Genetics of Neurodegenerative Movement Disorders in Pakistani population

By

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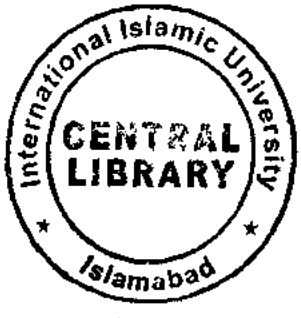
2012-2015



MS
611.01816
USG

- Biotechnology
- Pharmaceutical Chemistry
- Genetics
- Treatment

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Dated: March 20, 2015

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Mr. Muhammad Asaad Usmani and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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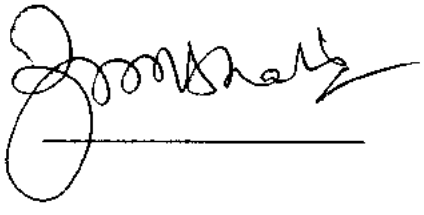

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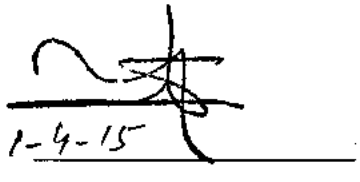

A thesis submitted to Department of Bioinformatics & Biotechnology,
International Islamic University, Islamabad

as a partial fulfilment of requirement for the award of

the degree of MS Biotechnology.

DEDICATED to

My family and all my teachers.

Especially to

My Parents, Shams-ul-Islam Usmani & Nuzhat Shamsi,

Dr. Aisha Akbar

&

My Co-supervisor Dr. Nafees Ahmad

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

Date 20-03-2015

Muhammad Asad Usmani

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ACKNOWLEDGEMENTS

All praise to Allah Almighty most Gracious and the Merciful. He honored man to his vicegerent, endued him with intellectual power and understanding, gave him spiritual insight so that he may discover himself, conquer nature and know his Creator through his wondrous sign. I bow in obeisance before my Lord for giving me strength to embark on my work and for His blessings due to which I was able to complete this work.

Sincere thanks to my supervisor Dr. Muhammad Arshad, Assistant Professor, Department of BI & BT, IIU, Islamabad for his moral support and guidance to complete this research. Words are few in honor of my worthy Co-supervisors Dr. Nafees Ahmad, Senior Scientific Officer, IBGE, Islamabad whose continuous guidance, support and help at every single step made this research to complete. I am very thankful to Dr. Rashda Abbasi and Mrs Sadia Rehman for their valuable suggestions and sympathetic attitude throughout my research.

I would express a special thanks to Dr. Wajid, Associate Professor, University of Education, Okara and my brother Dr. Umair Qamar Usmani for their kind help in field work.

Special thanks to all my friends Anwar Ullah, Sadia Saparas, Maleeha Ghafar and Madiha Sadiq for their cooperation and company during research work at IBGE.

I am very grateful to my dearest friends Dr. Arshia Maqbool, Muhammad Rizwan Riaz and Osama Raja Akbar for their moral support which encouraged me at every moment.

Words fail to express my overwhelming sense of affection and gratitude for my family who always encouraged me, support me and always pray for me.

Finally my deepest thanks to all.my teachers from school, college and universities, who made me able to come this far.

Muhammad Asad Usmani

LIST OF ABBREVATIONS

- AJ — Ashkenazi Jews
- CNS — Central Nervous System
- CT scan — Computerized axial tomography
- DBS — Deep brain stimulation
- DRD — Dopa-responsive Dystonia
- DT — Dystonia
- EFNS — European Federation of Neurological Societies
- GPi — Globus Pallidus internum
- GCH1 — ***GTP*** cyclohdrolase I gene
- MRI — Magnetic resonance imaging
- PD — Parkinson's disease
- Rpm — Revolutions per minute
- UDRS — Revised Unified Dystonia Rating scale
- UPDRS — Unified Parkinson's Disease Rating Scale

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ABSTRACT

Neurodegeneration is caused by gradual loss in neuronal cells, which result in nervous system dysfunction. Ageing and genetic polymorphisms are the two main factors for neurodegeneration. Strokes, oxidative stress, depression and many others also have role in causing loss of neuronal cells. In this study, two ND movement disorders; Parkinson's disease (PD) and Dystonia (DT) were included. In the world, PD is the 1st most common and DT is the 2nd most common neurodegenerative movement. Many genetic polymorphisms are reported causing DT and PD, Leucine-rich repeat kinase2 (*LRRK2*) gene is one of them. *LRRK2* is a large gene having 51 exons and encodes a multiple domain complex protein including ARM, ANK, LRR, ROC, COR, Kinase domain and WD40 repeats. Three polymorphs rs34637584 (G2019s), rs35870237 (I2020T) and rs34778348 (G2385R), were selected in this study.

12 families of DT and 13 triodes of PD were screened for heterozygous and minor alleles. The polymorphs rs34637584 (G2019s) and rs35870237 (I2020T) were not detected in any of the family. The SNP rs34778348 (G2385R) was detected in Pakistani Population. It is concluded that there are no evidence of existence of rs34637584 (G2019s) and rs35870237 (I2020T) in the population. In our population rs34778348 (G2385R) is present as heterozygous as well as minor allele in families affected with DT and PD. Furthermore it is suggested that large number of families should be collected and screened for other variants of LRRK2 for the better understanding of polymorphic forms of *LRRK2* in Pakistani population.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Neurodegenerative diseases are the result of the nervous system dysfunction caused by the gradual and progressive loss of neural cell (Rebecca *et al.*, 2005). Loss of selective neural cell in a particular region cause different types of disorders such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Dystonia, Huntington's disease and many others. There is no fundamental cure is available (Kim *et al.*, 2012).

Main risk factors known for neurodegenerative disorders are genetic polymorphisms and ageing, it may include other possibilities like endocrine conditions, oxidative stress, head trauma, depression, hypertension, stroke, etc. Among all these factors, some environmental factors are also included (Rebecca *et al.*, 2005).

Neurodegenerative disorders are estimated to few hundred and their classification is quiet challenging because clinically and pathologically, they overlap with one another. A disease may effects different part of brain especially in beginning, making it difficult to compare it with its final symptoms. Neurodegenerative disorders of Central Nervous System (CNS) can be classified as disorder of the brain stem, cerebellum, cerebral cortex causing abnormal behavior and basal ganglia, which results in movement disorder or the spinal cord (Przedborski *et al.*, 2003). Figure.1-1 showing the components of CNS and motor pathways

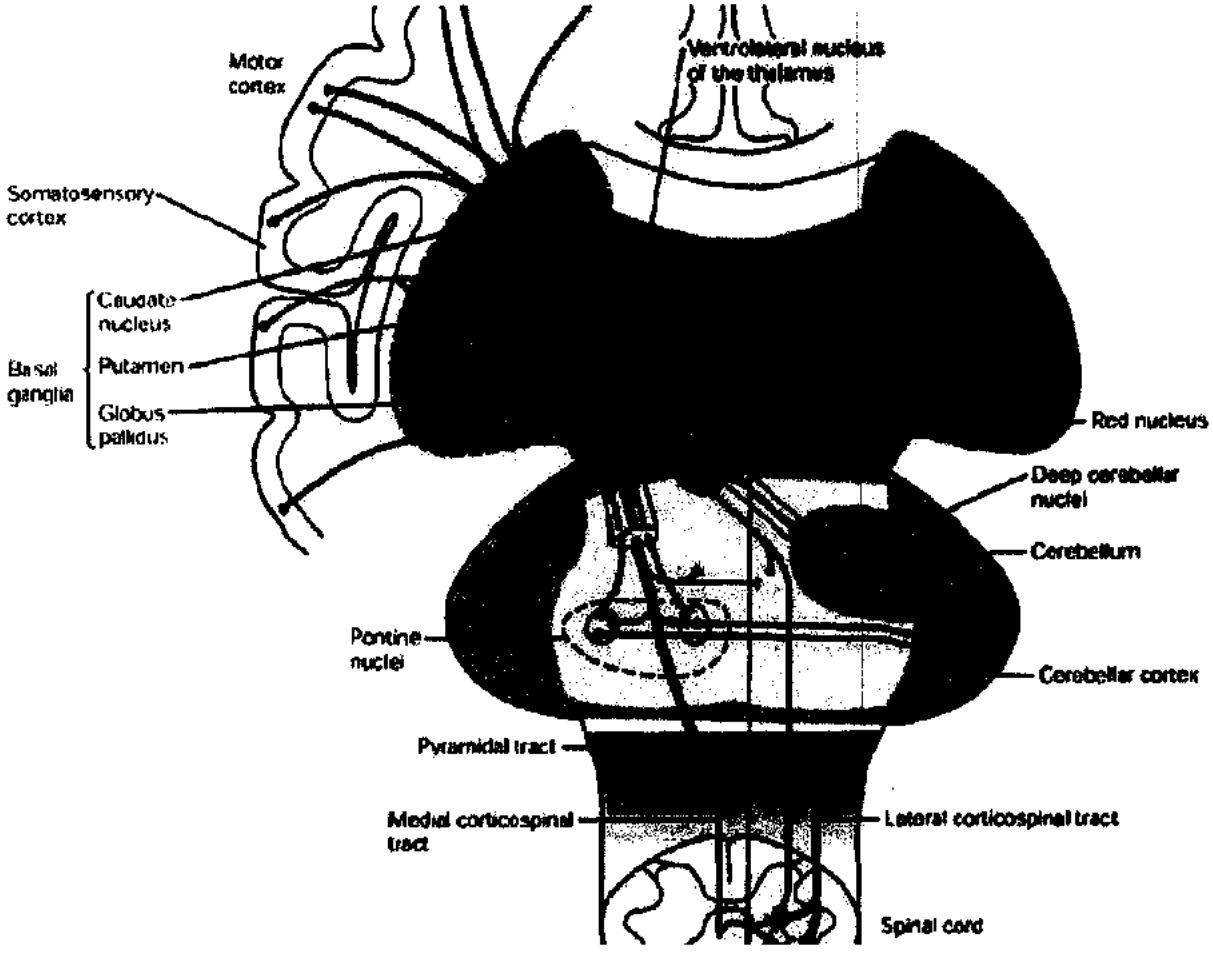

Figure 1-1: Components of Central Nervous System

Including motor cortex, basal ganglia, thalamus, cerebellum and spinal cord. Green line shows the descending projection and purple lines indicate the feedback projection (David, Neurosciences 3rd Edition).

The focus of this research was on genetic factors causing Neurodegenerative Movement Disorders. Neurodegenerative disorders are one of the most common causes of movement disability in the world. These disorders include Dystonia, Parkinson's Disease, Huntington Disease, etc. As we discussed, Movement disorders are caused by problem in Basal Ganglia including substantia nigra, globus pallidus, brain stem etc. Disease of the basal ganglia can be classified as hypo-kinetic, in which velocity and amplitude of movement is compromised or in some extreme cases non-extent, and hyperkinetic in which the normal voluntary movement face the interference of excessive abnormal movement. This study is focused on Dystonia and Parkinson's disease.

Dystonia is involuntary sustained muscle contraction, which results in repetitive movements, twisting of muscles or abnormal postures that trigger with action and abate during sleep or rest (Fahn *et al.*, 1987). In the World, Dystonia is 3rd most common movement disorder (Bressman *et al.*, 2006; Breakfeild *et al.*, 2008). It is 2nd most common Neurodegenerative disorder after Parkinson's disease. Hermann Oppenheim device "Dystonia", first time in his landmark work published nearly a century ago in 1911 in which he reported patients with involuntary muscular movement (Isador *et al.*, 1916). However, the identification and characterization of this hyperkinetic movement disorder began even earlier, in the first half of the 19th century (Pearce, 2005).

Dystonia is defined as a syndrome of involuntary sustained or intermittent muscle contractions leading to twisting or repetitive movements or abnormal postures. The Scientific Advisory Board of the Dystonia Medical Research Foundation first time proposed a formal definition of Dystonia in 1984, as *"a syndrome of sustained muscle*

contractions, frequently causing twisting and repetitive movements, or abnormal postures."(Matsumoto *et al.*, 2001)

Parkinson's disease is a progressive, neurodegenerative movement disorder. It causes disorder in motor system. Several evidences indicate that this disease has affected humanity since 2500 BC. In 1817, James Parkinson (a British physician) documented the main symptoms of the Parkinson's disease in his paper that published in 1817, what he called "the shaking palsy" (Stern, 1989). Parkinson's disease is considered as one of the most common movement disorders in the world affecting about 1% of people over the age of 60 and about 4-5% above the age of 85 (Lees *et al.*, 2009). This disease is present worldwide including Pakistan but the prevalence varies in different countries.

The striking feature of this movement disorder is the progressive degeneration of dopaminergic neurons of the substantia nigra of the midbrain. The neurons of substantia nigra produce a chemical messenger known as Dopamine for transmission of signals through nigrostriatal neurons from substantia nigra to corpus striatum that help in production of balanced, coordinated, purposeful and smooth motor activity.

Although the prevalence reports on Dystonia varied, different groups reported different prevalence rates in different regions, presently it is estimated that 220 per 100,000 people are affected by Dystonia worldwide (Stacy, 2012). The prevalence of Dystonia is repotted as 15-30 per 100,000 (Nutt *et al.*, 1988), with one study showing a prevalence rate of 732 per 100,000 in people over age of 50years (Muller *et al.*, 2002).

Parkinson Disease is second in prevalence after to Alzheimer's disease in world. About 1% population of the world with age more than 50 years are patients of Parkinson

Disease (Gibson *et al.*, 2012). The rough prevalence of Parkinsonism is approximately 1.5% of the population over 60 years of age in Pakistan. According to an estimate the number of PD patients in Pakistan is about 400,000 (AKUH, 2011).

Dystonia can be classified as primary or secondary. Primary torsion dystonia (PTD) is without an identified cause, no brain damage or any kind of injury, excepting tremor is seen, historically called as Oppenheim's Dystonia and dystonia musculorum deformans. Non-primary or secondary Dystonia is a heterogeneous group of syndromes and etiologies including acquired, inherited (with or without brain degeneration), and complex neurological disorders (Barrett and Bressman, 2011). It can also be classified on the bases of distribution in body, age of onset, and etiology as described in Table 1-1 (Fahn *et al.*, 1998).

Table 1-1: Classification of Dystonia

Sr. No.	**Classification of Dystonia**	
1	**Distribution in body**	Focal (Single Body Region)
		Multifocal
		Segmental (Contiguous regions)
		Generalized
2	**Age at on set**	Early-onset<26years
		Late-onset>26year
3	**Etiology**	Primary or Idiopathic Dystonia
		Secondary Dystonia

Classification of Dystonia on the bases of distribution in body, age of onset, and etiology.

The pathophysiology of Dystonia is not very well known at present time. It is cause by malfunction of various central neural circuits which control the movement like sensory motor cortex, basal ganglia cerebellum and interactions between these parts (Argyelan *et al.*, 2009); it is a heterogeneous condition whose pathogenesis is unknown. It is unknown that weather the change in these circuits is primary or secondary (Tarsey *et al.*, 2006).

The pathophysiology of Parkinson Disease is the progressive degeneration of dopaminergic neurons of the substantia nigra of the midbrain (Savitt et al., 2006). Patients with PD are not able to control or direct their movements in normal way due to loss of dopamine. Studies have shown that patients of Parkinson's disease have a loss of 80 percent or more of dopaminergic neurons in the substantia nigra (Davie, 2008). The reason of this death of dopaminergic neurons or impairment is unknown.

In 1994, by the finding of mutation in **GTP** cyclohdrolase **I** gene which cause **DRD** (dopa-responsive Dystonia), major step in understanding of molecular bases of movement disorders came. It was first hereditary progressive (Ichinose *et al.*, 1994) Dystonia causing gene. This disorder is a heterogeneous, Autosomal recessive form (Knappskog *et al.*, 1995). After that many genetic factors are being recognised as key player in the cause of Dystonia. These genes are involved directly or indirectly for causing Dystonia and are designated as DYT. It presents a heterogeneous form, in some cases Dystonia is the only phenotype known as primary Dystonia(DYT 1,2,4,13 etc) while in some cases Dystonia is a part of another syndrome, known as Dystonia plus

syndrome, such as parkinsonism (DYT 3, 11, 12, 15 etc) and paroxysmal forms of Dystonia (DYT 8,9,10 etc) (Ozelius *et al.,* 2011).

Mostly, Parkinson Disease cases are appeared to be sporadic but there is role of genetics in the disease, which is confirmed by identification of mutations in several genes (Martin et al., 2011). Many genes have been studied and associated with genetic form of PD (Xiong et al., 2012).

All of the known monogenic Parkinson's disease forms are autosomal dominant or autosomal recessive forms and attributed to be linked with autosomal chromosomes. (Klein and Westenberger, 2012). There are many genes, which show association with PD, some of the important genes are LRRK2, SNCA, PINK1, PARK2, and PARK7.

Till now there is no medical treatment which can give complete relief to this disorder. Dystonia can be treated by strategic management of oral medicine like anticholinergic drugs or benzodiazepines, botulinum toxin injections and with the help of physiotherapist. Deep Brain Stimulation surgery is also a good treatment for focal dystonia.

Parkinson's disease has no cure but symptoms of disease can be minimize by using medications. The most common treatment for PD is Dopaminergic Drugs. Neurosurgical treatment of PD is done with Deep brain stimulation (DBS) (Pollak *et al.*, 2002). These treatments only help to decrease the impact of disease. However, cure is not possible unless the exact causes of this neurodegenerative disease is identified

In Pakistan very little work has been done on Neurological Movement Disorders like DT and PD. Almost no data is available about dystonia in Pakistani families, very

little data is available on epidemiology, clinical presentation, and Genetic causes of Parkinsonism in Pakistani families. Therefore, efforts have to make to investigate the genetic variation in candidate genes for Dystonia and PD in Pakistan. It would be definitely helpful to find the cause and genetic risks in the families. These kinds of investigations are needed to understand the genetic variant in the populations and risk of developing the disease in lateral generations

The Current study was designed with the following objectives and aims.

- Investigation of genetic factors of DT and PD.
- Study of trends of genetic variants from Pakistani Families
- Identification of gene mutations / polymorphisms as a predictive marker or risk factor for developing DT and PD

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Prevalence

There is no published data on the prevalence of primary dystonia. Few studies based on population, are available in which survey was done by non-neurologists (Li *et al.*, 1985; Kandil *et al.*, 1994). Mostly epidemiologic studies were service-based but not on the bases of community (Nutt *et al.*, 1988; Nakashima *et al.*, 1995; Erjanti *et al.*, 1996; Duffey *et al.*, 1998; ESDE, 2000). The prevalence of primary dystonia is 15-30 per 100,000 in general population (Nutt *et al.*, 1988), in one study it was calculated as 732 per 100,000 in the general community, aged 50 and above, of Bruneck (Muller *et al.*, 2002). Focal dystonia-writer's cramp was known as occupational hazard akin to repetitive strain, before its recognition. In a book an author named it as Repetitive strain injury is its name (Lucire, 2003). The epidemics of writer's cramp were first reported among clerks of the British Civil Services, in 1830s. Sir Charles Bell described it as

"I have found the action necessary for writing gone, or the motion so irregular as to as make letters be written zig-zag, whilst the power of strongly moving the arm for fencing, remained...

"The nerves and muscle are capable of their proper functions and proper adjustment; the defect is in the imperfect exercise of the will, or in the secondary influence the brain has over the relations established in the body" (Bell, 1833)

The name scrivener's palsy (from the Latin scribere: to write) was used by Samuel Solly first time, for the writer's cramp, which he reported in 1864 (Solly, 1864).

Adult-onset focal dystonia is the most common form; about 90% of all types have a prevalence of 30 per 100,000 in general population (ESDE, 2000)

A Study was conducted in eight European countries to provide a better prevalence rate. Cases are diagnosed by Neurologists and special clinics for movement disorder. The exhaustive rate for primary dystonia was 152 per million, focal dystonia having the highest prevalence of 117 per million. The rates were higher in women than in men for focal and segmental dystonia. This study showed that prevalence for cervical DT was 57 per million as well as for blepharospasm and writer's cramp rates were 36 per million and 14 per million respectively (ESDE, 2000).

The dystonia is a group of hyperkinetic movement disorders, distinguished by involuntary sustained muscle contractions with abnormal postures, lead to twisting and repetitive movement which affect one or more sites of the body, According to an estimate, in UK, around 70000 people are affected by dystonia, which include about 8000 children and adolescents (Paudel *et al.*, 2012). In a study, conducted in USA, showed prevalence of early & late onset dystonia were 2 & 24 per million people per year respectively (Nutt et al., 1988. Prevalence studies on dystonia estimate that there are 2 to 50 cases per million for early onset and 30 to 732 cases for late onset. Cervical dystonia has prevalence of approximately 20–200 per million (Defazoi *et al.*, 2004).

The prevalence of Parkinson s' disease is all over the world including south Asia and has maximum rate of prevalence in Parsi community (Bharucha *et al.*, 1988; Moghal *et al.*, 1994). PD is second in prevalence after Alzheimer's disease among ND diseases worldwide and is found in about 1% population of the world with age more than 50years (Gibson *et al.*, 2012).

Prevalence of PD increased with increase in age and is categorized on the basis of age of onset. It is called as young onset if age is between 20-49 years, as middle onset if exist between 50-69 years and grouped as late onset if age is more than 70years. The age of onset is 50-60 years in most of the cases (Bower et al., 1999; Mehanna et al., 2014).

2.2 Classification

Classification of dystonia is not simple. It is a complex disease with remarkably variable spectrum. Dystonia is now considered as a heterogeneous disease (Vanek & Sarwar 2008). Disorder is a dynamic condition whose severity often changes with change in posture and on voluntary activity of the involved part. This changing occurrence makes the classification difficult. However, it is important to classify the disorder to provide prognostic information, genetic counseling, treatment as well as appropriate management of the disease.

The classification of dystonia can be done by clinically or on the basis of genetics. Clinically dystonia can be differentiate on one of the three conditions (i) age of onset; (ii) distribution of affected body parts; and (iii) etiology of the disease. On the bases of age, onset can be early onset mostly starts from the limb and become generalized later, it is usually associated with a known gene carrier, and late onset, which begins after 30years of age, mostly include focal dystonia. Dystonia can also be characterized as focal dystonia, segmental dystonia, generalized as well as multifocal dystonia. On the basis of third condition that is etiology, dystonia can be classified as primary or secondary dystonia (Defazio *et al*,. 2004, Vanek & Sarwar 2008, Ramdhani and Simonyan 2013). A

scheme of classification on the clinical bases recommended by European Federation of Neurological Societies is shown in Table 2-1 (Albanese *et al.*, 2011).

The classification done on the basis of genetics is referred to gene causing that syndrome or reference is given from list of loci (Charlesworth *et al.*, 2013) among which some are known and some are yet to be discover.

Table 2-1: E.F.N.S. based classification of Dystonia

Classification of dystonia, based on the European Federation of Neurological Societies current scheme		
By age at onset	Early onset (5-250 years of age)	Starts in a leg or arm and frequently progresses to involve other limbs and the trunk
	Late onset	Usually starts in the neck, cranial muscles or one arm. Remain localized with restricted progression to adjacent muscles
By distribution of affected body parts	Focal dystonia	Single body region
	Segmental dystonia	Contiguous body regions)
	Multifocal dystonia	Non-contiguous body regions
	Generalized	Both legs and at least one other body region
	Hemi-dystonia	Half of the body
By etiology	Primary dystonia	*Primary pure dystonia* Torsion dystonia is the only clinical sign. *Primary plus dystonia* Dystonia is associated with another movement disorder. Primary paroxysmal dystonia Torsion dystonia occurs in brief episodes with normalcy in between.
	Heredodegenerative dystonia	Dystonia is a feature, among other neurological signs, of a heredodegenerative disorder
	Secondary dystonia	Dystonia is a symptom of an identified neurological condition, such as a focal brain lesion, e.g. off-period dystonia in Parkinson's disease.

