject recruitment in the study population

S. No.	Subjects	Gender	Age (Mean $\pm$ SD)
1.	Case(n=23)	Male (18) Female (5)	37.33 ± 5.96 40.8 ± 5.58
2.	Control(n=23)	Male (18) Female (5)	38.20 ± 4.80 41.2 ± 5.35

Table 3 shows the karyotypic analysis of PD patients. Total 23 patients were seen with chromosomal anomalies. The abnormality seen in chromosome 22 with a deletion 46,XY,del(22q11.2) and 46,XX,del(22q11.2) in male and female respectively. The detailed chromosomal aberrations (CA) for individual samples were analyzed. Table 3a depicts the CA in all 23 cases with total mean $\pm$ SD of CTA, CSA and TCA. The mean $\pm$ SD values of chromosomal type aberrations (CSAs) and chromatid type aberrations (CTAs) were 1.61 ± 1.09 and 0.88 ± 1.07 in males whereas in females 1.4 ± 1.67 and 0.6 ± 0.54 is seen respectively. The TCA were found to be 2.5 ± 1.54 and 2 ± 2.12 in male and female respectively. The frequencies in male and female were significant at $P < 0.05$ level.

In Table 4, the genotype frequencies of *SNCA* and *Parkin* genes were examined. Genotypes of *SNCA* were 13.04 percent (n=3) for GG, 21.73 percent (n=5) for GA and 8.69 percent (n=2) for AA. Similarly for *Parkin* gene the genotype GG showed 17.39 percent (n=4), in GA 30.43 percent (n=7) and for AA 8.69 percent (n=2). The p value of the allele frequency in both the genes showed significant at $P < 0.05$ level.

DISCUSSION

The significance of genetic factors in etiology of PD has been debated for a long time and the current study aims to evaluate genetically based on the patient's personal medical record, lifestyle and other factors. The study intended

Table 2: Clinical features in early-onset cases

S. No.	Subjects	Onset of PD	Gender	Age	Form of PD	Clinical Features				
						Tremor	Bradykinesia	Rigidity	Depression	Dementia
1.	Case(n=23) EOPD		Male	34	SP	✓		✓	✓	
2.			Male	42	F	✓		✓		
3.			Male	48	SP	✓				✓
4.			Male	32	F	✓		✓		
5.			Female	38	F		✓		✓	
6.			Male	45	SP	✓		✓	✓	✓
7.			Male	32	F	✓			✓	
8.			Male	46	F	✓				
9.			Female	47	SP		✓	✓		✓
10.			Female	45	SP	✓				
11.			Male	33	F			✓		
12.			Male	40	SP	✓				
13.			Male	45	F		✓	✓	✓	
14.			Male	32	SP	✓		✓	✓	
15.			Male	37	F	✓			✓	
16.			Male	30	SP		✓	✓		
17.			Male	34	SP	✓			✓	
18.			Female	41	F					
19.			Male	36	SP	✓		✓		
20.			Male	30	SP			✓	✓	
21.			Female	33	F		✓		✓	
22.			Male	42	F	✓		✓		
23.			Male	34	F	✓				

Table 3: Displays the frequency of chromosomal alterations observed in PD patients

S. No.	Gender	Age	Karyotype results	Chromosomal alterations		
				CSA	CTA	TCA
1.	Male	34	46,XY,del(1q)	0	1	1
2	Male	42	46,XY,del(1q)	1	0	1
3	Male	48	46,XY,del(22q11.2)	2	1	3
4	Male	32	46,XY,del(1q)	4	1	5
5	Female	38	46,XX, del(22q11.2)	4	1	5
6	Male	45	46,XY,del(1q)	0	0	0
7	Male	32	46,XY,del(1q)	2	1	3
8	Male	46	46,XY,del(22q11.2)	3	0	3
9	Female	47	46,XX,del(22q11.2)	0	0	0
10	Female	45	46,XX,del(1q)	0	0	0
11	Male	33	46,XY,del(1q)	2	1	3
12	Male	40	46,XY,del(1q)	2	1	3
13	Male	45	46,XY,del(1q)	2	1	3
14	Male	32	46,XY,del(1q)	1	0	1
15	Male	37	46,XY,del(1q)	2	0	2
16	Male	30	46,XY,del(1q)	0	1	1
17	Male	34	46,XY,del(22q11.2)	1	3	4
18	Female	41	46,XX,del(1q)	1	1	2
19	Male	36	46,XY,del(1q)	3	0	3
20	Male	30	46,XY,del(1q)	1	0	1
21	Female	33	46,XX,del(22q11.2)	2	1	3
22	Male	42	46,XY,del(1q)	2	4	6
23	Male	34	46,XX,del(22q11.2)	1	1	2

with the idea of both genetic counseling and genetic testing in PD related genes (SNCA and