Classification of dystonia, based on E.F.N.S current scheme (Albanese et al., 2011)

2.3 Symptoms

Dystonia is a group of heterogeneous hyperkinetic movement disorders, characterized by torsion, involuntary sustained muscle contractions affecting one or more parts of body. Dystonia is the third most common movement disorder worldwide. (Charlesworth *et al.*, 2011).

Dystonia consists of directional movements, which are stereotyped and involve contraction of agonist and antagonist muscles. Dystonia may be affecting a single body region, known as localized, or may be in generalized form which affect multiple bogy regions. Symptoms of dystonia can be further characterized as Hemidystonia, affecting one side of the body, segmental which involves two affected contiguous body regions, or multifocal which affect two or more noncontiguous body regions (Barrett and Bressman, 2011).

Different symptoms of dystonia can be seen at different age of onset and etiology. Early onset is associated with more progressive disease (Greene *et al.,* 1995). In this age of onset, dystonia mostly starts first starts in a limb and then spreads into generalized form involving other limbs and axial muscles. Adult onset or late onset dystonia is typically focal dystonia usually begins in the arm, cranial muscles or neck it remain focal or segmental trough out life. Dystonia can also be characterized on etiology, as primary or secondary. Primary torsion dystonia or Oppenheim's dystonia is characterized as dystonia in isolation, without brain degeneration and without an identified acquired cause. Secondary or non-primary dystonia is a heterogeneous group of syndromes with complex neurological disorders and mostly is inherited (Barrett and Bressman, 2011).

Parkinson's disease is a clinical syndrome that has four principal symptoms, including bradykynesia, resting tremor, rigidity and instability of postural balance (Goldenberg, 2008). Many other non-motor symptoms whose intensity vary from patient to patient, are also present. These include emotional changes, urinary problem, depression, dysarthria, constipation, skin problems etc (Hauser 2006; Goldenberg, 2008).

There is considerable decrease in amount of dopaminergic neurons, about 4.7 to 9.8% per deca in PD patients, causing rigidity and akinetic state. This makes the patient unable to take his daily life work and unable to take care of themselves. (Gibbs, 1992; Reeve *et al.*, 2014).

2.4 Diagnoses

Dystonia is diagnosed phenotypically. The diagnosis depends on the occurrence of sustained, abnormal and repetitive postures which are with or without tremor as well as recognition of specific features, such as mirror movements, geste antagoniste or overflow movement as describe in the Table 2-2. The key feature for the diagnoses of dystonia is detailed history followed by a complete general and neurological examination. History detail should focus on the complete family history of three generations with attention to other relatives with neurological symptoms and signs, age of onset of abnormal postures of the case, with their pattern of progress and distribution in the body, specific triggers like alcohol, L-Dopa response and exposure to toxins and drugs (Vanek and Sarwar, 2008) (Charlesworth *et al.*, 2013).

On the bases of classification of dystonia it can be diagnosed as Primary dystonia or Secondary dystonia with recognition of specific phenotypic features of both types For

example sudden onset of dystonia over range of ages can be rapid-onset dystonia parkinsonism, a response to alcohol and improvement with L-Dopa support the diagnoses of myoclonus dystonia and dopa-responsive dystonia respectively (Laurie *et al.*, 2011) (Grimes *et al.*, 2002).

Table 2-2: Clinical Features of Dystonic movements

Sr. No.	Clinical feature	Detail
1	Mirror movements	Dystonic posture of affected body parts while performing motor tasks with unaffected parts
2	Geste antagoniste	Voluntary maneuver reduce the severity, for example by touching the face
3	Overflow movement	An unintentional movement of unaffected body part while performing a task with dystonic affected part

2.4.1 Primary dystonia

Following information is required for the diagnosis of primary dystonia:

- Early development a normal perinatal history.
- No history for any exposure to a drug which can cause dystonia, or any illness.
- No defects of intellectual, pyramidal, cerebellar, or sensory deficit.
- No underlying cause apparent after appropriate investigations.

(Muller *et al.*, 2002)

Different test should be conducted for the detection of primary dystonia that should starts with the analysis of metabolism by measuring serum caeruloplasmin and copper with a treatment of L-DOPA. Magnetic resonance imaging and testing for TOR1A and THAP1 mutations can also performed. If the etiology of Dystonia is unkown and occurred in early age, diagnosis of Wilson's disease or DOPA-responsive dystonia should be performed. Life-changing treatments can be performed by identification of these diseases at an early stage. (Charlesworth *et al.*, 2013). For a new form of Dystonia caused by deposition of brain manganese, a test for serum manganese measurement should be considered (Quadri *et al.*, 2012).

2.4.2 Secondary dystonia

Some important features that suggests the dystonia is non-primary dystonia are abnormal birth or perinatal history, Dysmorphia, Seizures, Hemidystonia, Sudden onset or rapidly progressive dystonia, Prominent oro-bulbar dystonia, Neurological signs

suggesting involvement of other neurological systems Signs suggesting disease outside of the nervous system and trigger further investigations (Charlesworth *et al.*, 2013). Table 2-3 gives an indication of some of the investigations that may be appropriate given the aetiology under consideration.

Table 2-3: Investigations for Diagnoses of Dystonia.

Investigation	Method	Indication/Example
Blood	Acanthocytes	Neuroacanthocytosis, neuronal brain iron accumulation
	Alpha fetoprotein	Ataxia telangiectasia
	Creatinine kinase	Neuroacanthocytosis
	Copper and caeruloplasmin	Wilson's disease, neuronal brain iron accumulation
	Lactate and pyruvate	Mitochondrial disorders
	Serum manganese	Dystonia with brain manganese deposition due to SLC30A10 mutation
	Serum ferritin	Neuroferritinopathy
	White cell enzymes	Lysosomal storage disorders
Urine	Urinary amino acids	Aminoacidaemias
	24 h urinary copper	Wilson's disease

Investigation	Method	Indication/Example
Neuroimaging	MRI	Most secondary causes, looking for structural lesions, iron/calcium deposition, caudate atropy, white matter abnormalities, etc
	Dopamine transporter (DaT) scan	Parkinsonism
Other	Nerve condition tests	Spinocerebellar ataxia, neuroacanthocytosis, metachromatic leukodystrophy
	Trial of L-DOPA	Early onset dystonia (5-30 years of age) of unknown aetiology
	Autonomic function tests	Multiple system atrophy
	Slit lamp Examination	Wilson's disease
	Liver biopsy	Wilson's disease
	Muscle biopsy	Mitochondrial disorders
	Electro-retinography	Neuronal brain iron accumulation

For neuro-imaging, generally the modality of choice is MRI, CT scan of basal ganglia is performed to distinguish iron deposition from calcium. To differentiate rapid-onset dystonia-parkinsonism or DOPA-responsive dystonia from other causes of Parkinsonism with secondary dystonia, a dopamine transporter (DaT) scan may be considered. (Romero-Lopez *et al.*, 2008) (Zanotti-Fregonara *et al.,* 2008).

Structural brain imaging studies have shown that not only the basal ganglia but also the sensory motor cortical regions and cerebellum may be instrumental in the pathophysiology of this disorder. While multimodal studies combining structural and functional imaging with clinico-behavioral evaluations, genetic analysis, and postmortem neuropathology are still rare, they have a tremendous potential to subvert the data discrepancies and provide a more coherent understanding of dystonia pathophysiology (Ramdhani and Simonyan, 2013).

The next step in the diagnoses of dystonia is that which gene is involved in the cause of particular disease type. This is also done by the phenotypic analysis of case. General strategy for the diagnoses of particular gene is explained by a strategic ideogram in Figure 2-1 (Charlesworth *et al.,* 2013).

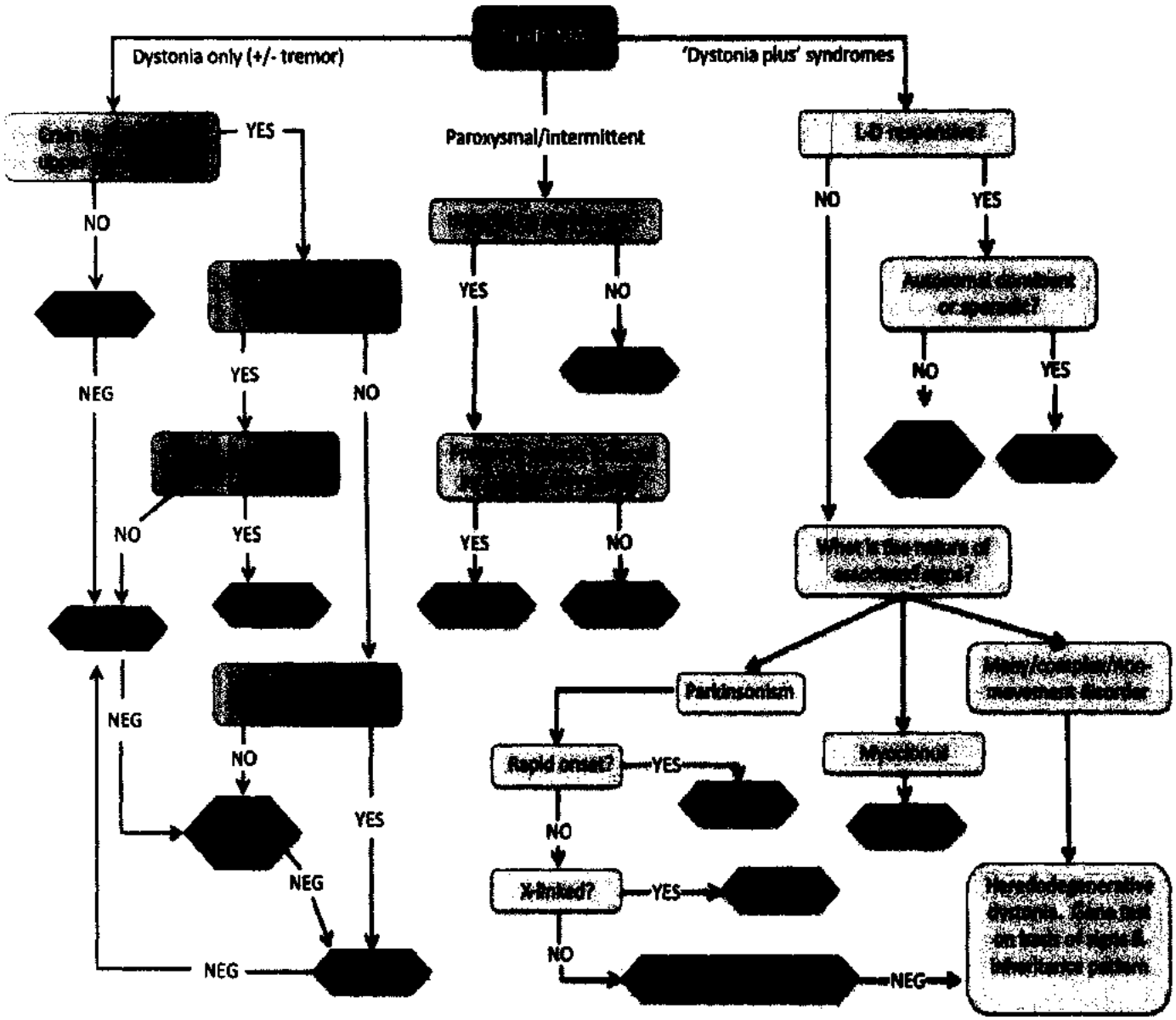

Figure 2-1: Strategy for the diagnoses of DT genes

Ideogram showing general strategy for the diagnoses of particular genes of Dystonia.

(Charlesworth *et al.*, 2013)

Parkinson s' disease is difficult to diagnose because of no proper test define for the disease. It can be diagnosed through clinical investigations which can be used to measure the severity of motor and non-motor symptoms associated with the disease. Some tests like conventional brain imaging i.e. CT scan and MRI can be helpful but are not compulsory and authentic tests (Davie, 2008). RTI of the nigrostriatal dopaminergic system is also used to diagnose the disease but is also not a valid test (Ravina, *et al.*, 2007).

2.5 Treatment

Dystonic treatment is mostly practice based. The empiric treatment is applied to improve the daily function, dystonic movements, relief from pain and psychiatric symptoms if any. Mostly dystonia is treated by oral medication, botulinum toxin injections or surgically by DBS (deep brain stimulation). There are currently no gene therapies are present but several dystonia forms have targeted causative mechanisms, whose diagnoses help in effective treatment.

In last 25 years options for the treatment of dystonia are incredibly changed. Introduction of botulinum toxin injection for focal dystonia than DBS for generalized dystonia changed the mod of dystonic treatment. Form long times treatment was relied on oral medication which was often not so effective and cause adverse effects (Ozelius *et al.*, 2011). Oral medications are still used for the treatment where muscular involvement is in excess, mostly in childhood onset and adolescent onset dystonia. Clinical phenotypes of dystonia are highly heterogeneous, because of unknown etiologies, incorrect outcome

measurements and small sample size, therefore objective data is very limited and so is the pharmacological data (Barrett and Bressman, 2011)

2.5.1 Oral Medication

A intends treatment by high-dose trihexyphenidyl for generalized primary dystonia was established by Burke and colleagues in 1986 (Burke *et al.,* 1986). Later other medications like tetrabenazine and clozapine for repetitive movements, levodopa for dopa-responsive dystonia and glutaric academia as one of the therapy for metabolic syndromes (Zimprich et al., 2001; Bernard et al., 2010). Oral medicines like baclofen, levodopa, anticholinergics and benzodiazepines are still essential treatment for primary dystonia like past decades.

Baclofen: Intrathecal baclofen is found to be better for the treatment of generalized dystonia but it is found to be more beneficial for secondary dystonia treatment especially for cerebral palsy. (Woon *et al.*, 2007). For the treatment of serve secondary dystonia, intraventricular baclofen is investigated by placing intraventricular catheters in the third ventricle by endoscopy (Albright and Ferson, 2009).

Botulinum Toxin: Previously focal dystonia was treated only by oral medication that has limited effect as well as had many adverse side effects, before the introduction of botulinum toxin (BoNT) treatment. First time blepharospasm was treated by BoNT, and Botox is approved by FDA for blepharospasm or cranial nerve VII disorders in 1989 (Simpson *et al.2008,*). It is first line of treatment for many forms of dystonia like upper focal dystonia, laryngeal dystonia, cervical dystonia (Tsui *et al.*, 1985) blepharospasm and is also frequently used for the treatment of oromandibular dystonia, hemifacial

spasm, and focal lower limb dystonia.(Simpson *et al.2008,*) it is also a choice of treatment for task specific dystonia.

Levodopa is also effectively used in Parkinson's disease patients as a precursor of dopamine to reduce the effects of the disease (Aminoff, 1994). After the duration of five to ten years the patients treated with Levodopa show lack of constancy in motor response as well as adverse effects like dyskinesia is observed in most of the cases. As the disease progresses effectiveness of Levodopa decreased (Manson *et al.*, 2012).

2.5.2 Supportive and Physical Therapies

Dystonia can be improved by managing proper medicine with some physical and occupational therapies. Braces and splints can be used to improve posture and muscle spasms. Dystonia is a chronic disorder which runs throughout life. The good therapeutic approaches should include counseling of patients and their families, genetic risk factors and psychosocial support (Vanek and Sarwar, 2008)

2.5.3 Deep Brain Stimulation (DBS) of the Globus Pallidus (GPI)

Over the past decade, deep brain stimulation, particularly involving the globus pallidus internum (GPi), has emerged as a viable surgical option for the management of dystonia and Parkinson's disease (Vanek and Sarwar, 2008; Pollak *et al.*, 2002). Primary generalized dystonia seems to respond the most to this surgery, better than secondary dystonia, although uncontrolled evidence also suggests that cervical, tardive and

pantothenate kinase-associated neurodegeneration associated dystonia may also improve. (Tarsey and Simon, 2006; Jankovic, 2006)

2.6 Genetics

Clinically it is important to recognize Genetic forms of a diseases. It provides valuable information about possible pathogenic mechanisms of the disorder. Many Genetic factors are recognized as important players in the pathogenesis of dystonia. With the advance technologies in the field of human genetic including PCR, Linkage mapping of Mendelian traits and sequencing, more than 20 monogenic loci of dystonia has been identified in last 25 years.

From a genetic point of view, hereditary dystonia can be classified either by the gene causing the condition, where it is known, or by reference to one of the ever expanding list of dystonia loci, of which there are currently. Historical designations of the DYT loci were based on phenotype or chromosomal location. (Muller, 2009).

2.6.1 Primary Torsion Dystonia (PTD)

The phenotype associated with PTD rang from early-onset generalized to adult onset focal. Loci have been defined for both types including DYT1, DYT2, DYT4, DYT6, DYT13, and DYT17 for early-onset forms (Ozelius and Bressman. 2011) and DYT7, DYT21, DYT23, DYT24 and DYT25 for later-onset forms(Norgen et al., 2011), but only 3 genes, DYT1 (Ozelius *et al.*, 1997), DYT6(Fuchs *et al.*, 2009) and DYT25,(Fuchs *et al.*, 2013) have been identified.

Early-Onset PTD

Two features of early-onset PTD were; its occurrence was familial and it was present frequently in Ashkenazi Jews (AJs). Linkage analysis in a large NJ family with early-onset disease identified. The first locus for early onset PTD i.e. DYT1 was identified in large NJ family on chromosome 9q34, which was also reported in other AJ and NJ families (Laurie *et al.*, 2011)

DYT1: Linkage analysis in a large NJ family with early-onset disease identified the first locus for Early onset PTD, DYT1 was identified in a large NJ family on chromosome 9q34 which was subsequently confirmed in other AJ and NJ families. Screening shows a 3-bp deletion in the TOR1A gene causing the removal of a single in-frame amino acid in the encoded protein, Torsin-A, in both AJ and NJ DT patients (Ozelius *et al.*, 1997). The phenotype of DYT1 dystonia can vary from severe dystonic storm to mild writer's cramp, begins in childhood or adolescence with involuntary posturing of the trunk, neck, or limbs (Nemeth, 2002).

DYT6: This locus is present on 8p11, the disease is caused by heterozygous mutation in THAP1 gene (Blanchard et al., 2011) It is an Autosomal dominant movement disorder characterized by early involvement of craniofacial muscles with secondary generalization often involving the arms, and laryngeal dystonia that causes speech difficulties (Djarmati et al., 2009).

DYT2 and DYT4: There are no chromosomal locations or genes are identified for DYT2 & DYT4. They are based on phenotype only. DYT2 is an autosomal recessive form of PTD reported in Spanish gypsy family (Gimenez *et al.*, 1988) Sephardic Jewish,

and Arab descent. DYT4 is an autosomal dominant form of PTD reported in a single large Australian family with prominent whispering dysphonia that begins in the second decade in most family members (range, 13–37 years) (Parker, 1985).

DYT13: is inherited as a dominant disorder DYT17 is an autosomal recessive trait. DYT13 was mapped to chromosome 1p36 in an Italian family with mainly segmental dystonia, prominent craniocervical involvement (Valente *et al.*, 2001). A consanguineous Lebanese family with 3 siblings was used to map DYT17 to chromosome 20p11.3 (Chouery *et al.*, 2008)

Late-Onset PTD

In Late-onset PTD the role of genes in the etiology of the adult clinical subtypes is not fully explained and it is more complex than early onset PTD. In addition individual genetic and environmental risk factors are involved, that occur in different combinations resulting in specific disease (Defazio *et al.*, 2007).

Studies based on the clinical examination of first-degree relatives of patients with focal dystonia have reported a risk of developing the same or another form of dystonia in the range of 23 to 36% (Schmidt et al., 2009).

DYT7 and DYT21: are both Autosomal dominant disorders each described in a single pedigree. DYT7 was assigned to chromosome 18p in a family from northwestern Germany with 7 affected members, all with adult-onset cervical dystonia (mean, 43 years; range, 28–70 years) (Leube *et al.,1997*) A family with 16 affected members from northern Sweden was linked to a region on chromosome 2q14.3–q21.3 and designated as DYT21. (Norgen *et al.*, 2011). The phenotype in this family is characterized by later

onset (mean, 27 years; range, 13–50 years) and mainly generalized/ multifocal PTD, with onset in the cranial/cervical muscles

DYT23 and DYT24: Both are attributed to the cause of cervical dystonia and are inherited as Autosomal dominant trait. DYT23 was reported in a large family of German origin at 9q34 location (Xiao *et al.*, 2012), show variable limitation of the neck, torticollis, and hypertrophy of the sternocleidomastoid muscles. DYT 24 is caused by heterozygous mutation in the ANO3 gene on chromosome 11p14 (Charlesworth et al., 2012), patients had jerky cervical dystonia, symptoms of blepharospasm, voice tremor, writing tremor, head tremor and arm tremor can also be seen (Munchau *et al.*, 2000).

DYT25: is caused by heterozygous mutation in the GNAL gene on chromosome 18p11. It is an Autosomal dominant neurologic disorder, reported in a German family, characterized by adult onset of focal dystonia, usually involving the neck, , progresses to involve other regions particularly the face and laryngeal muscles (Fuchs et al., 2013).

The multi-genetic inheritance, imparting a low to moderately increased risk of developing dystonia, in the result of two or more genetic variation and induce by environmental factors, may also underlie a significant proportion of the apparent heritability of late-onset dystonia.

2.6.2 Dystonia Plus

There are 6 forms of dystonia plus, 4 forms include Parkinsonism as a phenotype (DYT3, DYT5/14, DYT12, and DYT16) and 2 have myoclonus in addition (DYT11 and DYT15). All Genes have been identified for these loci except DYT15 (Muller, 2009).

Dystonia plus Parkinsonism

DYT3: It is the only form of dystonia, inherited as an X-linked trait. The disease originated from Philippines and was mapped to Xq13.1 (Nemeth *et al.*, 1999).

DYT5a/ DYT14: The disease was initially named as hereditary progressive dystonia with marked diurnal fluctuation (HSP) by Segawa and later as dopa-responsive dystonia (DRD) The inherited is Autosomal dominant trait and gene was mapped to chromosome 14q13, mutation in GCH1(GTP cyclohydrolase 1) gene was confirmed. Its phenotype include early limb-onset dystonia with worsening symptoms as the day progresses, improvement after sleep, and dramatic response to L-dopa therapy (Laurie *et al.*, 2011)

DYT5b: DRD can also be inherited as Autosomal recessive trait, assigned to chromosome 11p15. It is caused due to a homozygous mutation in tyrosine hydroxylase gene (TH)

DYT12: DYT12 is a rapid onset dystonia Parkinsonism found in large Indiana family. It is inherited as large autosomal dominant disorder in young adulthood. It is caused by mutation in gene encoding alpha-3 subunit of N, K-ATPase on chromosome 19 at 19q12-q13.2

DYT16: An Autosomal recessive form of dystonia-parkinsonism, assigned to chromosome 2q31 in families and a sporadic case from Brazil. The phenotype is characterized by early limb onset which later progress to generalized dystonia, including prominent bulbar involvement with spasmodic dysphonia, dysarthria, dysphagia and Parkinsonism limited to bradykinesia (Camargos *et al.*, 2008).

Myoclocic Dystonia

DYT11: The DYT11 locus was mapped to chromosome 7q21in a large North American family, inherited as an Autosomal dominant trait, 5 heterozygous mutations were identified in the epsilon sarcoglycan (SGCE) gene this form of dystonia is known as Myoclonic Dystonia, M-D is characterize by myoclonic jerks and dystonia that usually begins in childhood. Onset occurs earlier in girls than in boys regardless of mutation type (Kinugawa et al., 2009).

DYT15: M_D is reported, without mutations in SGCE gene suggesting genetic heterogeneity. The inheritance is Autosomal dominant, a second locus for M-D is map to chromosome 18p in a Canadian family, and it is typical alcohol-responsive M-D (Grimes *et al.*, 2002).

2.6.3 Paroxysmal Dystonia/Dyskinesia

This is a heterogeneous disorders characterized by sudden transient attacks of involuntary movements. They are subdivided into kinesigenic (DYT10 and DYT19), non-kinesigenic (DYT8 and DYT20), and exercise-induced forms (DYT9 and DYT18). The genes for DYT8 and DYT18 have been identified (Muller *et al.*, 2009).

Paroxysmal non-kinesigenic dyskinesia

DYT8: Also known as PNKD1, is inherited as an Autosomal dominant trait, was localized to chromosome 2q33–36 in 2 families in a 5-generation Italian family and a large Polish-American family. Three miss-sense mutations A7V, A9V and A33P were reported in the myofribillogenesis regulator (MR-1). The phenotype of PNKD is attacks

of dystonia, chorea, ballismus, or athetosis in episodes that remain from minutes to hour and with a frequency of once per day to once or twice in a year (Ghezzi et al., 2009).

DYT20: This locus was assigned to chromosome 2q31 in a Canadian family, designates a second form of paroxysmal non-kinesigenic dyskinesia (PNKD2) (Spacey et al., 2006), just proximal to the DYT8/MR-1 gene. No mutations were detected in the MR-1 gene, and thus a new PNKD locus was described. The clinical symptoms in this family are very similar to those described in the DYT8/MR-1 families.

Paroxysmal kinesigenic dyskinesia

DYT 10: In Japanese families, using linkage analysis locus was mapped on chromosome 16p11.2–q12.1, showing Autosomal dominant inheritance of PKD. It is also confirmed in African American family. The disease is characterized by short (secs to mins) and frequent (up to 100 times per day) attacks of dystonic or choreiform movements with sudden unexpected movements (Chen et al., 2011).

DYT19: was assigned to chromosome 16q13–q22.1 in an Indian family, for paroxysmal kinesigenic dyskinesia (PKD2). Attacks could last up to 2 minutes, occurred with a frequency of 1–20 per day, and produced dystonic or choreic movements in response to sudden movements (Valente et al., 2000).

Paroxysmal exercise-induced dyskinesia

DYT9: This locus was mapped on chromosome 1p21–p13.3 in a large German family, with Autosomal dominant inheritance and symptoms included episodes (20minutes and twice a day or year) of involuntary dystonia of the limbs, double vision, episodic ataxia, paroxysmal choreoathetosis and dyskinesia (Weber et al., 2011).

DYT18: Paroxysmal exercise-induced dyskinesia (PED) is caused by heterozygous mutation in the SLC2A1 gene which encodes the GLUT1 transporter, on chromosome 1p34, shows an Autosomal dominant inheritance. PED is characterized by exercise-induced attacks of dystonic, choreoathetotic, and ballistic movements affecting the exercised limbs that last from a few minutes to an hour (Brockmann, 2009).

In many forms of dystonia, there is a significant genetic contribution. In early onset dystonia Monogenic inheritance is seen most often but reduced penetrance of some of genes means that many apparently sporadic cases may also fall into this category. Furthermore, negative genetic testing of familial dystonia does not prove that it is not genetic because number of genes responsible for familial dystonia remains to be discovered (Charlesworth *et al.*, 2013). All the known genes as well as assumed loci are shown in the Table 2-4

Table 2-4: Types of Dystonia and their related Genes/Loci.

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
1 Primary Torsion Dystonia 1.1 Early onset PTD	DYT1	9q34	128100	Autosomal Dominant Trait	13y	Serve dystonic storm to writer's cramp	Ashkenazi Jews (80%) & Non-Jewish (50%)	TOR1A	3 bp GAG deletion and 2 missense mutation
	DYT6	8p21-22	602629	Autosomal Dominant Trait	16y (5y-62y)	Onset in arms 50% followed by neck 25% & Cranial muscles 25%, Rarely in legs 4%	Amish-Mennonite families(60%), Europ. , Chinese & Brazilian	THAP1	Insertion and deletion in THAP1, 45 heterozygous mutations & 2 homozygous missense mutations
	DYT2	Un-known	224500	Autosomal Recessive Trait		Early limb onset to generalized Dystonia	Spanish Gypsy, Sephardic Jews & Arabs		

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
	DYT4	Un-known	128101	Autosomal Dominant Trait	13y-37y	Whispering Dysphonia	Single large Australian family		
	DYT13	1p36	607671	Autosomal Dominant Trait		Segmental DT, Prominent carniocervical involvement	Italian family		
	DYT17	20p11.22-q13.12	612406	Autosomal Recessive Trait	17year 14y-19y	Onset in neck, progress to segmental or generalized DT	2008, Lebanese family		

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
1.2 Late-Onset PTD	DYT7	18p	602124	Autosomal Dominant Trait	43year (28y-70y)	Cervical dystonia, brachial and cranial involvement.	1996, northwestern German family		
	DYT21	2q14.3-q21.3	614588	Autosomal Dominant Trait	27 year (13y-50y)	Generalized / Multifocal PTD	Northern Swede family		
	DYT23	9q34	614860	Autosomal Dominant Trait	40y-60y	Variable limitation of neck, torticollis, & hypertrophy of sternocleidomastoid muscles	1993, German origin	CIZ1	

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
	DYT24	11p14	610110	Autosomal Dominant Trait	29 year (19y-40y)	Focal dystonia affecting the neck, laryngeal upper limbs muscles	2000, British family	ANO3	May be heterozygous mutation in ANO3 involved, but not confirm
	DYT25	18p11	615073	Autosomal Dominant Trait	28.4year (7y-50y)	Focal DT involving neck, progressed to other region face laryngeal muscles	1994, German Ancet., African American family	GNAL	2 heterozygous mutation
2 Dystonia Plus Syndrome									
2.1 Parkinsonism	DYT3	Xq31.1	314250	X-linked Inheritance	35 year (y12-52y)	Start as focal dystonia, progress to multifocal or generalized, 50% develop parkinson	Philippines	TAF1	48-bp deletion in exon, 5, single b-pair changes in TAF1 gene, and a retrotransposon insertion in an intron of TAF1

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
	DYT5 / DYT14 .	128230	14q13	Autosomal Dominant Trait (Rarely recessive)	6 year	Childhood limb-onset, improve after sleep, High response to low-dose levodopa, Parkinsonism, Hyper flexia	Japan	GCH1	100 mutations of all types (nonsense, missense, splicing, promoter region, and large genomic deletions)
	DYT12	128235	19q13	Autosomal Dominant Trait	Early 20s (4y-58y)	Rapid-onset dystonia, parkinsonism		ATP1A3	6 hetrogeneuos mutation, 10 novel mutation(8 missense, a 3bp inform insertion & a 3bp inform deletion)
	DYT16	612067	2q31	Autosomal recessive Trait	2y-18y	Early limb onset to generalized DT, Parkinsonism limited to bradykinesia	Brazil & Germany	PRKRA	Missense mutation (P222L)

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
2.2 Myoclonus Dystonia	DYT11	159900	7q21	Autosomal Dominant Trait	Early onset	Myoclonus jerks & DT begin in childhood	North American, Germany	SGCE	5 heterozygous mutations in Germany, 50 mutations of all types in more than 100 probands
	DYT15	607488	18p11	Autosomal Dominant Trait	9.6year (7y-15y)	alcohol-responsive M-D	Canadian family		
3 Paroxysmal Dystonia 3.1Exercise-induced forms	DYT9	601042	1p21-p13	Autosomal Dominant Trait	2y-15y	Involuntary DT of the limbs, for 20 min, dysarthria episodic ataxia	German family		

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
3.2 Kinesigenic Dystonia	DYT18	612126	1p35-31	Autosomal Dominant Trait	Early-onset	exercise-induced attacks of dystnic choreoathetotic, &ballistic movements	Belgian family	SLC2A1	Heterozygous mutation
	DYT10	128200	16p11.2-q12.1	Autosomal Dominant Trait	Adolescence-onset	Short (sec-mins), frequent (up to 100 day) attacks of dystonic or choreiform, sudden unexpected mov..	Japanese family, African American family		
	DYT19	611031	16q13-q22.1	Autosomal Dominant Trait	7y-13y	2 Minutes attach, 1–20/dy, dystonic or choreic movements in response to sudden movemnt.	2000, Indian		

Dystonia Type	DYT Designation	Chromosomal Position	OMIM Number	Inheritance	Mean Age at Onset	Phenotype	Ethnicity / Origin with penetrance	Gene	Mutation
3.3 Non-Kinesigenic Dystonia	DYT8	118800	2q33-q36	Autosomal Dominant Trait	Adolescence-onset	Attacks of DT, chorea, ballismus, provoked by alcohol or caffeine, Episodes remain 1min-hour	Italy, Polish-American Family	MR-1	3 missense mutation (A7V. A9V, A33P)
	DYT20	611147	2q31	Autosomal Dominant Trait	Childhood-50y	Symptoms similar DYT8/MR-1 families	2006, Canadian		

DYT22 is not designate to any of the dystonic form. It is undescribed form of dystonia designation may have been reserved

(Dystonia overview, NCBI Bookshelf)

Parkinson's disease mostly is a multifactorial disorder caused by combination of environmental and genetic factors (Schulte and Gaseer 2011). The genes causing Parkinsonism can be divided into several groups. Autosomal dominant form which resemble with the sporadic disease, recessive form which shows early age of onset with pure-parkinsonism and others group which exhibit additional clinical feature with Parkinsonism. Approximately 10%-20% PD patients have family history (Farrer, 2006). There are numbers of genes linked with PD and their list is updated continuously on PD gene database (http://www.pdgene.org).

Monogenic forms of PD are identified through linkage analysis of large families in which affected and unaffected individuals are used (Bras and Singelton 2011). Till now more than 500 genetic mutations / variants have been identified which show biological relatedness with the pathogenicity of PD (Nuytemans *et al.,* 2010). These genetic variants only include 18% copy number variants remaining 82% are simple mutations like missense, silent and nonsense mutations.

The PD genomics start with the discovery of a missense mutation in SNCA gene in 1997. After the discovery many other genes were discovered through Linkage mapping techniques (Schulte and Gaseer 2011). List of most important genes related to Parkinsonism with their inheritance and position is shown in Table 2-5.

Table 2-5: List of Genes causing Parkinson's disease.

Locus	Gene	Chromosomal Location	Inheritance	Type of Parkinsonism
PARK1/PARK4	SNCA	4q12	Autosomal Dominant + risk	Early onset/ Late onset, Dementia
PARK2	Parkin	6q25-q27	Autosomal Recessive	Early onset
PARK3	Unknown	2p13	Autosomal Dominant	Late onset
PARK5	UCHLI	4p14	Autosomal Dominant	Late onset
PARK6	PINK1	1p36	Autosomal Recessive	Early onset
PARK7	DJ1	1p36	Autosomal Recessive	Early onset
PARK8	LRRK2	12q12	Autosomal Dominant + risk	Late onset
PARK9	ATP13 A2	1p36	Autosomal Recessive	Early onset, Kufor-Rakeb syndrome
PARK10	Unknown	1p32	Unknown	Late onset
PARK11	GIGYF2	2q37	Autosomal Dominant	Late onset
PARK12	Unknown	Xq21-25	X-Linked	Late onset
PARK13	HTRA2	2p12	Autosomal Dominant	Late onset
PARK14	PLA2G6	22q13	Autosomal Recessive	Early onset

Locus	Gene	Chromosomal Location	Inheritance	Type of Parkinsonism
PARK15	FBXO7	22q12-13	Autosomal Recessive	Early onset
PARK16	Unknown	1q32	Risk	Late onset
PARK17	GAK	4p16	Risk	Late onset
PARK18	HLA	6p21	Risk	Late onset
------	EIF4GI	I3q27	Autosomal Dominant	Late onset
------	GBA	1q21	Risk	Late onset
-----	MAPT	17q21	Risk	Late onset
-----	BST1	4p15	Risk	Late onset

Important genes/loci related to Parkinson disease, their mode of inheritance and associated clinical features (Schulte and Gaseer 2011).

LRRK2:

The *LRRK2* is a gene present on chromosome 12q12 as shown in Figure 2-2. The heterozygous mutation in this gene result into autosomal dominant Parkinson's disease. It is a large gene of 51 exon and encodes a protein called Dardarin.

Dardarin protein belong to ROCCO protein family. *LRRK2* is a large and complex multi-domain protein consists of 2527 amino acids. Multi-domain of *LRRK2* protein are Armadillo repeats (ARM), Ankyrin repeat (ANK), Leucine rich repeats (LRR), a Ras of complex (ROC), a C terminal of Roc (COR), kinase domain and WD40 repeats as shown in Figure 2-3. Most of the PD mutations are present in central core of the protein, one is present in LRR, two are found in ROC domain, one is COR and two are present in kinase domain (Cookson 2010; Cookson and Bandmann 2010).

LRRK2 is of the most common gene causing familial PD. Its frequency in family cases is 5% to 15% and is 1% in sporadic cases (Berg *et al.*, 2005; Fonzo *et al.*, 2006; Latourelle *et al.*, 2008). *LRRK2* may play a major role in pathogenesis of many important ND disorders which are associated with PD (Zimprich *et al.*, 2004). This cause advancement in understanding and treatment of familial PD (Gilks, 2005).

Mutation in *LRRK2* gene encoding the LRR protein (leucine rich repeat protein 2) are the most frequent cause of PD known to date (Kruger R., 2008). Several interaction pattern and potential mediated pathways of *LRRK2* have been identified but cellular function *LRRK2* and progression of PD caused by *LRRK2* remain unknown. Mutation in the *LRRK2* domain (ARM, ANK, LRR & WD40 repeats) containing many protein-protein interaction play a role in pathogenesis ofPD. These domains are involved in

assembly of signaling complex and mutation in these can affect the binding of protein and cause pathogenic effect by the composition of these signaling protein complex. Mutation in ROC and COR domain have catalytic effect and decrease GTPASE, while mutation in kinase domain causes gain or loss of kinase activity (Gomez *et al.*, 2012).

LRRK2 gene frequently is inherited as autosomal dominant mutation in late onset PD. The clinical and pathological; features of *LRRK2* associated PD cannot be differentiated from idiopathic late onset PD (Goldenberg, 2008). Study of *LRRK2* gene is important as it is hot candidate gene of familial PD (Berg *et al.*, 2005; Fonzo *et al.*, 2006; Latourelle *et al.*, 2008; Fonzo et al., 2005; Mata *et al.*, 2006). Mutation in *LRRK2* gene can be identified by using genome wide association studies, candidate gene sequencing and high resolution recombination (Zimprich *et al.*, 2004; Lesage and Brice, 2012).

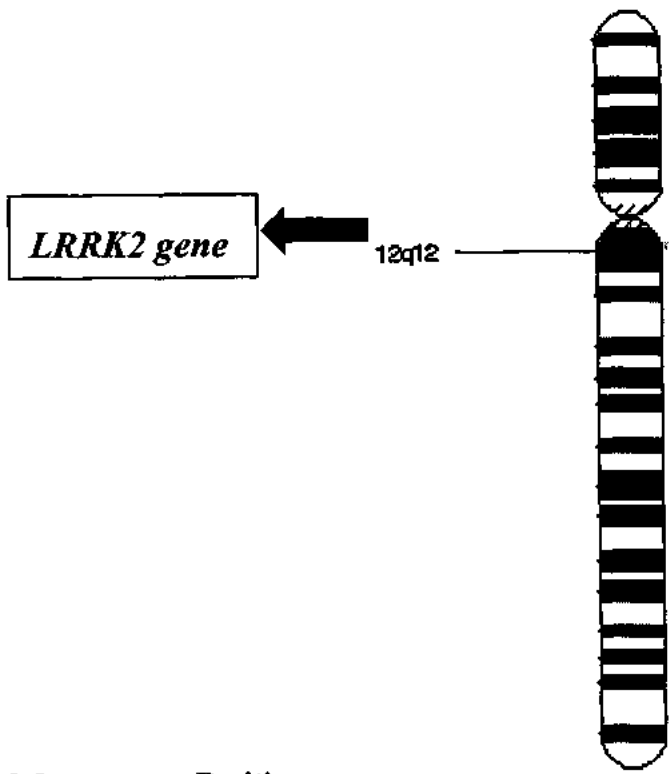

Figure 2-2: Position of LRRK2 gene on chromosome 12.

(http://ghr.nlm.nih.gov/gene/*LRRK2*)

CHAPTER 3

MATERIAL AND METHODS

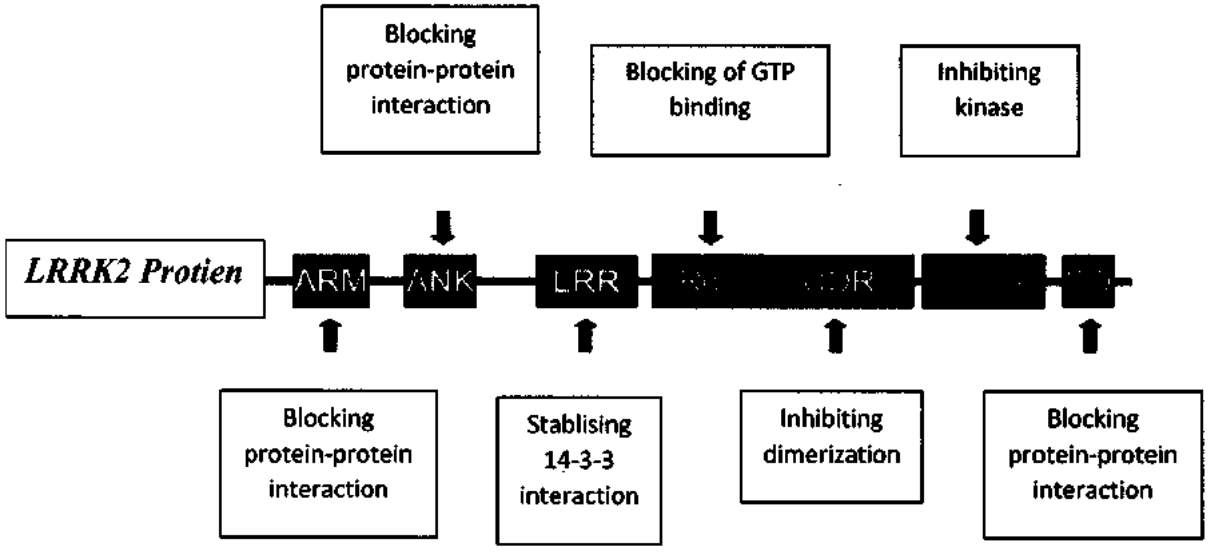

Figure 2-3: Domain topology of human LRRK2

Showing the different domains of the protein and disorders caused by the mutation in the respective domain (Ho *et al.*, 2014)

3. MATERIAL AND METHODS

3.1 Collection of families

Twelve families of dystonia and thirteen nuclear families of Parkinson's disease were collected from different regions of Punjab and Khyber Pakhtunkhwa. The families were collected on personal contact basis and detailed disease histories as well as their mode of inheritance were ascertained by visiting these families at their residential places. All families were informed about the study procedure and written consent forms were obtained from the study participants (Appendix (i)). The study was approved by Institutional ethics committee of Institute of Biomedical and Genetic Engineering (IBGE), Islamabad and was in concordance to Helsinki Declaration "Declaration of Helsinki is a set of ethical principles regarding human experimentation developed for the medical community by the World Medical Association (WMA)".

3.2 Blood Sampling and Pedigree drawing

Blood samples of affected as well as normal individuals of families were collected in 8ml Vaccutainer tubes (Becton Dickinson (BD), Franklin Lakes NJ, USA) containing ACD (acid citrate dextrose) as an anti-coagulant. The collected blood samples were stored at 4 °C.

The family history and mode of inheritance was studied by pedigree analysis. Pedigrees of all families were drawn using Cyrillic Program (http//:www.cherwell.com).

This program gives a graphical output as well as provides "Buttons" for different indications and information for the particular individual. Blank circles and squares represent the normal female and male respectively while filled circles and squares represent affected females and males. A horizontal line between a square and circle indicates a non-consanguineous marriage while a double line represents the consanguineous marriage in the family. A vertical line down from the marriage line shows the offspring. A diagonal line across a square or a circle indicates a dead member. An arrow on a particular individual is used to indicate the proband.

3.3 Genomic DNA Isolation

The Genomic DNA was extracted from the collected blood samples through organic method (Pikor *et al.*, 2011). In organic method, the DNA is extracted by phenol-chloroform extraction. It is a liquid-liquid base DNA extraction method that separates mixtures of molecules based on the differential solubility of the individual molecules in two different immiscible liquids. This method is widely used to separate DNA, RNA and proteins.

In this method, organic solvents are used to separate out DNA from other contaminants by liquid-liquid separation. All the contaminants separate out in the organic phase of the two immiscible liquid and the Nucleic acid move in the saline environment. Therefore, correct salt concentration and pH must be used during extraction to ensure that contaminants are separated into the organic phase and that DNA remains in the aqueous phase.

The separation of DNA from cellular components can be divided into three stages:

1. Disruption & Lysis
2. Removal of proteins and contaminants
3. Recovery of DNA

In this method first the disruption and Cell lysis was performed. First of all blood was transferred from vaccutainers to labeled 50ml tubes (Biologix Research Company, U.S.A) than three times cell lysis buffer (KHCO3, NH4Cl, and EDTA) was added in the blood. The cells were ruptured by osmotic shock method. The mixture was centrifuged for 10mins at 1200rpm and centrifuged at 4 °C (Mistral 3000i Centrifuge, Fisons, UK). The supernatant was discarded in glass beaker containing bleach so that the entire infectious agent could be killed; pellet was re-suspended in cell-lysis buffer and centrifuged as previously. The step was repeated until the red pellet turns white.

The whole pellet was re-suspended in STE buffer. It provided the saline environment to lysate mixture. After this 250µl of 10% SDS (sodium-dodecyl-sulphate) was added to denature the proteins and Protinease K was added to digest the linear strands in the amino acids. The samples were then placed in 55 °C water bath (Orbit Shaker Bath, Lab-Line, USA) for over-night.

Next day, 5ml equilibrated Phenol (pH 8.0) was added in all samples, the samples were then shake for 10minutes, and placed on ice for 10 minutes. The samples were than centrifuged at 3200*rpm* for 30minutes at 4 °C; the mixture separated out in two layers, the upper aqueous layer and the lower organic i.e. phenol layer. All the contaminants and

protein, shifted in the phenol layer due to their high molecular weight and the nucleic acid remained in the aqueous layer. The aqueous layer was then removed in new labeled 15ml tube (Biologix Research Company, USA) by cut tips (so that genomic DNA do not denature). After transfer of aqueous layer 5ml Chloroform:Isoamyl-Alcohol in 24:1 was added. The sample were again shacked for 10minutes than placed on ice for 10minutes and centrifuged at 3200rpm for 30 minutes and at 4 °C. The mixture separated out in two layers, upper aqueous layer was removed in new tubes. RNase A was added in all samples and placed in 37 °C water shaker bath for 2 hours. After 2 hour incubation 250µl of 10% SDS and Proteinase K was added, so that it will degenerate the RNase A and remaining proteins as well and placed in 55 °C water-bath shaker for 1 hour.

After one hour incubation samples were treated with Phenol and Chloroform:Isoamyl-Alcohol as previously. DNA was precipitated out by adding 500µl 10M ammonium acetate and 5ml chilled Iso-propanol. The samples were shacked until the DNA became visible as white threads. Samples were then placed at-20 °C overnight so that maximum amount of DNA could be precipitated.