Parkin) in 23 cases of early-onset. In the present study we set out to investigate the CA and ge-

Table 3a: Statistical data of CSA, CTA and TCA of PD subjects

<i>Genes</i>	<i>Subject</i>	<i>Gender</i>	<i>Total</i>	<i>CSA Total age (Mean $\pm$ S.D)</i>	<i>CTA Total age (Mean $\pm$ S.D)</i>	<i>TCA Total age (Mean $\pm$ S.D)</i>
SNCA / Parkin	Case (n=23)	Male	18 (78.26%)	1.61 $\pm$ 1.09	0.88 $\pm$ 1.07	2.5 $\pm$ 1.54
		Female	5 (21.73 %)	1.4 $\pm$ 1.67	0.6 $\pm$ 0.54	2 $\pm$ 2.12

Table 4: Exhibits the allele frequency of SNCA and Parkin in PD cases

<i>Subjects</i>	<i>SNCA</i>			<i>Parkin</i>		
	<i>GG</i>	<i>GA</i>	<i>AA</i>	<i>GG</i>	<i>GA</i>	<i>AA</i>
Case (n=23)	3(13.04 %)	5(21.73 %)	2(8.69%)	4(17.39 %)	7(30.43 %)	2(8.69%)
Odds ratio	0.130	0.217	0.087	0.173	0.304	0.087
P value	0.002	0.007	0.002	0.004	0.022	0.002
95% CI	0.0343 to 0.4955	0.0705 to 0.6707	0.0183 to 0.4123	0.0519 to 0.582	0.1092 to 0.8479	0.0183 to 0.4123
	Z= 2.991	Z=2.655	Z=3.076	Z=2.836	Z=2.276	Z=3.076

netic changes in Tamil Nadu patients. SNCA is the prime gene implicated in the development of PD and the three point mutations and gene multiplications in it have been reported to be rare and are estimated to contribute <1 percent of monogenic cases of PD. The first missense mutation A53T of SNCA in Italian kindred with familial form of PD was identified (Polymeropoulos et al. 1997). In 2013 study, the similar mutation was found to be replicated in Greek population (Bozi et al. 2013). Astonishingly, in some Brazilian studies there were no SNCA-A53T mutation (Teive et al. 2001; Camargos et al. 2009; Moura et al. 2012). In another finding, 7 exons with A53T mutation and other mutations of SNCA gene in 30 European and American children with autosomal dominant PD was assessed (Vaughan et al. 1998). Similarly, the researchers' data also reported with A53T variation with the transition G-A in exon 4. However in India, three studies did not reveal with SNCA mutation (Nagar et al. 2001; Vishwanathan et al. 2012; Kadalakol et al. 2014). Taken together this is the first study with A53T variation in Tamil Nadu population. In addition, previous studies reported with copy number variations of SNCA duplication (Chartier-Harlin et al. 2004) and triplication (Singleton et al. 2003) where SNCA is associated with familial PD. These two forms are rare culprits in familial form of PD (Vishwanathan et al. 2012).