The third and final day was the washing of the samples. First, tubes were centrifuged at 3200rpm for 1hour and at 4 °C so that DNA could be palette out, supernatant was discarded and the pellet was re-suspended. Than sample were washed with 5ml of 70% Ethanol, dried and re-suspended in 150µl-300µl of 10mM Tris-Cl. The samples were tapped for several hours so that the entire DNA re-suspends equally in the 10mM Tris-Cl. After re-suspension the DNA was transferred in labeled 1.5ml tubes;

optical density was taken by spectrophotometer (Nanodrop 2000C, Thermo-Scientific, USA), concentration of each sample was note down and samples were stored at -20 °C.

For the amplification of DNA by Polymerase Chain Reaction, 100ng/µl dilutions were made and also stored at -20 °C for further use.

3.4 Linkage Analysis

Linkage Analysis was done to trace the genetic defects in selected families. Initial step was to "link" the gene or locus to already known gene or locus. Samples were screened for the linkage of most hot candidate regions of the gene involved in neurodegenerative disorders (*LRRK2* gene) by using microsatellite markers around these genes. The detail of microsatellite markers is shown in Table 3-1.

3.4.1 PCR for Linkage analysis

PCR was performed in total volume of 20µl containing 5ng of genomic DNA, by using Thermal Cycler (PxE 0.2, Thermo electron Corporation). First, initial denaturing of the double stranded DNA was done at 94 °C for 5min, than at stage 2 cyclic denaturing at 93 °C for 30sec was done followed by annealing and cyclic extension at 58 °C for 30sec and 72 °C for 30sec respectively. The stage 2 was repeated for 30times. At stage three final extension was done for 5min at 72 °C. The details about the cyclic condition is shown in Figure 3-1.

Table 3-1: List of Markers used for Linkage Analysis

Gene Region	Locus	STR Markers	Genomic Position	Approx. Size
LRRK2 gene	12q12	D12S2194	55.5cm	20bp
LRRK2 gene	12q12	D12S87	53.32cm	20bp
LRRK2 gene	12q12	D12S1048	56.52cm	20pb
DYT12	19	D19S587	59cm	25pb
DYT12	19	D19S900	70.16cm	23bp
DYT1	9q34	D9S63		20bp
DYT1	9q34	D9S64		22pb

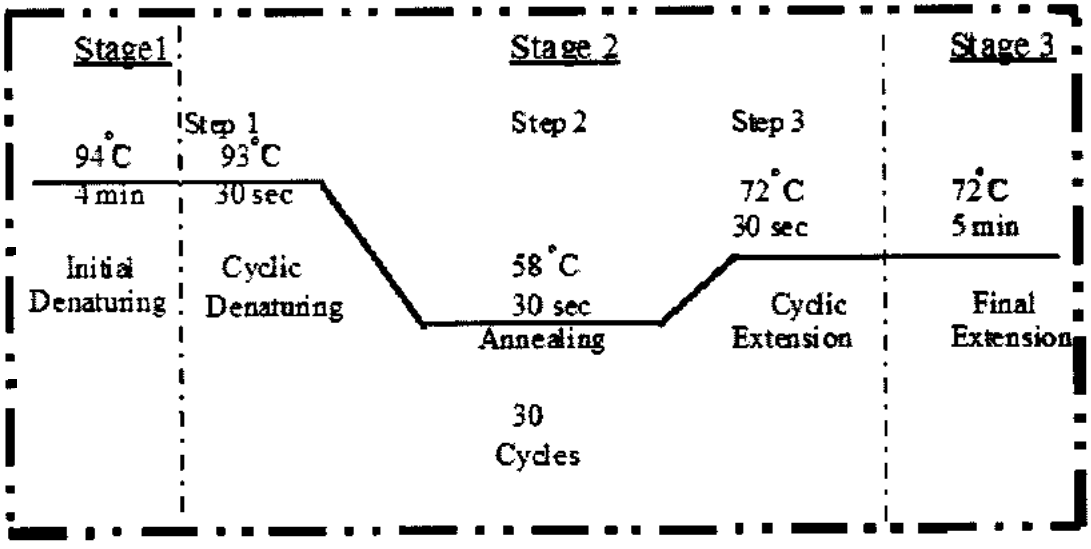

Figure 3-1: Cyclic conditions for Linkage Analysis PCR:

For amplifying DNA template 30cycles were performed. Initial denaturation was performed at 94 °C. In a cycle denaturing, annealing and extension was done at 93 °C, 58 °C and 72 °C respectively.

3.4.2 Polyacrylamide Gel Electrophoresis

8% PAGE was prepared (containing acrylamide; bis-acrylamide; ammonium per sulfate; TEMED) and left overnight for polymerization. Next day gel was transferred to Gel tank (sequi-Gen® GT sequencing cell, Bio-Rad, USA). 10µl of PCR products containing 4µl of gel loading dye was loaded in the wells of acrylamide gel. Loading dye contains "Xylene Cyanol" which moves at 165bp and "Bromophenol Blue" which runs at 45bp in the gel. In first well 3µl of 100 bp DNA Ladder (GeneRuler, ThermoScientific INC., USA) was loaded for size estimation of the amplified PCR product. Electrophoresis was performed at 100-watt current using Power supply (P200B, Sigma Chemical Company, St. Louis, USA).

The gel was stained in ethidium bromide solution for 2min, visualized under UV trans-illuminator and photographed using Gel Documentation System (UVITECH, Cambridge, UK).

3.4 Genotyping

3.4.1 Primer Designing

Primer is a single strand chain of nucleic acids from which DNA synthesis starts, usually of 18-24 nucleotides long chain, which binds with DNA at specific region. Primers were designed to amplify the specific region of *LRRK2* gene. DNA sequence of *LRRK2* gene (Gene ID: 120892) was retrieved from "Ensemble" (http://www.ensemble.org). Three single nucleotide polymorphism of *LRRK2;* rs34637584 (G2019s), rs35870237 (I2020T)

and rs34778348 (G2385R), were selected in this study and their primers were designed by using online primer designing tool "Primer3" (http://primer3.ut.ee/). These primers were designed for allele specificity. The details of sequences, annealing temperatire and size of their products are shown in Table 3-2, 3-3 and 3-4 for rs34637584 (G2019s), rs35870237 (I2020T) and rs34778348 (G2385R) respectively. These primers were used for ASP (Allele specific PCR).

Table 3-2: Primer sequence for rs34778348

No.	Primer ID	Sequence	Tm (°C)	Band type	Product Size (bp)
1	LK2- 48CF	TATGCAGCTTTCAGTGATTCC	62°	Control	442
2	LK2- 48CR	TCAAAGCTAGCAGAGACTCCA			
3	LK2- 48GF	GGATAAGAAAACTGAAAAACTCTCTG	62°	Wild	350
4	LK2- 48CR	TCAAAGCTAGCAGAGACTCCA			
5	LK2- 48CF	TATGCAGCTTTCAGTGATTCC	62°	Minor	138
6	LK2- 48AR	GTGCACGCAGTCTATTAGCCT			

Table 3-3: Primer sequence for rs34637584

No.	Primer ID	Sequence	Tm (°C)	Band type	Product Size (bp)
1	LK2- 84CF	GCAGATACCTCCACTCAGCC	62°	Control	736
2	LK2- 84CR	TCAGTAGGGTCACGGCCA			
3	LK2- 84CF	GCAGATACCTCCACTCAGCC	62°	Wild	133
4	LK2- 84GR	CTACAGCAGTACTGAGCAATGCC			
5	LK2- 84AF	TGCAAAGATTGCTGACTCCA	62°	Minor	645
6	LK2- 84CR	TCAGTAGGGTCACGGCCA			

Table 3-4: Primer sequence for rs35870237

No.	Primer ID	Sequence	Tm (°C)	Band type	Product Size (bp)
1	LK2-37 CF	GCAGATACCTCCACTCAGCC	64°	Control	736
2	LK2-37 CR	TCAGTAGGGTCACGGCCA			
3	LK2-37 TF	GCAAAGATTGCTGACTACGCAT	64°	Wild	644
4	LK2-37 CR	TCAGTAGGGTCACGGCCA			
5	LK2-37 CF	GCAGATACCTCCACTCAGCC	64°	Minor	139
6	LK2-37 GR	CCCATTCTACAGCAGTACTGAGAAG			

3.4.2 Allele specific polymerase chain reaction

Allele specific PCR (ASP), also known as amplification refractory mutation system (ARMS) was introduced by Newton *et al.*, in 1989. It comprise of two PCRs, each containing one allele specific primer to amplify that particular allele in the reaction. Further, control primers are also used to amplify any DNA region in the template and are meant to make sure that there is nothing wrong in the reaction and PCR setup. The specificity of allele specific primer is maintained by 3' nucleotide, which is complementary to one of the allele but not for the second allele. ARMS is very efficient and economical method for the detection of known mutations. No further enzymatic manipulation or hybridization is required

3.4.3 ARMS Reaction

For the amplification of our desired genomic regions for *LRRK2* gene, APS was performed in 20µl reaction volume in two reactions; each containing one of the allele specific primer and the respective 2nd primer (forward or reverse) to amplify that particular allele. Therefore, for each sample two PCR reactions were performed to determine the genotype of sample. Each PCR reaction contained 0.75µM of each primer, 1.0U of *Taq* DNA polymerase, 125 µM each of the dNTP, 1X PCR reaction buffer and 100ng of genomic DNA. The details of reagents used in reactions are shown in Table 3-5. The annealing temperature for the reaction was 62℃. The details of cyclic conditions are shown in Figure 3-2.

Table 3-5: Details of reagents for ARMS

Sr. No	Reagents	Stock Conc.	Final Conc/Rxn	Vol(µl)/Rxn
1	*Taq* buffer	10X	1X	2
2	$MgCl_2$	50mM	1.5mM	0.6
3	dNTPs	2.5mM	125µM	1
4	Forward Control Primer	10µM	0.75µM	1.5
5	Reverse Control Primer	10µM	0.75µM	1.5
6	*Taq* DNA Polymerase	5U	0.0625U	0.25
7	Genomic DNA	100ng	--	1
8	dH_2O	--	--	12.15
	Total Volume	--	--	20

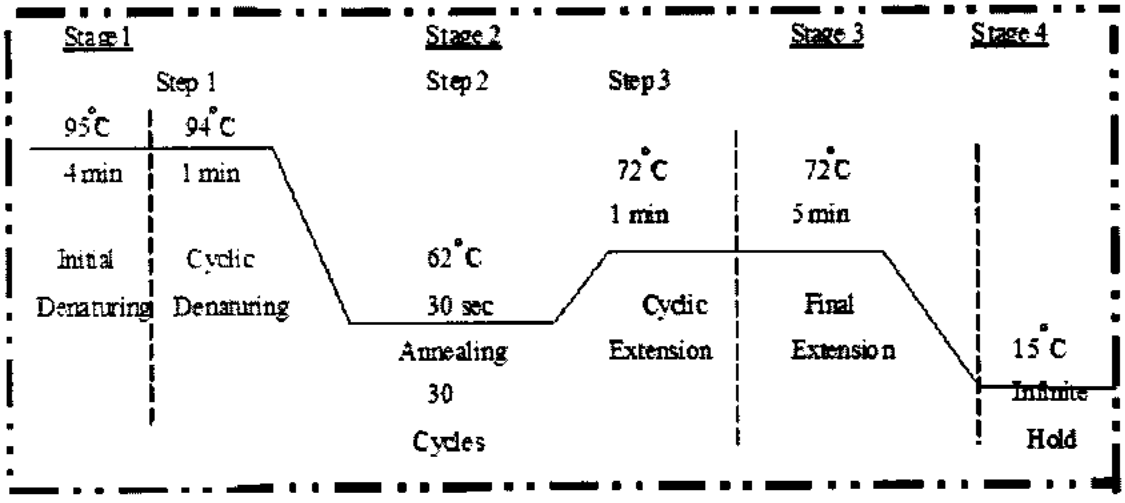

Figure 3-2: Cyclic conditions for ARMS PCR

Total volume of 20µl reaction was amplified. The annealing stage was at 62 °C. Total 30 cycles were repeated for "stage 2".

3.4.3 Agarose gel electrophoresis

After the amplification of ARMS the product was electrophoresed on 2% agarose gel containing 0.02µg/ml ethidium bromide as final concentration, for visualization. 10µl of PCR product was mixed with 2µl of 6X gel loading dye and loaded on the agarose gel. The gel was run at 120volts for 35 min on electrophoresis Gel System (Maxicell EC 360 M, St. Petersburg, Florida, USA) by Electrophoresis power supply (Power Pac 3000, Bio-Rad, USA). Size of the nucleic acids was determined by 100bp DNA Ladder (GeneRuler, ThermoScientific INC., USA). The gel was visualized under UV transilluminator and photographed using Gel Documentation System (UVITECH, Cambridge, UK).

3.5 DNA Sequencing

For DNA sequencing automated sequencing system (ABI 3130, Applied Biosystems, USA) was used along with ABI Prism Ready Reaction Dye-Deoxy Terminator cycle sequencing kit (BigDye™). In it each of four dideoxynucleotides were labeled with a different fluorochrome and is based on chain terminating chemistry (Lee *et al.*, 1992). The ABI 3130 is set to coincide with the emission of four different fluorescent dyes and can detect fluorescent at four different wavelength simultaneously.

3.5.1 PCR reaction and purification

For the amplification of template to sequence the gene region for rs34778348, PCR was performed in 40ul reaction volume with control primers. The reaction contains 0.75µM each of the primer, 1.0U of *Taq* DNA polymerase enzyme and 100ng genomic DNA sample. Initial denaturing was performed at 95 °C for 4mins. Stage 2 was repeated

for 30 times in which denaturing, annealing and extension was done at 94 °c for 1min, 62 °C for 30sec and at 72 °C for 30sec, respectively. After the reaction, 5µl of PCR product was run on agarose gel to confirm the amplification. The remaining amplified product was precipitated by using 4µl of 10M ammonium acetate and 80µl absolute ethanol. The tubes were placed on ice for 20min to enhance the precipitation. After that, the tubes were centrifuged at 14000rpm for 10mins. Supernatants were discarded and pellet was washed with 70% ethanol, dried and re-suspended in 20µl deionized water.

3.5.2 Sequencing reaction and its purification

Sequencing reaction was set up by using 5µl of purified PCR product, 2µl BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA), 1µl reverse or forward primer and 2µl deionized water, for making total volume up to 10µl. The Cyclic conditions for the reaction are shown in the Figure 3-3.

The unincorporated labeled nucleotides were removed by adding 2.5µl of 125mM EDTA and 30µl Absolute ethanol in 10µl amplified PCR product. A short spin was performed and then the mixture was placed on ice for 15mins so that the reaction could precipitate. These were than centrifuged at 14000rpm for 20min. The supernatants were carefully discarded and pellet was washed with 100µl 70% ethanol, air-dried. Just before the electrophoresis 10µl HI-DI formamide was added in the sample and heated at 95 °C for 4min to denature the DNA.

The sequence data collected from the system was then processed by associated software to get an electropherogram. The sequences were aligned using BioEdit program.

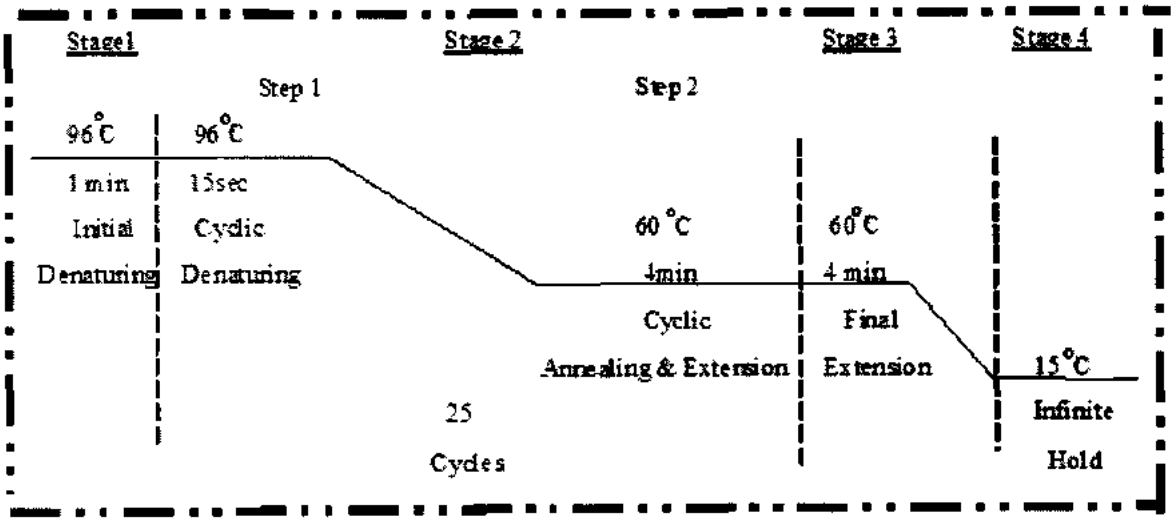

Figure 3-3: Cyclic Conditions for Sequencing PCR

CHAPTER 4

RESULTS

4. RESULTS

4.1 Clinical Features

In this study, twelve families of Dystonia named as DT-1 to DT-12 and thirteen nuclear families of Parkinson named as PK3-D01 to PK3-D13 were collected from different regions of Punjab and KPK. Proband along with his relative were carefully interviewed for the maximum accuracy about the detail of families. The families were ascertained based on the information from different resources and then analyzed according to the Revised Unified Dystonia Rating scale (UDRS) for Dystonia (Appendix (ii)) and Unified Parkinson's Disease Rating Scale (UPDRS) for Parkinsonism (Appendix (iii)), before the sample collection (Cynthia *et al.*, 2003; Goetz et al., 2006)).

Two factors were included in UDRS;

1. Duration of the disease
2. Motor severity

In Dystonia, duration of the disease was observed at 9 points, starting from '0' to '4', with an increase in level '0.5' in each stage. '0' indicates none and '4' shows the Constant duration period. A pie chart representation of duration factor found in patients is show in the Figure 4-1. The Motor Severity Factors have 5 points, starting from '0', showing no sign, up to '4' indicating extreme severity. UPDRS has total 42 questions and each question has 5 options, 0, 1, 2, 3, 4 & 5. The '0' shows no sign of particular symptom, '1' shows mild and '4' is for maximum severity.

Motor severity was further divided into eye and upper face, lower face, jaw and tongue, larynx, neck, trunk etc. (Table 4-1). In case of UPDRS, features like Intellectual Impairment, Sleep Disorder, Thought Disorder, Depression, Handwriting, Swallowing, Tremor Rigidity, Speech etc, were included (Table 4-2).

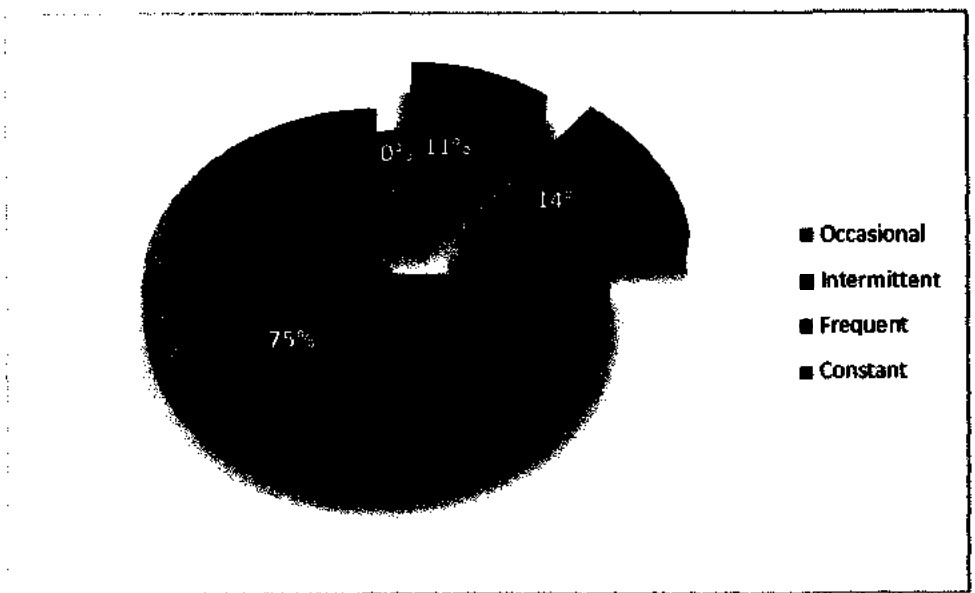

Figure 4-1: Pie-chart for the DT duration factor.

A pie-chart representation of the percentage of duration factor for Dystonia in familial patinets.

- None of the family is with Occasional duration
- 11% are with Intermittent
- 14% have Frequent duration
- 75% of the mamilies have Constant duration factor

Table 4-1: Clinical Features of Dystonic Patients

Clinical Features of Dystonia Patients					
1	**Duration Factor**	Occasional	Intermittent	Frequent	Constant
		0	3	4	21
2	**Motor Severity Factor**	None	Mild& Moderate	Severe	Extreme
I	EYE & UPPER FACE	21	4	3	0
Ii	LOWER FACE	20	7	0	1
Iii	JAW & TONGUE	23	4	0	1
Iv	LARYNX	25	3	0	0
V	NECK	26	1	0	1

Clinical Features of Dystonia Patients					
Vi	SHOULDER & PROXIMAL ARM	14	10	3	1
Vii	DESTAL ARM & HAND	6	18	3	1
viii	PELVIS & PROXIMAL LEG	6	6	10	6
Ix	DISTAL LEG & FOOT	0	10	10	18
X	TRUNK	17	5	1	5

Table 4-2: Clinical Features of Parkinsonism Patients

Clinical Features of Parkinsonism Patients					
	Clinical Features	**No Sign of Disease**	**Mild Symptom**	**Moderate Symptom**	**Serve Symptom**
1	Intellectual Impairment	3	6	4	0
2	Depression	6	4	3	0
3	Thought Disorder	4	8	1	0
4	Motivation	6	3	3	1
5	Salivation & Swallowing	5	8	0	0
6	Dressing & Hygiene	0	6	7	0
7	Falling	0	5	7	1

Clinical Features of Parkinsonism Patients					
8	Walking Difficulties	1	5	6	1
9	Tremor at Rest	1	5	7	0
10	Speech Impairment	5	4	4	0
11	Rigidity	0	4	8	1
12	Body Bradkinesia & Hypokinesia	1	6	5	1
13	Postural Instability	0	8	4	1
14	Presence of Early Morning Dystonia	10	2	1	0
15	Any Sleep Disturbance	10	3	0	0
16	Painfull	1	0	10	2

4.2 Pedigree Analysis

The clinical features were recorded carefully. It was tried to get maximum information about elder generations to trace the founder individual. The pedigrees were drawn by Cyrillic software (http//:www.cherwell.com). The square boxes indicates the male individuals while circle is showing the female individuals, dark color boxes show the infected member of particular family and clear boxes are representing the normal individuals. Single line between male and female shows the non-consanguineous marriage while double lie shows the marriage between two consanguineous male and female. After carefully drawing the pedigree according to standard, the mode of inheritance was studied according to Mendel's Law of Inheritance. Age of onset and clinical type of Dystonia affecting the families were carefully identified, a graph is shown in Figure 4-2, representing the Clinical type of disease in families. All the families of DT and PD are summarized in Table 4-3 and Table 4-4 respectively.

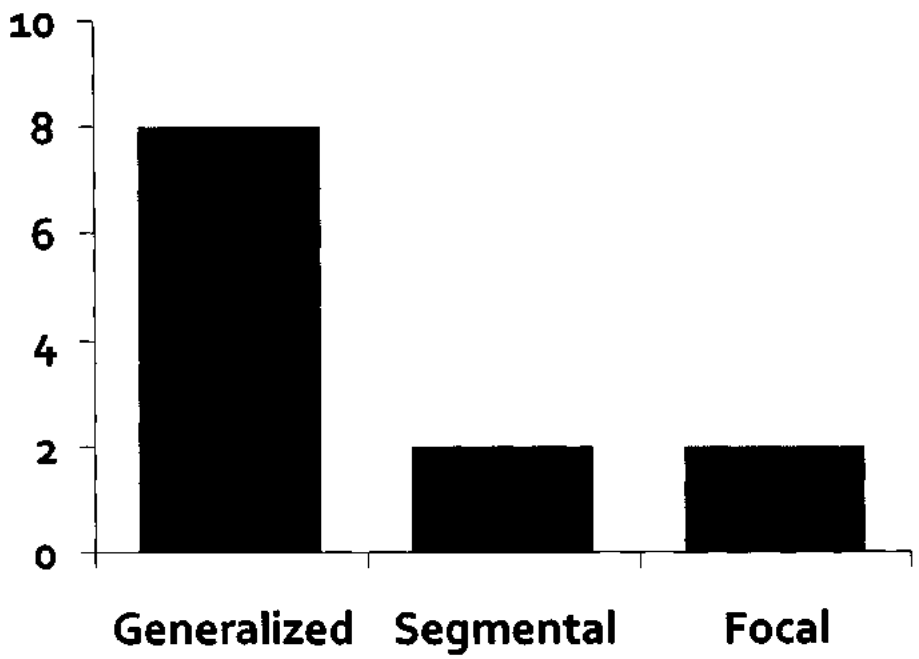

Figure 4-2: Clinical types of Dystonia

In 12 families of DT clinical type was diagnosed.

- 8 families are with Generalized Dystonia
- 2 are with segmental and 2 are with focal dystonia

Table 4-3: Detail of DT Families

Family	Caste	Age of Onset	Mod of Inheritance	Type of Dystonia
DT-01	Rajput	Early Age Onset	Autosomal Recessive	Generalized Dystonia
DT-02	Arain	Early Age Onset	Autosomal Recessive	Generalized Dystonia
DT-03	Khan	Early Age Onset	Autosomal Recessive	Focal Dystonia
DT-04	Khral	Early Age Onset	Autosomal Dominant	Segmental Dystonia
DT-05	Sheikh	Early Age Onset	Autosomal Recessive	Generalized Dystonia
DT-06	Kharl	Early Age Onset	Autosomal Dominant	Segmental Dystonia
DT-07	Arain	Early Age Onset	Autosomal Recessive	Generalized Dystonia
DT-08	Jatt	Early Age Onset	X-Linked	Generalized Dystonia
DT-09	Mochi	Onset After 18Years	X-Linked	Generalized Dystonia
DT-10	Lohar	Early Age Onset	Autosomal Dominant	Generalized Dystonia
DT-11	Masi	Early Age Onset	Nuclear family	Generalized Dystonia
DT-12	Arain	Early Age Onset	X-linked	Focal Dystonia

Table 4-4: Detail of PD Families

Family	Ethnicity	Age of Onset	Duration of disease	Stage of Parkinson
PK3-D01	Pathan	Late age of Onset	25	1
PK3=D02	Pathan	Early age of Onset	11	1
PK3-D03	Pathan	Early age of Onset	6	1
PK3-D04	Pathan	Early age of Onset	3	1
PK3-D05	Pathan	Late age of Onset	2	4
PK3-D06	Pathan	Early age of Onset	6	1
PK3-D07	Pathan	Late age of Onset	5	3
PK3-D08	Siraiki	Late age of Onset	1	1
PK3-D09	Pothohari	Late age of Onset	3	2
PK3-D10	Pothohari	Late age of Onset	1	3
PK3-D11	Siraiki	Late age of Onset	5	1
PK3-D12	Pathan	Late age of Onset		2
PK3-D13	Pathan	Late age of Onset	3	2

DT-1

A large consanguineous family with DT was collected from central Punjab. There were mostly inter-marriages; data till the fourth generations were collected from the proband. All the members of first generation was dead and no information about their diseases status was known. In the second generation, one female member was affected. Her three sisters and a brother were normal. In third generation all the individuals were normal. There were three cousin marriages, which are shown by double lines in the pedigree. At least one of their child is affected with DT. There were six affected individuals in the fourth generation. They have early age of onset. The mode of inheritance is most probably autosomal recessive.

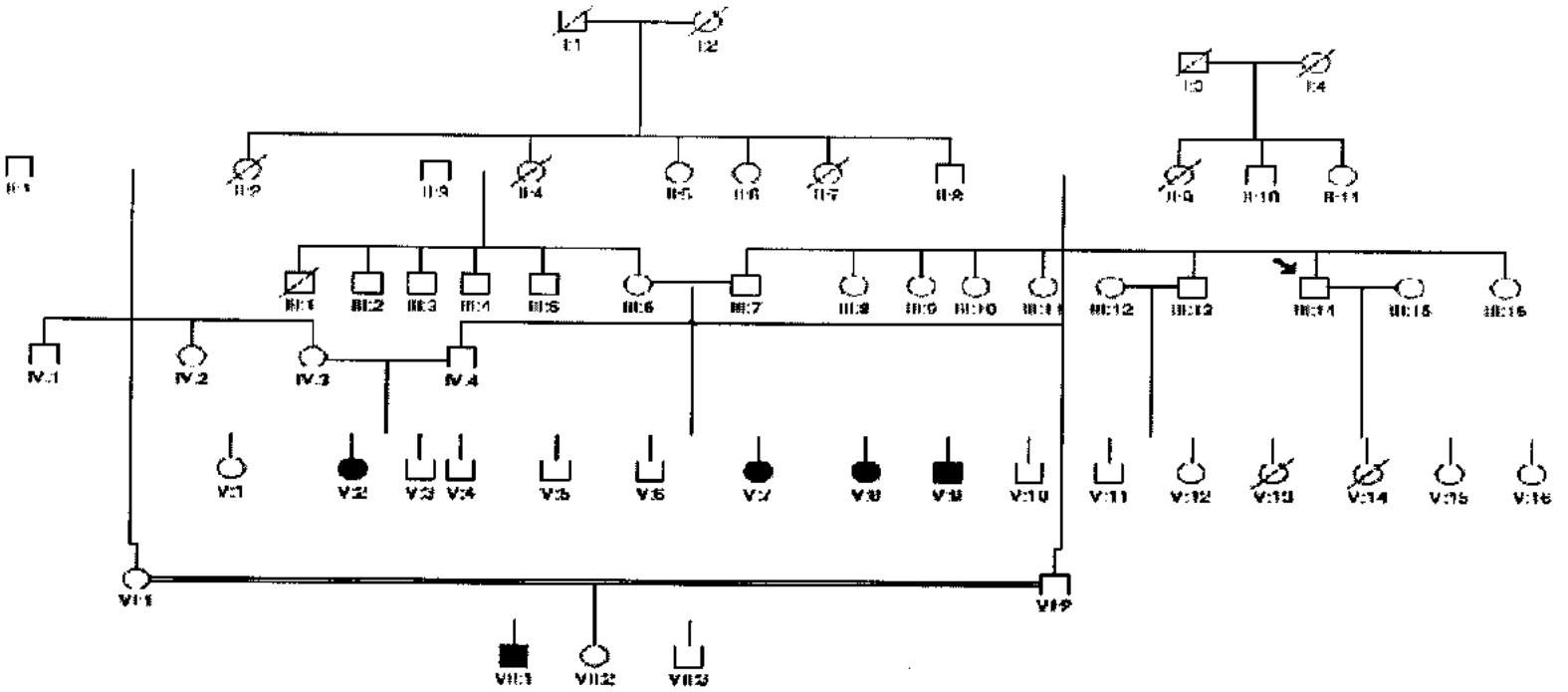

Figure 4-3: Pedigree of DT-1

A large family collected from central Punjab region. The mode of inheritance was autosomal recessive. Individuals have early age of onset having Generalized Dystonia, starting from lower limb and becoming generalized.

DT-2

A family with three individuals affected with DT was collected from Central Punjab. The proband (individual identifier) provided the information about the five generations. There was an affected male in 2nd generation with normal brothers and sisters. Individual of third and fourth generation were normal there was a cousin marriage between a third generation male and fourth generation female both parents were phenotypically normal. Their two male children were affected with Dystonia. The age of on set was around three years. The pedigree analysis shows that the inheritance is autosomal recessive.

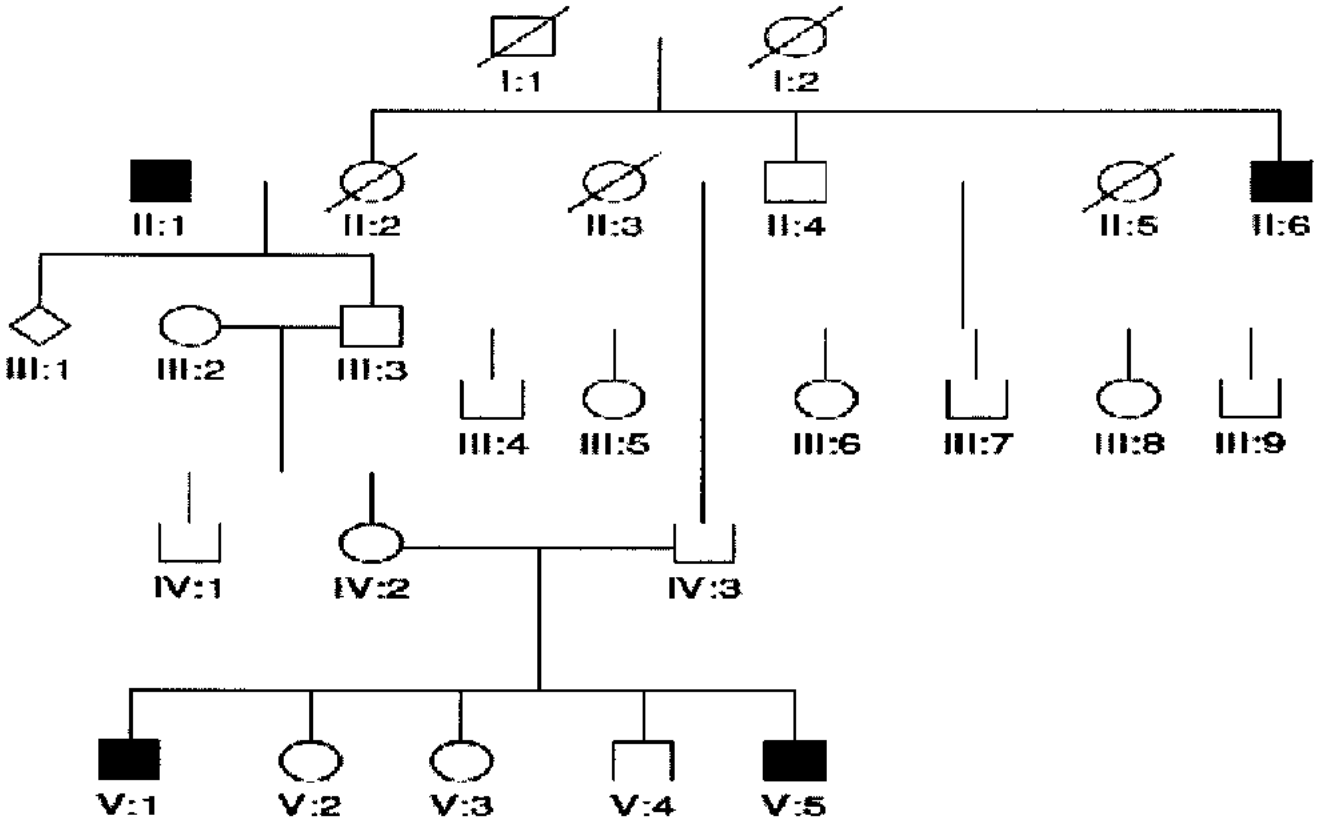

Figure 4-4: Pedigree of DT-2

A family having four males affected with Dystonia was identified. They have early age of onset. In 4th generation a consanguineous marriage has two affected male individuals. Pedigree analysis shows that mode of Inheritance is autosomal recessive.

DT-3

A family was identified as DT, having seven affected individuals in four generations among which six were male and a Female. The proband give the information about four generations in which 29 individuals were present. There was no affected individual in first generation and they all were dead. In 2nd generation, 2 male individuals were affected; both affected members belong to different loops. In third generation, again two males were affected and in fourth generation two males and a female were affected. The parents of both males and a female were phenotypically normal. They have early age of disease onset. The inheritance is look like autosomal recessive.

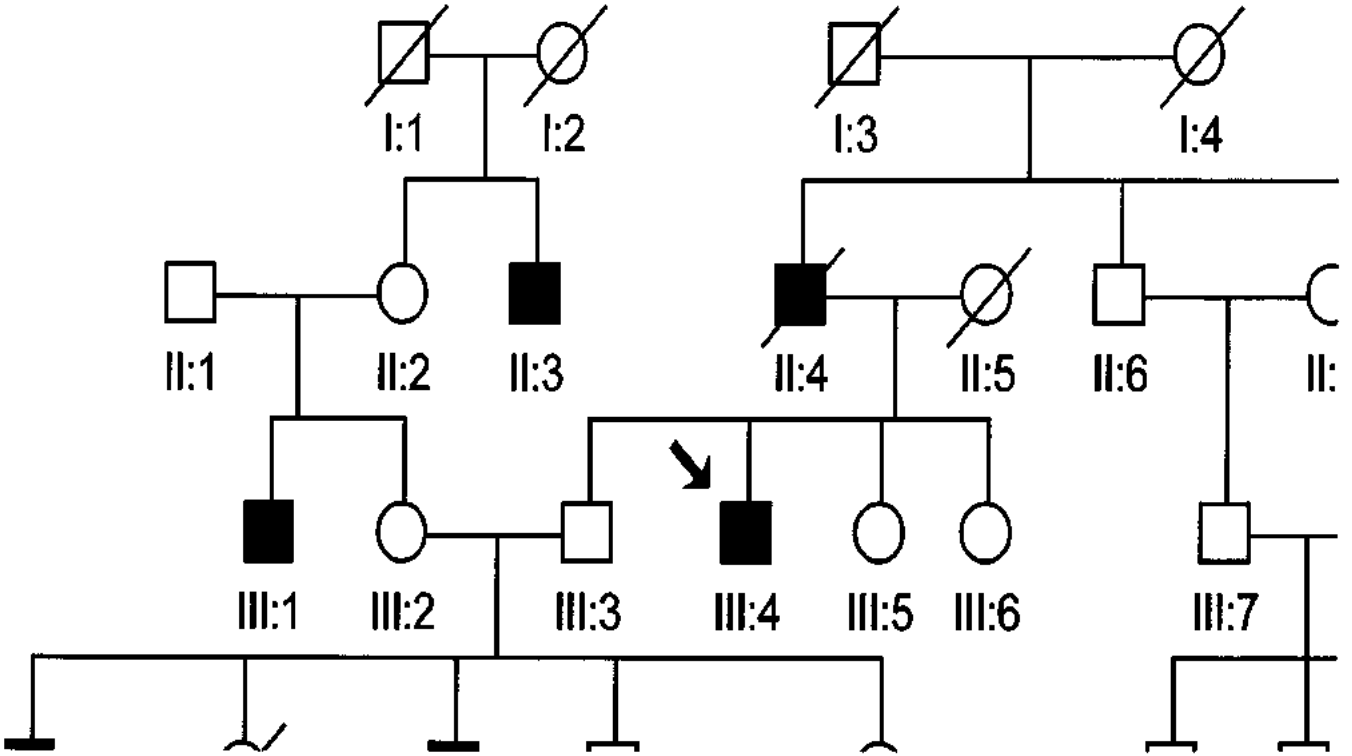

Figure 4-5: Pedigree of DT-3

A family having six males and a female affected with Dystonia was identified. They have early age of onset Pedigree analysis shows that mode of Inheritance is autosomal recessive. They had Focal Dystonia

DT-4

A family was identified with DT. The proband provided the information about the four generations. He was interviewed about the 23 individuals, out of which three members were affected. In first generation there were no affected member. In 2nd generation one male and a female were affected. These both patients were dead. In third generation one male was affected. He was the son of affected male in 2nd generation He has a non-cousin marriage with a normal female having four normal children. They were affected with segmental Dystonia. The pedigree analysis shows that the mode of inheritance is autosomal dominant.

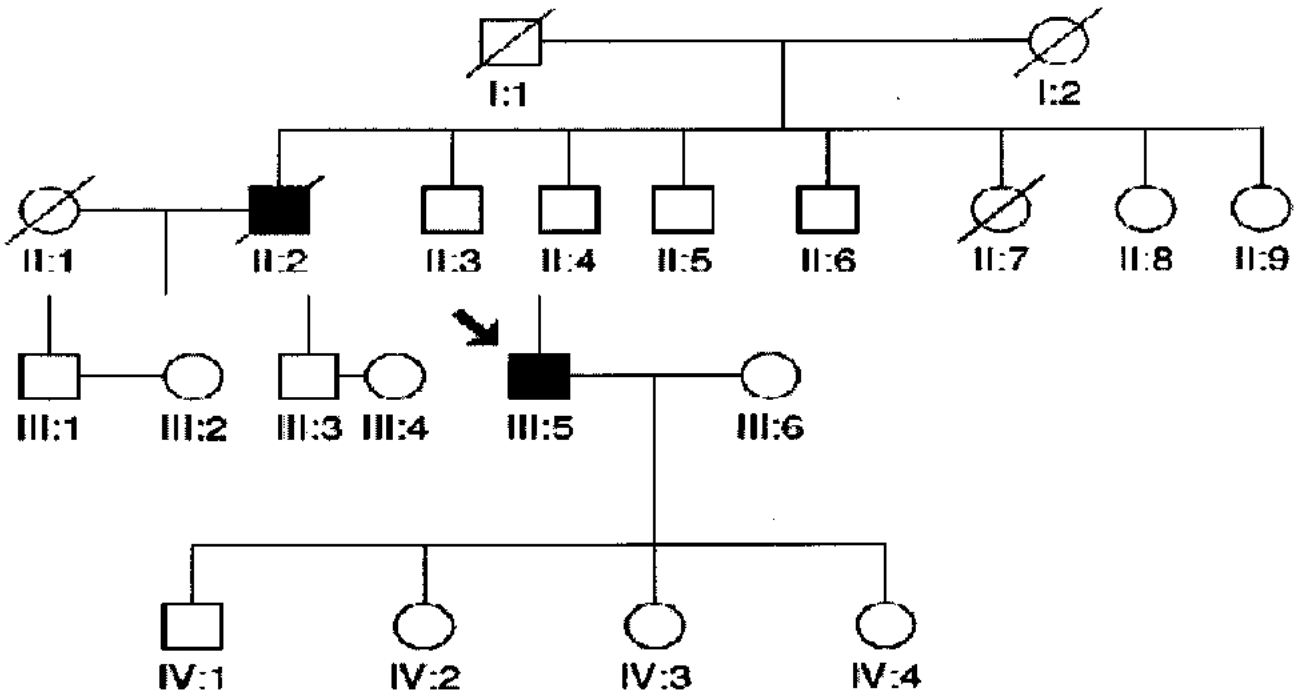

Figure 4-6: Pedigree of DT-4

A small family having two affected males was identified. They have early age of onset. There is an affected male in 2nd generation three offspring, an affected male and two normal daughters. Pedigree analysis shows that mode of Inheritance is autosomal dominant.

DT-5

A large family with dystonia was identified from central Punjab region near Gujranwala. The proband provided the information about four generations. There were number of consanguineous marriages in every generation. All the members of first three generations were normal. There were six affected individuals in 4th generation. Four males and two females were affected. A loop has an affected male and a female with four normal siblings. In 2nd loop again a male and female was affected having five normal sisters. In 3rd loop a male was affected. All these affected individuals have normal parents with consanguineous marriages. In 4th loop a male was affected having a normal brother and three sisters, parents had non-consanguineous marriage. The analysis of pedigree shows that they have autosomal recessive mode of inheritance. All the individuals have early age onset between 8-10 years.

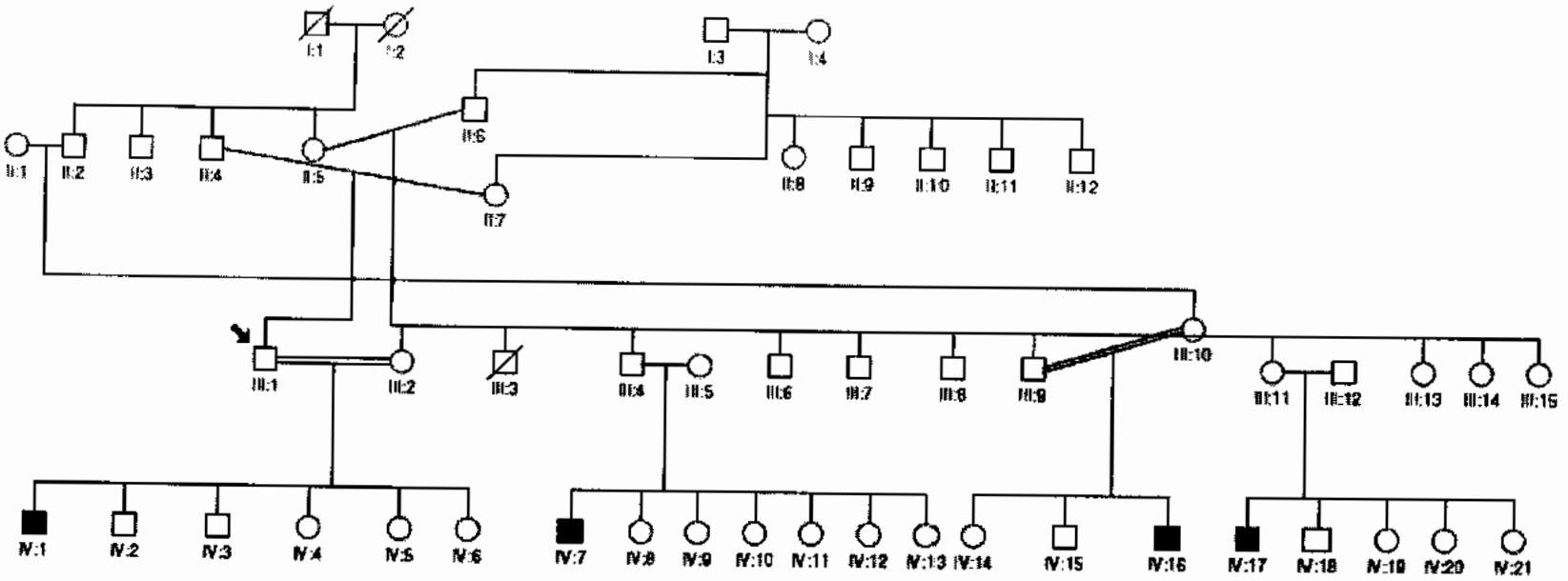

Figure 4-7: Pedigree of DT-5

We identified a family having four affected males in 4th generation. All the parents of affected individuals were phenotypically normal. They have early age of onset. Pedigree analysis shows that mode of Inheritance is autosomal recessive.

DT-6

A consanguineous family with Dystonia was identified. Five generation information was provided by proband. All members of first two generations were normal. In third generation a male and a female was affected with disease. The male individual was married with her cousin who is shown by the double line. They have 9 offspring among which six were female and three were male, one male was affected with the disease. The affected male has two marriages, a non-consanguineous and a consanguineous marriage. In fifth generation the affected male has an affected daughter with Dystonia from her cousin marriage. The age of onset was from birth. The pedigree analysis shows that the mode of inheritance is Autosomal Dominant.