The other gene assessed for polymorphism is Parkin and it is a causative for autosomal re-

cessive juvenile Parkinsonism (AR-JP) which was the first to be seen in Japanese families (Takahashi et al. 1994). The frequency of Parkin mutations in early-onset is about 2 percent, 10 percent in familial form and 25 percent in autosomal recessive, whereas, in an Indian study high frequency with 68 percent was seen in early onset cases (Padmaja et al. 2012). The Parkin mutations in early-onset is estimated to be as high as 40-50 percent (Lucking et al. 2000; Hedrich et al. 2002; Klein et al. 2003) and 10-20 percent in sporadic cases (Lucking et al. 2000; Hedrich et al. 2002; Khan et al. 2003; Periquet et al. 2003). Varying frequencies of wide range of mutations has been reported across different ethnic groups (Chaudhary et al. 2006). The association between parkin mutations and AR-JP in early onset PD is well established (Hattori et al. 1998; Lucking et al. 2000; West et al. 2002; Poorkaj et al. 2004; Bertoli-Avella et al. 2005). While, the polymorphism in exon 10 of Parkin gene was suggested to be a protective factor against PD because of the presence of a mutant allele in lower frequency of PD patients while the S167N and V380L polymorphisms were reported not to be associated with PD (Wang et al. 1999). Conversely, in sporadic PD, the patients were seen with heterozygosity of S167N polymorphism which was associated with increased risk of PD (Satoh and Kuroda 1999). In another study, 12 exons in Parkin gene were sequenced and investigated for the polymorphism in exon 4, 10, and 11 were it resulted with no evidence of mu-

tation or associated polymorphism in sporadic and familial form of PD (Annesi et al. 2000). It is said in a report that the formation of inactive protein by substitution, truncation or deletion of the amino acid(s) is due to the mutation in Parkin gene (Kitada et al. 1998). In a Chinese population, the frequency of G/A (S167N) transition in exon 4 is 42 percent and 34 percent in PD and controls, respectively, when compared with Caucasian PD patients (2 percent – 3 percent). From the above studies, the present findings resulted similar polymorphism Ser167Asn (S167N) with the nucleotide change G–A transition in exon 4. The frequency data of Parkin in early-onset showed 49 percent incidence in other countries (Lucking et al. 2000; Haylett et al. 2012) when compared with Indian population that revealed relatively lesser incidence (Pratibha et al. 2016). The detection of Parkin mutations depends on sample size, racial origin, criteria for cases and the methods used for mutation detection.

The present report analyzed Parkinsonian features in patients carrying SNCA and Parkin mutations. Although the clinical features associated with PD in all 23 cases of early onset, in which three cases revealed the presence of dementia that correlates with Hurtig et al. (2000) study where α -Synuclein inclusions are responsible for dementia and strongly linked with PD. Additionally, dementia is identified to be linked with duplication and triplication of SNCA hence it depends on the gene and protein dose (Singleton et al. 2003; Chartier-Harlin et al. 2004). These two preceding studies confirmed the association between dementia and PD.

In this study, chromosomal alterations have also been analyzed and it resulted with a deletion in chromosome 22q. In a case report with 22qDS, Parkinsonian features and symptoms seen with schizophrenia have been described with muscle stiffness in infancy and developed multiple extra-pyramidal signs when provided with antipsychotic drugs (Krahn et al. 1998). Similarly, Butcher and colleagues, reported 22q11.2 deletion syndrome in early onset PD and the epidemiological studies revealed that the usage of antipsychotic treatment may be associated with the risk of developing PD (Butcher et al. 2013). In a recent study, the deletions showed 0.49 percent frequency in patients with early-onset (<45 years) when compared with 0.04 percent frequency in those with an age of onset

above 45 years (Mok et al. 2016). Hence in the researchers' data, deletion in 22q11.2 was observed and it is a novel deletion syndrome in PD research. Physicians caring for 22qDS patients should be aware of the association with PD and therefore they should be provided with swift diagnosis and treatment.

CONCLUSION

Genetic testing is a powerful tool in families with Mendelian aggregation of disease due to the increasing number of genes related to PD. The advent of next-generation sequencing (NGS) has been implemented in diagnostic services where it can be challenging in field of genetic risk assessment. Hence, genetic testing can be conducted in a successful and multidisciplinary manner where it is supported by the expertise in this field. Genetic counseling is essential and important since it provides with information based on the onset of the disease and helps to make informed medical and personal decisions. Genetic risk assessment have been less developed especially those with early-onset of PD cases. Psychological impact and counseling can be helpful in dealing with PD hence prevention and treatment strategies should be improved since the pathogenesis of the patients with early onset in 22qDS is unknown but ultimately it may contribute to the understanding of PD etiology if elucidated.

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