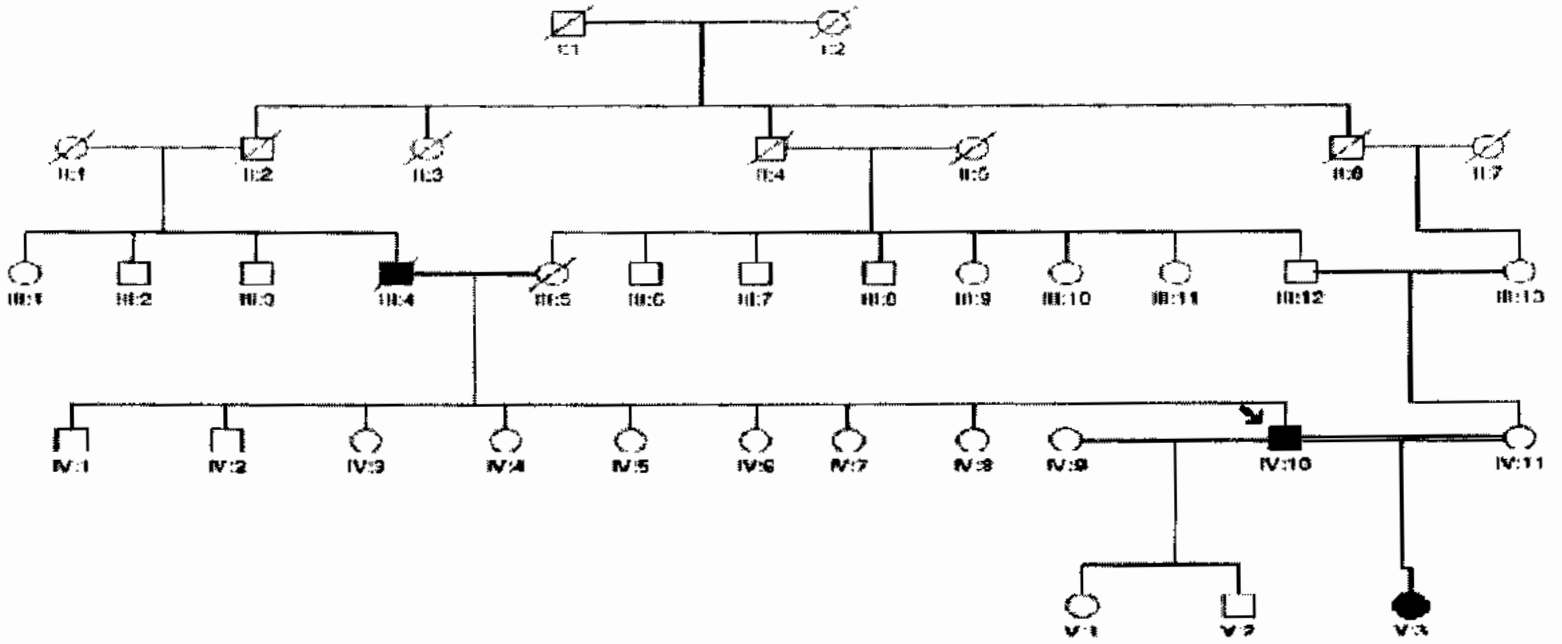

Figure 4-8: Pedigree of DT-6

A family having two affected males and a female was identified. They have early age of onset. There is an affected male in 3rd generation having nine offspring, an affected male and two normal sons and six daughters. Affected son has an affected daughter. Pedigree analysis shows that mode of Inheritance is Autosomal Dominant.

DT-7

A large family with dystonia was identified from Kasur and Sahiwal region in Punjab. The family has two main loop linked with two sisters in 4th generation. In 1st loop, proband provided the information about five generations. All the members of 1st three generation were dead and no information regarding disease was available. In 4th generation a male and female has consanguineous marriage. They have three affected males and two normal daughters. The affected individuals have early age of onset. In 2nd loop the proband was interviewed along with his relatives and data about six generations was obtained. All the individuals from 1st three generation were dead and no information about the disease was obtained. There were many cousin marriages in this loop. In a male was affected having normal parents with two brothers and a sister. In another loop a consanguineous marriage parents have an affected male with a normal son and three daughters. In 6th generation again a male was affected having normal parents there mode of inheritance was Autosomal recessive.

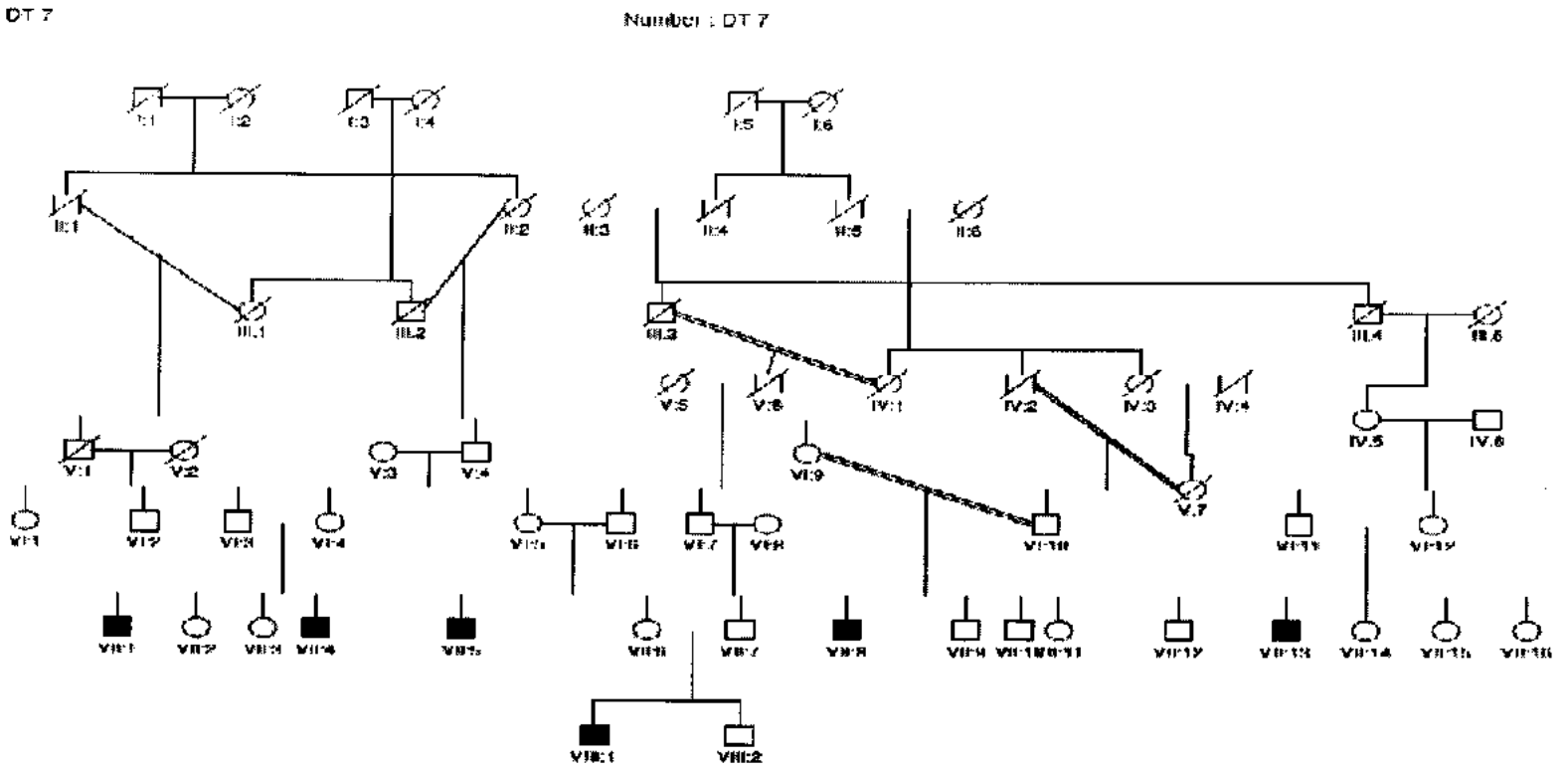

Figure 4-9: Pedigree of DT-7

A complex family having six affected males was identified. Family has two main loops linked by two sisters in fourth generations. They have early age of onset having Generalized Dystonia. The mode of Inheritance is concluded as Autosomal Recessive after Pedigree analysis.

DT-8

A family from central Punjab with three affected females was identified. The proband was interviewed and provided the information about the five generations. The first 2 generations are dead and there was no information regarding disease was known by the informer. In 3rd generation there were three marriage lines, making three loops, all the individuals were phenotypically normal. A loop has two male offspring which were married with the female of other two loops, showing double marriage line. All the four members of fourth generation were phenotypically normal. In fifth generation two females in one loop with five normal members and a female in second loop with two normal members were affected with the disease. They have Generalized Dystonia and their age of onset was from birth. The mode of Inheritance was concluded as X-linked after the pedigree analysis.

Family name : DT-8 Number : DT-8

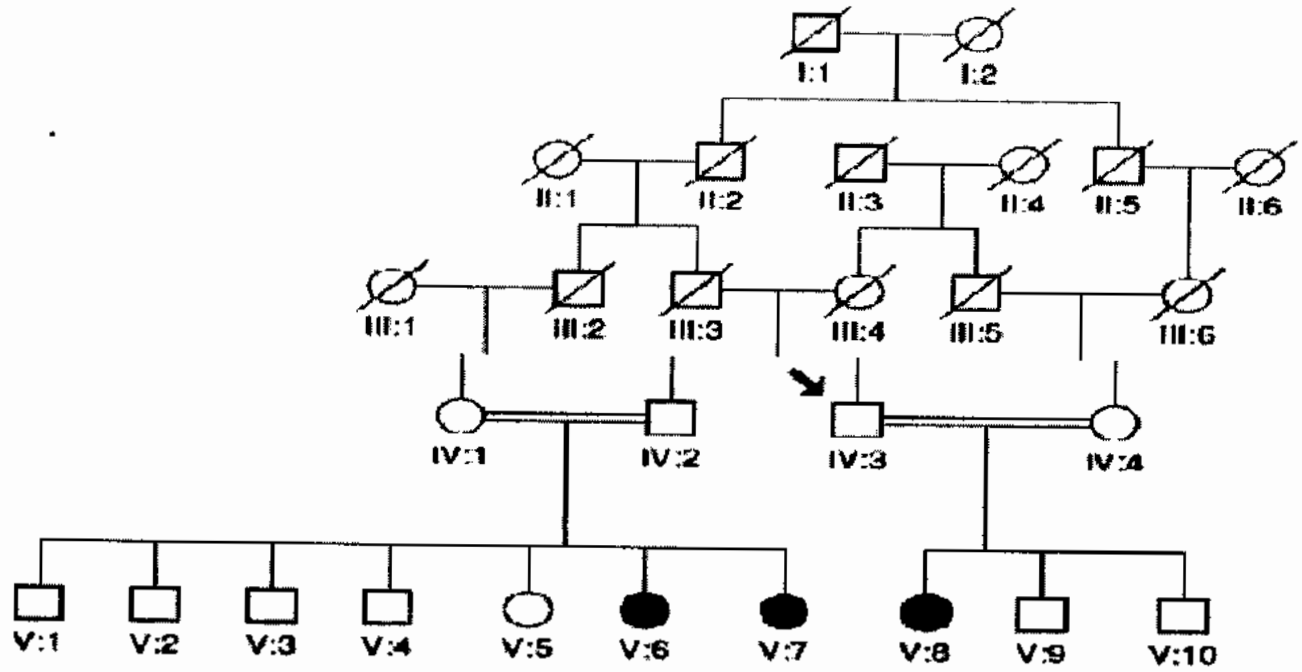

Figure 4-10: Pedigree of DT-8

We identified a family near Okara Region, having three affected females in 5th generation. All the parents of affected individuals were phenotypically normal. They have early age of onset. Pedigree analysis shows that mode of Inheritance is X-linked. They have Generalized Dystonia.

DT-9

A family with complex consanguineous marriages was identified with Dystonia in Pakistani population. Their age of onset was 18years. The disease started affecting the lower limbs and become generalized after time. Three male members were affected with the disease in the family. The proband DT9-V:8 provide us the information about the five generations according the information all the members of first three generations were normal. In third generation DT9-IV:9 married with DT9-IV:10 and had one affected offspring DT9-V:8. Later they got separated and DT9-IV:9 married with DT9-IV:5. They had one daughter and five sons out of which two males were affected. DT9-IV:10 married with DT9-IV:11, has three normal siblings. The mode of inheritance in the pedigree was X-linked.

Family name : DT-9 Number : DT-9

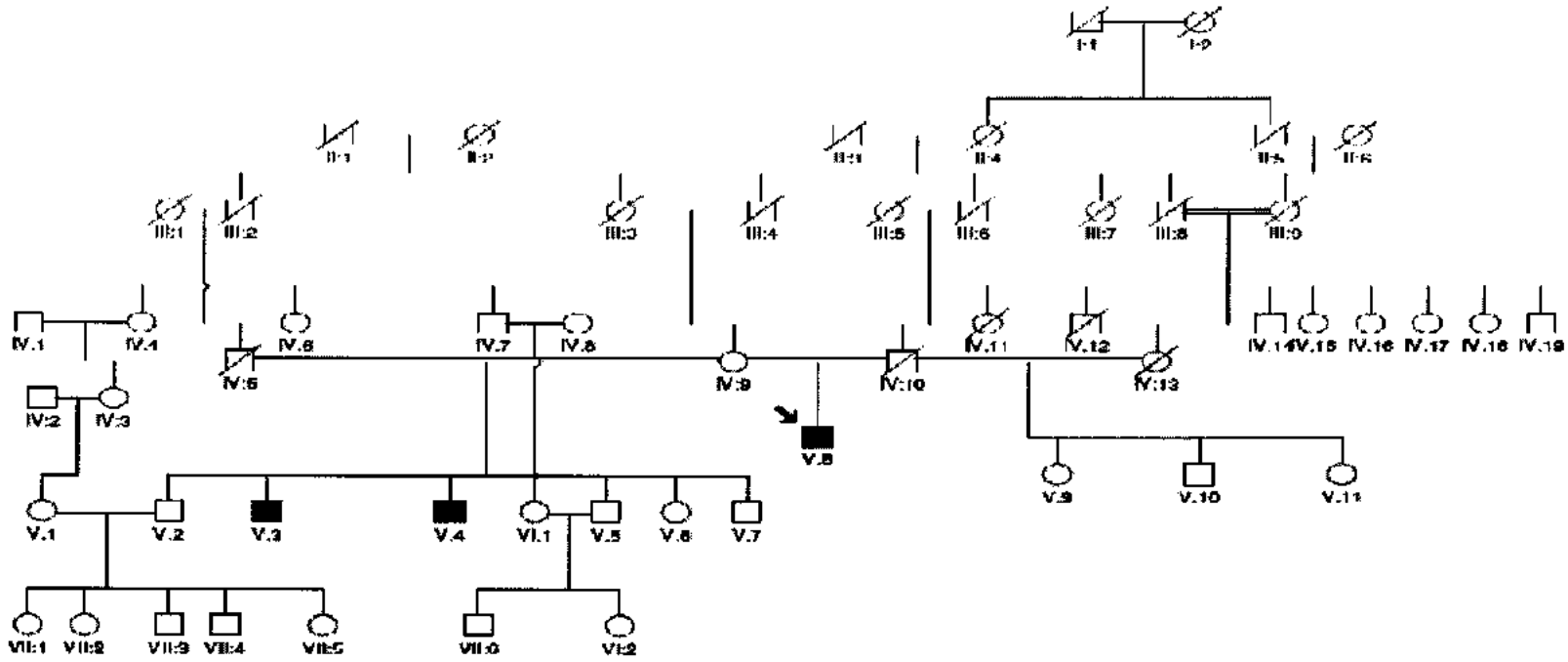

Figure 4-11: Pedigree of DT-9

A complex family having three affected males was identified. In 4th generation a female has two marriages having an affected male from first husband and two affected males along with four normal offspring from 2nd husband. Their age of onset was 18years. The mode of Inheritance is concluded as X-linked after Pedigree analysis

DT-10

A large family with Dystonia was identified in Punjabi population from Central Punjab. The proband provided the information about the five generations in which 68 members were present out of which 25 members were affected with the disease. In 1st generation a female was affected. All the members of 1st generations were dead. In 2nd generations four siblings, two males and two females were affected. They were the offspring of affected female in first generation. In 3rd generations there were eight affected individuals in three loops. In a loop there were six offspring, a female and five males. All the males were affected with the disease. Their father was phenotypically normal married with affected female from 2nd generation. In other two loops a male from an affected female and an individual from affected male, whose sex was not clearly known were affected. In fourth generation 11 members out of 19 individuals were affected in which four males and seven females were included. In fifth generation a female was affected having two normal sisters and a brother. Their age of onset was from birth. After the analysis of pedigree we conclude that their mode of inheritance is Autosomal dominant.

Family name : DT-10 Number : DT-10

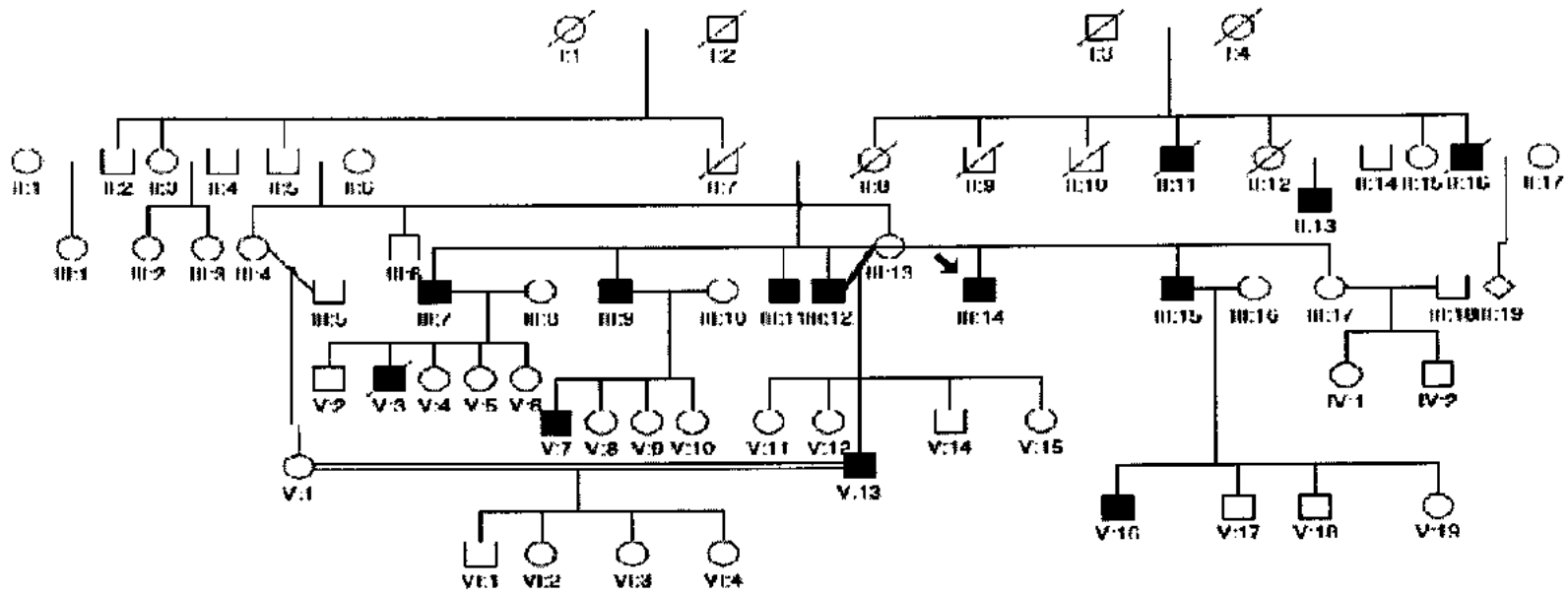

Figure 4-12: Pedigree of DT-10

A large family was collected from central Punjab region. The mode of inheritance was Autosomal Dominant. Individuals have early age of onset having Generalized Dystonia, starting from lower limb with affecting other body regions. All members of 1st generation were normal. In 2nd, 3rd, 4th and 5th generations there were two, seven, three and two affected individuals respectively.

DT-11

A small Christine family having one affected individual was identified. The affected male was from fifth generation, having four normal siblings. Both parents were phenotypically normal and there were no trace of disease in earlier generations. The double marriage line of parents shows that they have consanguineous marriage. Mode of inheritance was not clear because only one individual is affected.

Family name : DT-11 Number : DT-11

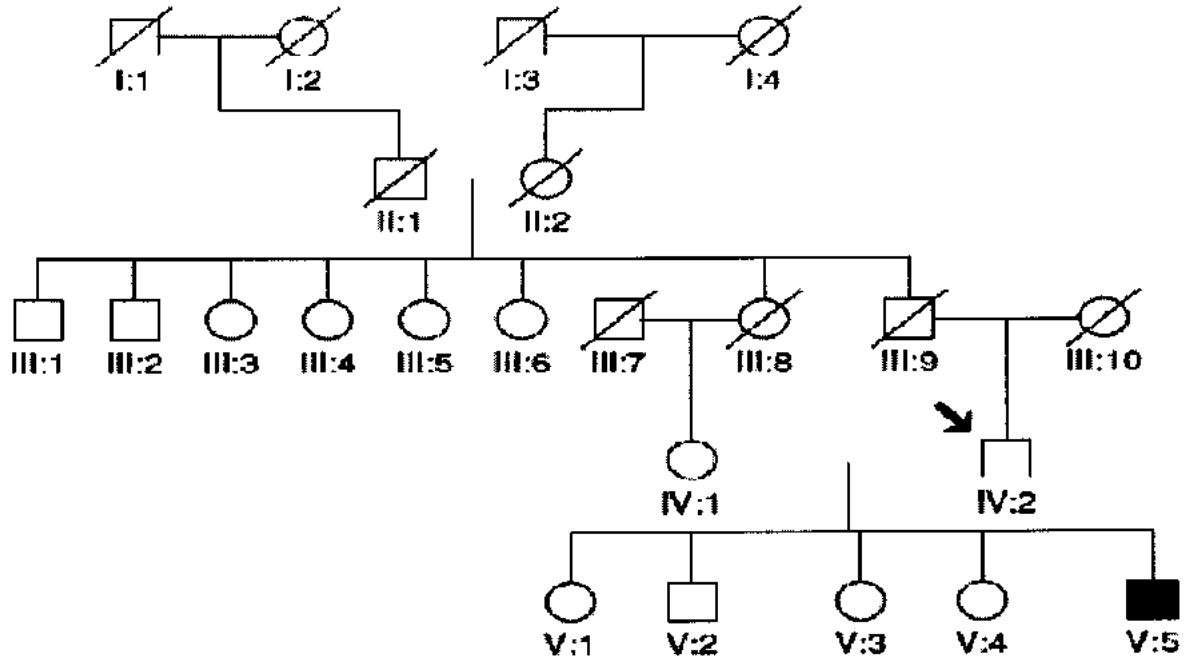

Figure 4-13: Pedigree of DT-11

A small family having an affected male was identified. He has early age of onset. The mode of Inheritance is not clear as only one individual is affected.

DT-12

A family with four affected individuals was identified in Islamabad. The proband was interviewed and provided information about three generations. All individuals of first generation were dead. There were five female in 2nd generation. Three of them were married having non-consanguineous marriages. At least one of the kids of these females was affected with the disease. There were four affected males in third generation, among which one was in 1st loop. In 2nd loop there were three offspring, two normal and one affected individual and in 3rd loop two males were affected with the disease. The mode of inheritance is X-linked and have early age of onset.

Family name : DT 12 Number : DT 12

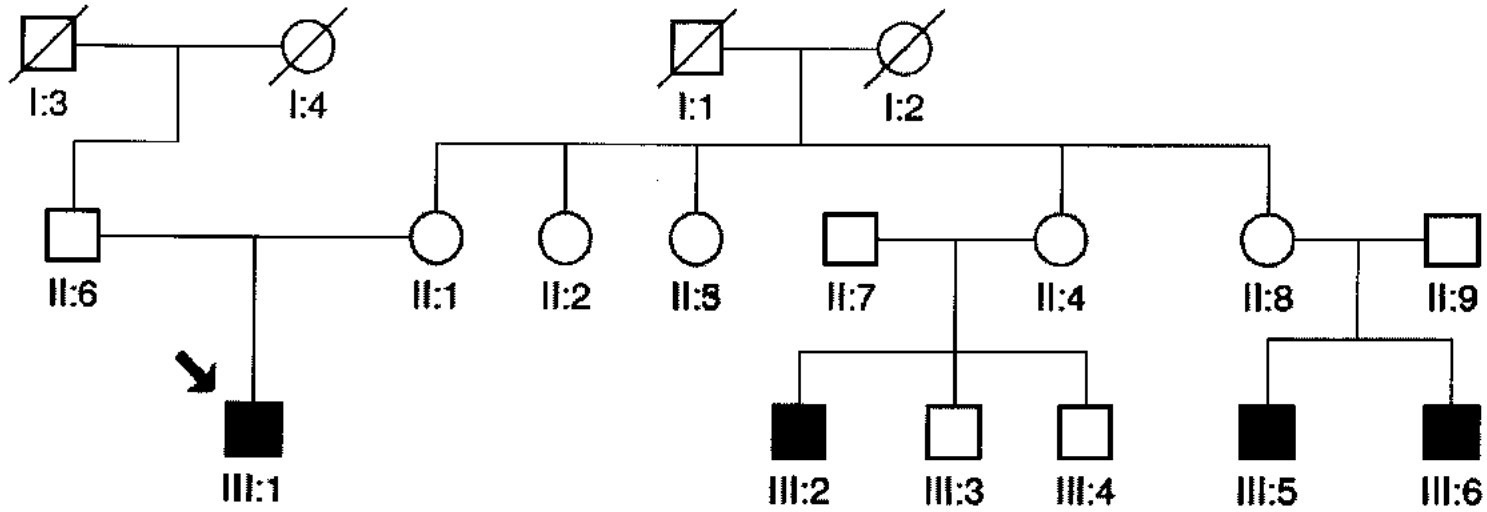

Figure 4-14: Pedigree of DT-12

We identified a family having three affected males in 3rd generation. All the parents of affected individuals were phenotypically normal. They have early age of onset. Pedigree analysis shows that mode of Inheritance is Autosomal Recessive. They have Focal Dystonia

4.3 Linkage Analysis

Linkage analysis is a useful approach in family-based studies in genetic mapping of diseased gene in heterogeneous population. Linkage analysis was done for the selected families of Dystonia.

Seven markers were selected for linkage analysis. The detail of markers, the gene region of amplification and their product size are shown in Table 4-5. Five markers, D12S2194, D12S87, D12S1048, D19S587 and D19S900 were used for DT-1, DT-7 and DT-8. These markers were used to link the families with Parkinsonism and Dystonia Parkinsonism. Four markers D19S587, D19S900, D9S63 and D9S64 were used for the linkage studies of DT-10. D9S63 and D9S64 are only linked of *DYT1* gene and for the families that have autosomal dominant mode of inheritance. None of the family was linked with STR markers used.

Table 4-5: Details of STR Markers

STR Markers	Gene Region	Approx. product Size (bp)	Disorder
D12S2194	*LRRK2* gene	250-262	Parkinsonism
D12S87	*LRRK2* gene	169	Parkinsonism
D12S1048	*LRRK2* gene	209-229	Parkinsonism
D19S587	*DYT12*	143–155	Dystonia-Parkinsonism
D19S900	*DYT12*	141-177	Dystonia-Parkinsonism
D9S63	*DYT1*	116	Dystonia
D9S64	*DYT1*	99	Dystonia

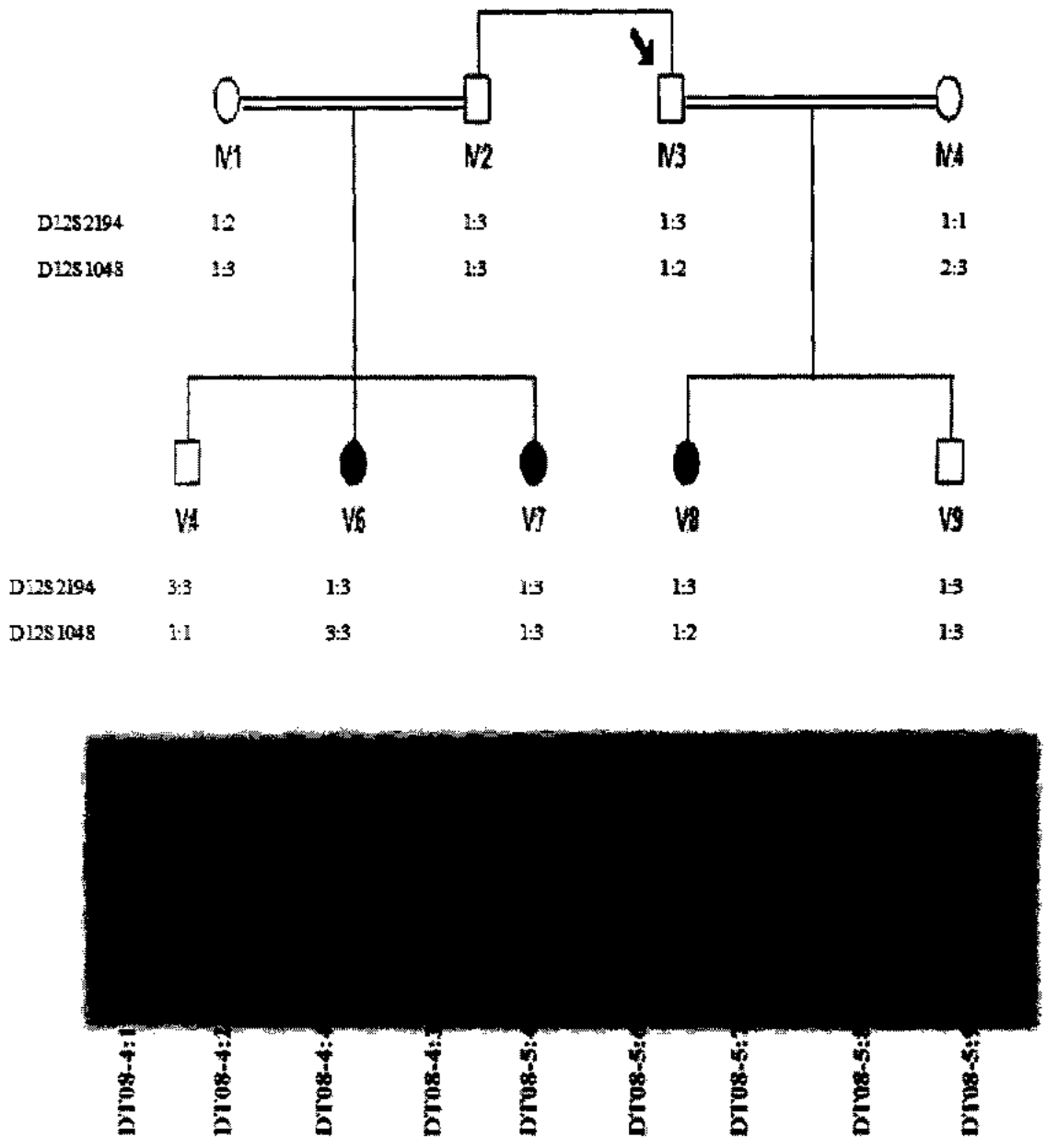

Figure 4-15: Image of Linkage analysis for D12S2194

Ideogram showing the result of linkage analysis for D12S2194 with the edited pedigree of DT-08, along with the haplotypes for D12S2194 and D12S1048. The image shows that none of the individual was linked.

4.4 Genotyping

4.4.1 Detection of Genetic Variants by ARMS

To study the genetic variants of ***LRRK2*** gene in Pakistani populations, three SNPs were selected, to evaluate in Dystonia families and PD triodes. The structure of domain of **LRRK2** protein indicating positions of the genetic variants is shown in the Figure 4-15. ARMS method was used for the detection of SNP. The variants were detected on the bases of band size on agarose gel and alleles were assigned according to their band size.

The details of SNPs of the variants as well as genotyping results of families are given in the Table 4-6. The genetic variant, rs34637584 (G2019s) was not detected in any of the family. All the affected as well as normal individuals are homozygous for the major allele: **"AA"** and none of them was detected homozygous **"GG"** or heterozygous **"AG"** for the minor allele (Figure 4-6).

For the genetic variant rs35870237 (I2020T), genotyping was performed and no polymorphism was detected in any of the family. All the families were homozygous for the major allele: "TT" whose band size is 139bp. No individual of any family was detected heterozygous "TC" or homozygous "CC" for the minor allele. The band size for minor allele was 644bp. An image of electrophoresis gel is shown in Figure 4-17, representing the result of genetic variant rs35870237 (I2020T).

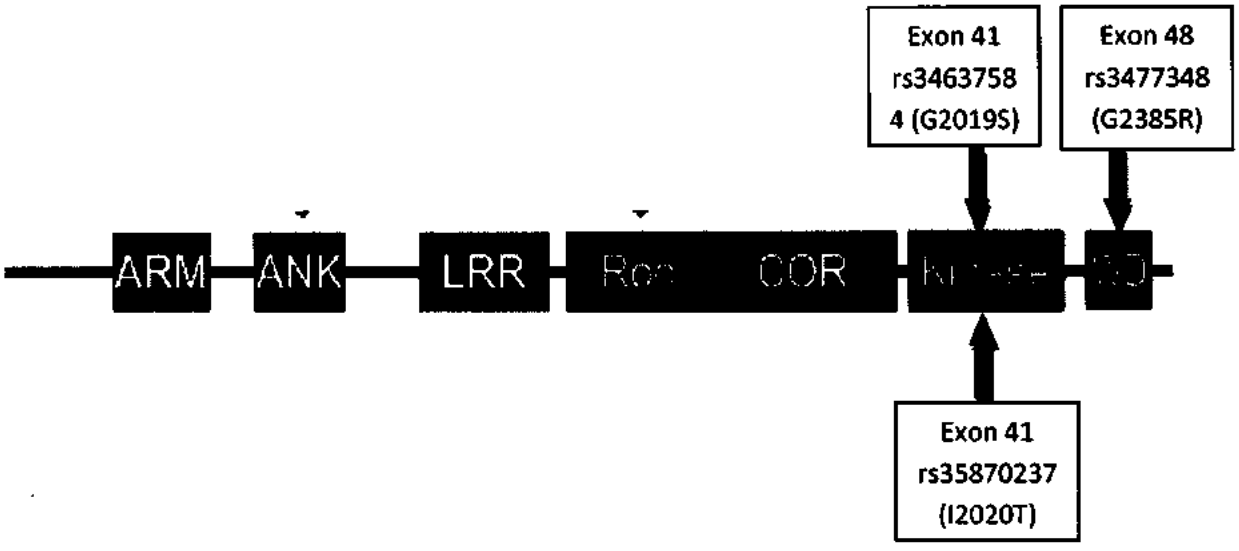

Figure 4-16: Domains of *LRRK2* with SNPs positions.

The structure of domain of LRRK2 protein indicating positions of the genetic variants. Two variants rs34637584 (G2019s) and rs35870237 (I2020T) are present on Exon 41 at kinase domain and the third variant rs34778348 (G2385R) is present on Exon 48 at WD40 repeats.

Table 4-6: Gene Frequencies of SNPs of the variants & genotyping results

Genetic Variant	Genotype	Dt Families Result	PD Triodes Result
rs34637584 (G2019s)	AA	All Families	All Families
	GA	----------	----------
	GG	----------	----------
rs35870237 (I2020T)	TT	All Families	All Families
	TC	----------	----------
	CC	----------	----------
rs34778348 (G2385R)	GG	All Families	All Families
	GA	DT1, DT8, DT9	PK3-D4
	AA	DT8, DT9	PK3-D5, PK3-D10

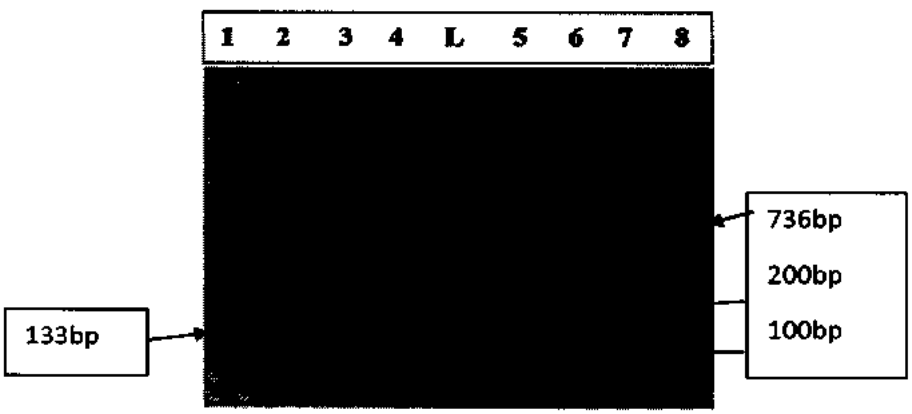

Figure 4-17: Image of Gel Electrophoresis for rs34637584 (G2019s).

Bands in lane 1-4 at 133bp represents the wild type allele of genetic variant rs34637584 (G2019s), in lane 5-8, there are no bands for minor allele which indicates the absence of the minor allele. In all lane control bands are shown at 736bp. L is for 100bp gene ruler.

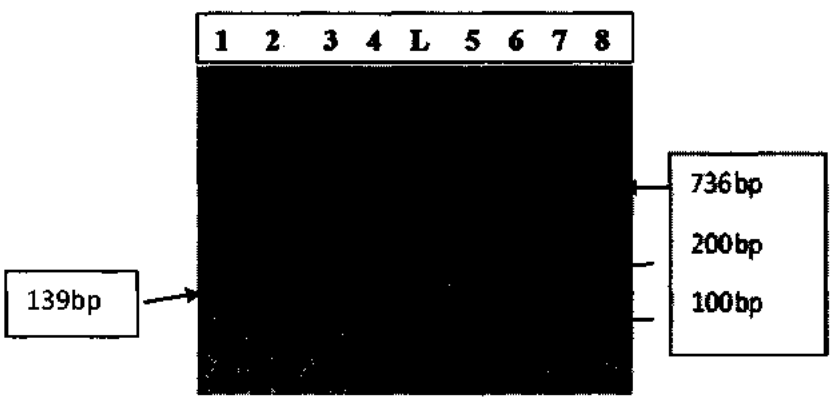

Figure 4-18: Image of Gel Electrophoresis for rs35870237 (I2020T)

Bands in lane 1-4 at 139bp represents the wild type allele of genetic variant rs35870237 (I2020T), in lane 5-8, there are no bands for minor allele which indicates the absence of the minor allele. In all lane control bands are shown at 736bp. L is for 100bp gene ruler.

In three families of Dystonia i.e. *DT-01, DT-08 and DT-09* and three triodes of Parkinson s' Disease which were PK3-D, *LRRK2* genetic variant rs34778348 (G2385R) was detected. The wild type allele **"G"** is 350bp in size and the size of mutant allele **"A"** is 138bp. All the remaining families of DT and PD have only major allele **"GG"** in them. The minor allele "AA" and heterozygous allele "**GA**" were not detected in them. The gel images of DT and PD are shown in Figure 4-18 and Figure 4-19.

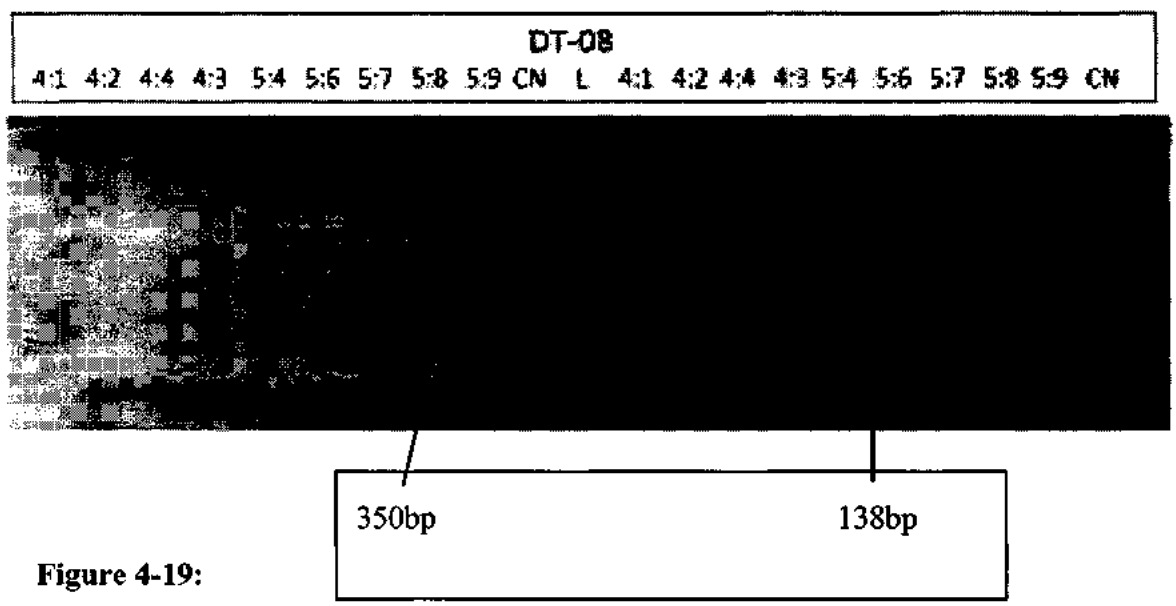

Figure 4-19:

Image of Gel Electrophoresis of DT-08 family for rs34778348.

Gel Electrophoresis of DT-08 family for rs34778348 (G2385R). On left hand side bands are for wild type allele "G" and their size is 350bp while on right side bands for mutant genetic variant "A" are shown having 138bp. CN is for control band and L is for 100bp Gene Ruler. All the individuals were heterozygous, except 3 individuals which were 4:1, 5:6 and 5:7.

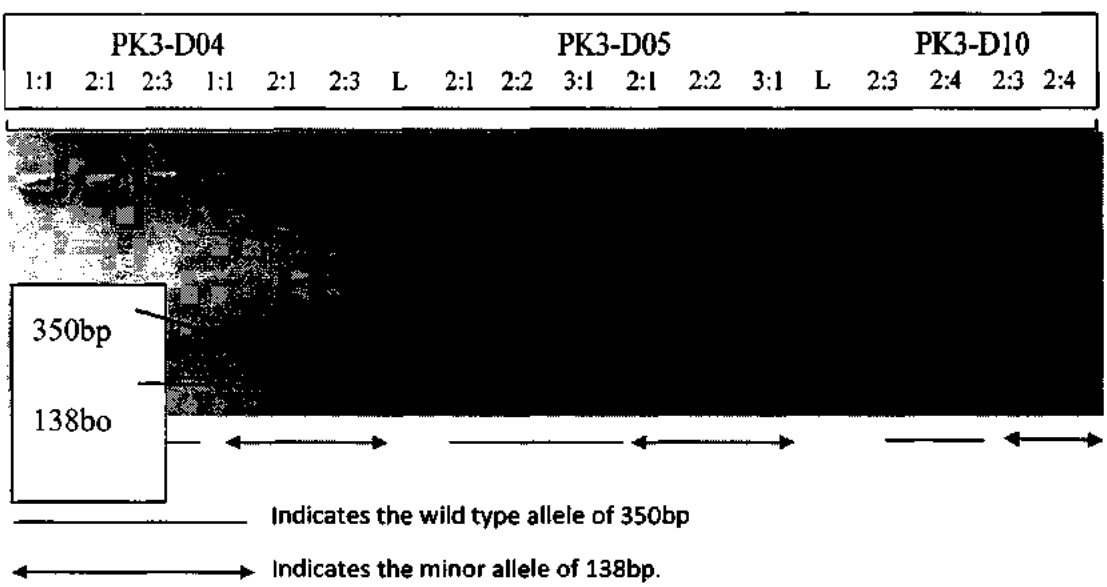

Figure 4-20: Image of Gel Electrophoresis of PK3-D family for rs34778348.

Results of three PK3-D family for *LRRK2* genetic variant rs34778348 (G2385R). In PK3-D04 1:1 was normal and other two members 2:1 and 2:3 were heterozygous. In PK3-D05 an individual 2:1 was homozygous for mutant variant and in PK3-D both members were homozygous for mutant allele.

4.4.2 Sequencing of the rs34778348 (G2385R)

Only rs34778348 (G2385R) is detected in the families of DT and triodes of PD. Genetic variants rs34637584 (G2019s) and rs35870237 (I2020T), present on Kinase domain (exon 41) were not detected in any family during genotyping by ARMS, therefore these variants were dropped for further analysis. It is expected that these polymorphism are absent in families of Pakistani population.

The genetic variant rs34778348 (G2385R), present on WD40 domain (exon 48) was detected in three families of DT and three families of PD. Therefore, to confirm the presence of SNPs, sequencing of these families were performed. In rs34778348 (G2385R), heterozygous (GA) allele was found in three families of DT and in one triode of PK3-D, and a patient in a family of DT and two of PK3-D were found to be homozygous (AA) for mutant allele which were confirmed by the sequencing. The electropherogram of rs34778348 (G2385R) for heterozygous GA and homozygous minor allele (A) are shown in the Figure 4-20 and 4-21 for heterozygous forward strand and reverse strand respectively and 4-22 for homozygous mutant allele.

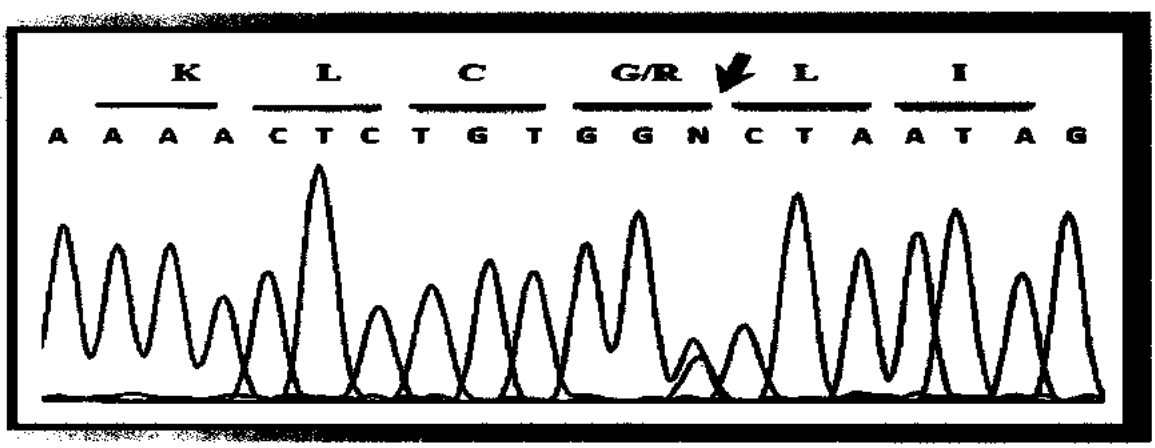

Figure 4-21: Electrophorogram of Heterozygous rs34778348 (G.A) for F. strand

The sequencing electrophorogram of *LRRK2* gene variant rs34778348 (G2385R) for heterozygous allele (G.A) of forward strand.

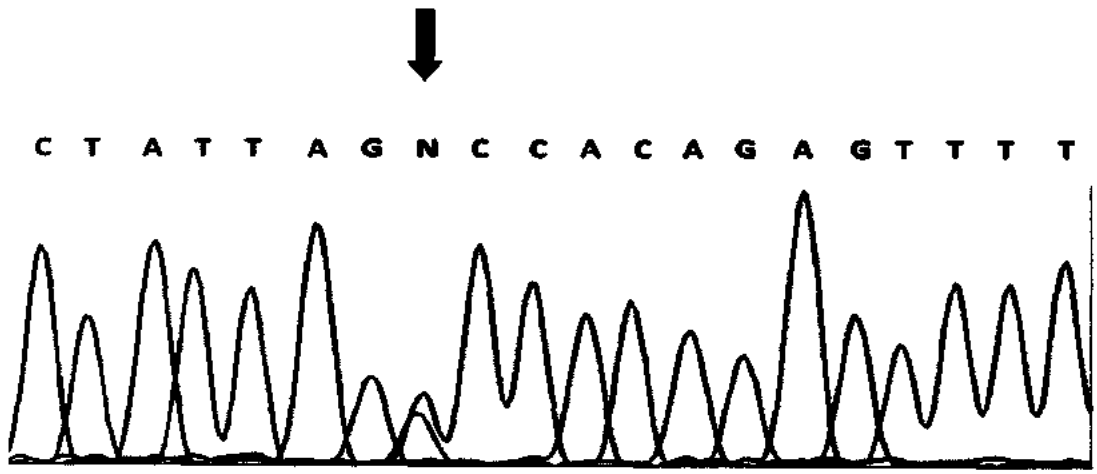

Figure 4-22: Chromatogram of Heterozygous rs34778348 (T.C) for R. strand

The sequencing chromatograms of *LRRK2* gene variant rs34778348 (G2385R) for heterozygous allele (T.C) of reverse strand.

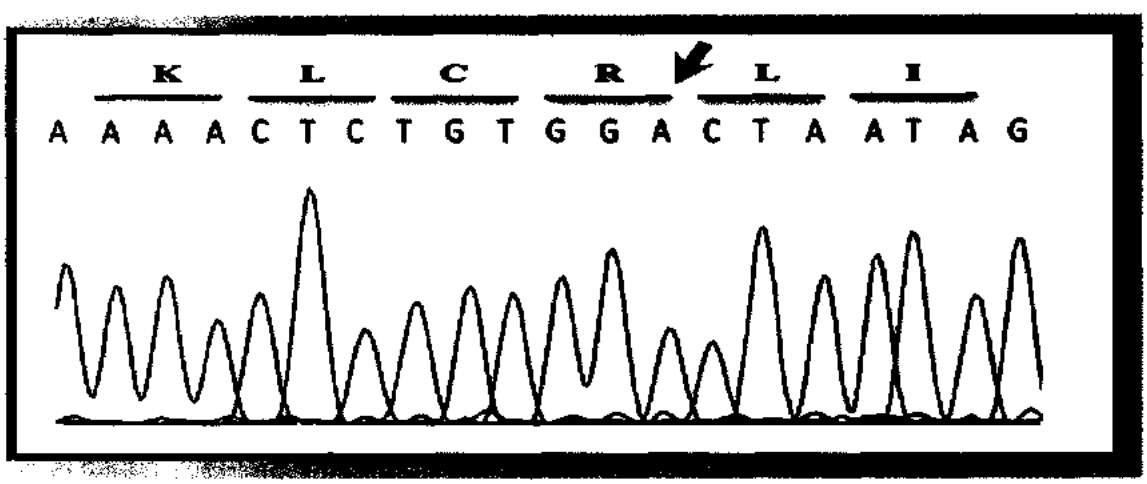

Figure 4-23: Chromatogram of minor allele rs34778348 (A.A).

The sequencing chromatograms of *LRRK2* gene variant rs34778348 (G2385R) for minor allele of forward strand (A.A)

CHAPTER 5

DISCUSSION

5. DISCUSSION

LRRK2 (Human leucine-rich repeat kinase 2, OMIM 609007) gene belongs to ROOCO protein family and encodes a multi-domain protein dardarin (PARK8), which is involved in protein-protein interaction, interferon-gamma response, kinase activity and response of individuals to pathogens. It is a large gene of 51 exons located at the 12q12 chromosomal region (Zimprich *et al.*, 2004). LRRK2 is a multi-domain protein with 2527 amino acids, includes are Armadillo repeats (ARM) and Ankyrin repeat (ANK) for protein-protein interactions, Leucine rich repeats (LRR), a Ras of complex (ROC) performing GTPase activity, a C terminal of Roc (COR), kinase domain and WD40 repeats. (Mata et al., 2006)

Mutation in the *LRRK2* gene is one of the most common cause of familial Parkinson's disease. Its frequency is 5% to 15% in familial cases and around 1% in sporadic cases in Caucasian ancestry (Berg et al., 2005, Fonzo et al., 2006; Latourelle et al., 2008). More than 50 variants of *LRRK2* gene are known, out of which only 10% have been proven as pathogenic. Their frequency is approximately 2% in sporadic and 10% in familial PD cases (Berg et al., 2005; Fonzo et al., 2005; Mata et al., 2006). Among many missense mutations pathogenicity is only clearly known for six variants i.e., R1441C, R1441G, R1441H, Y1699C, G2019S, and I2020T (Healy et al., 2008).

The prevalence of *LRRK2* variants in other neurodegenerative disorders and movement disorders are completely unknown. Whether these variants are specifically associated with Parkinson's disease or they have some risk for other diseases remains

totally unexplored. Investigation of large number of patients with other movement disorders and ND diseases are very much important. Therefore, risk factors of these variants are tested in Dystonia families as well as in triodes of Parkinson's disease. Pathogenic mechanism of Primary Dystonia and Parkinson's disease might be same, like protein mutation in DYT1 associates with a-synuclein in lewy bodies as well as other molecular disorders and protein quality control systems (Sharma et al., 2001).

Parkinson's disease is thought to be a multifactorial disease. It is caused by combination of many genetic as well as environmental factors. Involvement of *LRRK2* gene has been substantiated in recent years with the finding of many risk-variants but their frequency varies in different populations. A genetic variant G2019S, is common in Parkinson's disease families of Caucasian origin; occurring up to 2%–7% of cases (Kachergus *et al.*, 2005; Nichols *et al.*, 2005). While in some cases the frequency of G2019S is very high, being up to 20% in Ashkenazi Jews and 40% in North African Arabs (Ozelius *et al.*, 2006; Lesage *et al.*, 2006) and it was rarely seen in Asians (Chinese, Japanese, Korean, and Indian) (Healy *et al.*, 2008). Two variants, G2385R and R1628P, were identified as risk factors in the Asian population (Fonzo et al., 2006; Ross et al., 2008). Based on occurrence of *LRRK2* variants in specific ancestry, three genetic variants rs34637584 (G2019s), rs35870237 (I2020T) and rs34778348 (G2385R) were selected in this research. The polymorphs rs34637584 (G2019s) and rs35870237 (I2020T) are present on kinase domain and rs34778348 (G2385R) on WD40 repeats.

The SNPs rs34637584 and rs35870237 lies in the kinase domain of the protein and is translated by 41th exon. The exon 41 (Gly2019S, I2020T) are mutational hotspots and affect the activation segment of the kinase resulting in pathology. The kinase domain

phosphorylates and activates other protein to interact with each other. G2019S is one of the most frequent LRRK2 mutation and is based on the potential change from glycine (encoded by rs34637584 (G) allele) to serine (encoded by the rs34637584 (A) allele) at position 2019 of the LRRK2 protein (Nichols et al., 2005). One copy of minor allele (A) is sufficient to greatly increase risk for PD (Healy et al., 2008). It association with familial PD is estimated to 5% to 6% and 1% to 2% of idiopathic cases in populations of European descent (Tan and Skipper 2007; Singleton, 2005). No study was found on this variant regarding other movement disorders. Its polymorphism was also not detected in any individual of Dystonia families or in triodes of PD. All patients and their normal family members were homozygous for the major allele (rs34637584 (G)) and none of sample was positive for heterozygous or homozygous genotype for minor allele (rs34637584 (A)). It was in accordance with previous studies i.e. G2019A mutation is rare (at least < 0.1%) in Asian cohort (Tan et al., 2005). It is found only in Chinese population among Asian's ancestry. Gly2019S mutation is present across the Mediterranean populations, with high prevalence among patients from Portugal, Spain, and Italy, and very high prevalence among those from the North African and Middle Eastern populations (Guedes et al., 2010). The frequency of genetic variant rs35870237 (I2020T) is <1% in most populations (Tan et al., 2007). Our results are similar to this study. No minor allele for this polymorphism was detected in this study.

The genetic variant rs34778348 (G2385R) on WD40 repeats were detected in both DT as well as PD families. Families were positive for both heterozygous (GA) and minor allele (A) genotypes. An individual with homozygous minor allele (A) was identified in a 'DT-9' family and two individuals were identified in triodes of PD. Three families of DT

out of twelve families, and two families out of thirteen triodes of PD were found positive for heterozygous allele (GA). The function of WD40 repeats remains unknown. Mutations in this domain decrease the kinase activity as well as limits the neurotoxicity (Jorgensen et al., 2009). Many mutations in WD40 repeats have been identified out of which rs34778348 (G2385R), shows a genetic association with Dystonia and Parkinson's disease. (Song et al., 2008; Fung et al., 2006; Funayama et al., 2007). Due to this variant, change in amino acid, Glycine to Arginine, take place at position 2385 of LRRK2 protein. However, how this change affects the activity of protein is not well understood. The frequency of rs34778348 (G2385Arg) genotype was 4.18% in Taiwan (Song et al., 2008). In a chines population its frequency was estimated to 4% in all cases of PD (Tan and Skipper 2007). The Gly2385 variant was purposed as an important risk factor for PD in a Japanese sample and in Chinese populations of Singapore and Taiwan (Tan and Skipper 2007; Farrer et al., 2007; Fung et al., 2006; Funayama et al., 2007). This confirms the occurrence of Gly2385r in our population and supports our results. On the other hand the mutant variant Gly2385Arg is not present in Europeans (Berg et al., 2005; Fonzo et al., 2006). From these studies, there are now convincing evidence that a LRRK2 variant (G2385R) have risk for Dystonia and Parkinson's disease in Asian population. A routine testing for this mutation at least in our population and some Chines ancestry, may be cost-effective. It is very important to completely analyze the entire 51 exons of *LRRK2* before recommendations and guidelines for screening of LRRK2 for Neurodegenerative movement disorders like Dystonia and Parkinson's disease for diagnostic purposes and genetic counselling of inherited families

CONCLUSION AND FUTURE WORK

In the current research, three genetic variants rs34637584 (G2019s), rs35870237 (I2020T) and rs34778348 (G2385R) of *LRRK2* gene was screened for familial Dystonia and Parkinson's disease triodes, in our population. The minor alleles of rs34637584 (G2019s) and rs35870237 (I2020T) are not found in both DT and PD families from Pakistani population. In case of rs34778348 (G2385R), heterozygous (GA) allele was found in three families of DT and in one triode of PK3-D, and a patient in a family of DT and two of PK3-D were found to be homozygous (AA) for mutant allele. Therefore, rs34778348 (G2385R) could be a risk factor for DT as well as PD cases in Pakistan. However, the families included in the current study are low in number, as these were collected on the rating criteria from UDRS and UPDRS. These families might also have difference in their ethnicity. Therefore, the results need to be replicated in large cohorts for the final conclusion.

There was no study performed on these polymorphisms in context of Dystonia in Pakistan, as well as very few studies are present on other ancestries. Therefore, we cannot compare our results to justify them. The association of the LRRK2 polymorphisms with PD was not consistent among different studies, which might be attributed to the environmental factors, patient's ethnicity, age at onset etc.

Increasing evidence for incomplete penetrance of *LRRK2* mutations presented a major challenge for diagnostic testing and genetic counselling and highlighted the unmet need of validated estimates of population frequency and expressivity of the disease trait. It is, therefore recommended that the study must be conducted on a larger number of

families and in case-control set up from Pakistani population for all the genetic variants of *LRRK2* with neurodegenerative movement disorders to evaluate the risk factors of polymorphisms in this gene in local population.

CHAPTER 6

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6. REFERENCES

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Appendix (i)

Institute of Biomedical and Genetic Engineering (IBGE), Islamabad

24-Mauve area, G-9/1, P.O. Box 2891, Islamabad 44000, Pakistan

CONSENT FORM

(رضامندی فارم)

"Control Samples for Human Genetics Research"

I _____, son/daughter of _____, hereby, fully agree to contribute in the above mentioned study. I understand that the study is designed to add to the medical/scientific knowledge. I have been informed about the nature of procedure and possible risks/ discomforts involved. I had the opportunity to ask any question about the study & I agree to donate my blood sample as requested by the representative of Institute of Biomedical and Genetic Engineering Islamabad, Pakistan. I have no objection in case the data obtained from my sample investigation is published in scientific journal, maintaining confidentiality.

میں _____ ولد _____ یہاں پوری طرح مندرجہ بالا تحقیق میں شرکت کرنے سے اتفاق کرتا / کرتی ہوں۔ میں سمجھتا / سمجھتی ہوں کہ یہ تحقیق طبی اور سائنسی علم میں اضافے کے لیے کی جا رہی ہے۔ مجھے اس کے طریقہ کار کی بنیاد اور اس سے متعلقہ شکوک و اضطراب سے آگاہ کیا گیا ہے۔ مجھے اس تحقیق سے متعلق سوالات کرنے کا موقع بھی دیا گیا ہے۔ اور میں اس سلسلے میں خون کا نمونہ دینے کا اقرار کرتا ہوں جیسا کہ حیاتیاتی اور جینیاتی انجینئرنگ ادارے، اسلام آباد، پاکستان کے نمائندے کی جانب سے کہا گیا ہے جو کہ اس پر تحقیق کر رہے ہیں۔ مجھے اس چیز پر اعتراض نہیں ہے کہ میرے سے متعلق یہ تحقیق کسی رسالے میں بغیر ذاتی معلومات کھولے شائع ہو۔

Patient Name: _____

Signature: _____ **Signature of Investigator:** _____

Date: _____ **Date:** _____

Institute of Biomedical and Genetic Engineering (IBGE), Islamabad

24-Mauve area, G-9/1, P.O. Box 2891, Islamabad 44000, Pakistan

Questionnaire for Control Samples

Name: _____ **Age:** _____

Gender: _____ **Marital status:** _____

Ethnic Group: (e.g. Punjabi/Sindhi/Bloch/Pashtun) specify: _____

Cast: _____ **Sub-Cast:** _____

No of Children: _____ **Ph.no:** _____

Address _____

_____

Disease history _____

Hypertension: yes No

Diabetes: yes No

Obesity: yes No

Any other disease: _____

Family history of any disease: (including 1st cousin, 2nd cousin): _____

Informed Consent: I am donating my blood for research purpose and not for commercial use.

Signature: _____ **Date:** _____

Signature of investigator: _____ **Date:** _____

Appendix (ii)

Unified Dystonia Rating Scale (UDRS)

I. Duration Factor

0 none

0.5 occasional (< 25% of the time); predominantly submaximal
1.0 occasional (< 25% of the time); predominantly maximal
1.5 Intermittent (25-50% of the time); predominantly submaximal
2.0 Intermittent (25-50% of the time); predominantly maximal
2.5 Frequent (50-75% of the time); predominantly submaximal
3.0 Frequent (50-75% of the time); predominantly maximal
3.5 Constant (> 75% of the time); predominantly submaximal
4.0 Constant (> 75% of the time); predominantly maximal

2. Motor Severity Factor

EYES AND UPPER FACE

0. none
1. mild: increased blinking and/or slight forehead wrinkling (< 25% maximal intensity)
2. moderate: eye closure without squeezing and/or pronounced forehead wrinkling (> 5% but < 50% maximal intensity)
3. severe: eye closure with squeezing, able to open eyes within 10 seconds and/or marked forehead wrinkling (> 50% but < 75% maximal intensity)
4. eye closure with squeezing, unable to open eyes within 10 seconds and/or intense forehead wrinkling (> 75% maximal intensity)

LOWER FACE

0 none
1 mild: grimacing of lower face with minimal distortion of mouth (< 25% maximal)
2 moderate: grimacing of lower face with moderate distortion of mouth (> 25% but < 50% maximal)
3 severe: marked grimacing with severe distortion of mouth (> 50% but < 75% maximal)
4 extreme: intense grimacing with extreme distortion of mouth (> 75% maximal)

JAW AND TONGUE

0 none
1 mild: jaw opening and/or tongue protrusion < 25% of possible range or forced jaw clenching without bruxism
2 moderate: jaw opening and/or tongue protrusion > 25% but < 50%of possible range or forced jaw clenching with mild bruxism secondary to dystonia
3 severe: jaw opening and /or tongue protrusion > 50% but < 75%of possible range or forced jaw clenching with pronounced bruxism secondary to dystonia
4 extreme: jaw opening and/or tongue protrusion > 75% of possible range or forced jaw clenching with inability to open mouth

LARYNX

0 none
1 mild: barely detectable hoarseness and/or choked voice and/or occasional voice breaks
2 moderate: obvious hoarseness and/or choked voice and/ or frequent voice breaks
3 severe: marked hoarseness and/or choked voice and/or continuous voice breaks
4 extreme: unable to vocalize

NECK

0 none
1 mild: movement of head from neutral position < 25% of possible normal range
2 moderate: movement of head from neutral position > 25% but < 50% of possible normal range
3 severe: movement of head from neutral position > 50% but < 75% of possible normal range
4 extreme: movement of head from neutral position > 75% of possible normal range

SHOULDER AND PROXIMAL ARM (Right and Left)

0 none
1 mild: movement of shoulder or upper arm < 25% of possible normal range
2 moderate: movement of shoulder or upper arm 25% but < 50% of possible normal range
3 severe: movement of shoulder or upper arm 50% but < 75% of possible normal range
4 extreme: movement of shoulder or upper arm 75% of possible normal range

DISTAL ARM AND HAND INCLUDING ELBOW (Right and Left)

0 none
1 mild: movement of distal arm or hand < 25% of possible normal range
2 moderate: movement of distal arm or hand 25% but < 50% of possible normal range
3 severe: movement of distal arm or hand 50% but < 75% of possible normal range

4 extreme: movement of distal arm or hand 75% of possible normal range

PELVIS AND PROXIMAL LEG (Right and Left)

0 none

1 mild: tilting of pelvis or movement of proximal leg or hip < 25% of possible normal range

2 moderate: tilting of pelvis or movement of proximal leg or hip 25% but < 50% of possible normal range

3 severe: tilting of pelvis or movement of proximal leg or hip 50% but < 75% of possible normal range

4. extreme: tilting of pelvis or movement of proximal leg or hip 75% of possible normal range

DISTAL LEG AND FOOT INCLUDING KNEE (Right and Left)

0 none

1 mild: movements of distal leg or foot < 25% of possible normal range

2 moderate: movements of distal leg or foot 25% but < 50% of possible normal range

3 severe: movements of distal leg or foot 50% but < 75% of possible normal range

4 extreme: movements of distal leg or foot 75% of possible normal range

TRUNK

0 none

1 mild: bending of trunk < 25% of possible normal range

2 moderate: bending of trunk 25% but < 50% of possible normal range

3 severe: bending of trunk > 50% but < 75% of possible normal range

4 extreme: bending of trunk > 75% of possible normal rangea

Appendix (iii)

UNIFIED PARKINSON'S DISEASE RATING SCALE

I. MENTATION, BEHAVIOR AND MOOD

1. Intellectual Impairment
0 = None.
1 = Mild. Consistent forgetfulness with partial recollection of events and no other difficulties.
2 = Moderate memory loss, with disorientation and moderate difficulty handling complex problems. Mild but definite impairment of function at home with need of occasional prompting.
3 = Severe memory loss with disorientation for time and often to place. Severe impairment in handling problems.
4 = Severe memory loss with orientation preserved to person only. Unable to make judgements or solve problems. Requires much help with personal care. Cannot be left alone at all.

2. Thought Disorder (Due to dementia or drug intoxication)
0 = None.
1 = Vivid dreaming.
2 = "Benign" hallucinations with insight retained.
3 = Occasional to frequent hallucinations or delusions; without insight; could interfere with daily activities.
4 = Persistent hallucinations, delusions, or florrid psychosis. Not able to care for self.

3. Depression
1 = Periods of sadness or guilt greater than normal, never sustained for days or weeks.
2 = Sustained depression (1 week or more).
3 = Sustained depression with vegetative symptoms (insomnia, anorexia, weight loss, loss of interest).
4 = Sustained depression with vegetative symptoms and suicidal thoughts or intent.

4. Motivation/Initiative
0 = Normal.
1 = Less assertive than usual; more passive.
2 = Loss of initiative or disinterest in elective (nonroutine) activities.
3 = Loss of initiative or disinterest in day to day (routine) activities.
4 = Withdrawn, complete loss of motivation.

II. ACTIVITIES OF DAILY LIVING (for both "on" and "off")

5. Speech
0 = Normal.
1 = Mildly affected. No difficulty being understood.
2 = Moderately affected. Sometimes asked to repeat statements.
3 = Severely affected. Frequently asked to repeat statements.
4 = Unintelligible most of the time.

6. Salivation
0 = Normal.
1 = Slight but definite excess of saliva in mouth; may have nighttime drooling.
2 = Moderately excessive saliva; may have minimal drooling.
3 = Marked excess of saliva with some drooling.
4 = Marked drooling, requires constant tissue or handkerchief.

7. Swallowing
0 = Normal.
1 = Rare choking.
2 = Occasional choking.
3 = Requires soft food.
4 = Requires NG tube or gastrotomy feeding.

8. Handwriting
0 = Normal.
1 = Slightly slow or small.
2 = Moderately slow or small; all words are legible.
3 = Severely affected; not all words are legible.
4 = The majority of words are not legible.

9. Cutting food and handling utensils
0 = Normal.
1 = Somewhat slow and clumsy, but no help needed.
2 = Can cut most foods, although clumsy and slow; some help needed.
3 = Food must be cut by someone, but can still feed slowly.
4 = Needs to be fed.

10. Dressing
0 = Normal.
1 = Somewhat slow, but no help needed.
2 = Occasional assistance with buttoning, getting arms in sleeves.
3 = Considerable help required, but can do some things alone.
4 = Helpless.

11. Hygiene
0 = Normal.
1 = Somewhat slow, but no help needed.
2 = Needs help to shower or bathe; or very slow in hygienic care.
3 = Requires assistance for washing, brushing teeth, combing hair, going to bathroom.
4 = Foley catheter or other mechanical aids.

12. Turning in bed and adjusting bed clothes
0 = Normal.
1 = Somewhat slow and clumsy, but no help needed.
2 = Can turn alone or adjust sheets, but with great difficulty.
3 = Can initiate, but not turn or adjust sheets alone.
4 = Helpless.

13. Falling (unrelated to freezing)
0 = None.
1 = Rare falling.
2 = Occasionally falls, less than once per day.
3 = Falls an average of once daily.
4 = Falls more than once daily.

14. Freezing when walking
0 = None.
1 = Rare freezing when walking; may have starthesitation.
2 = Occasional freezing when walking.
3 = Frequent freezing. Occasionally falls from freezing.
4 = Frequent falls from freezing.

15. Walking
0 = Normal.
1 = Mild difficulty. May not swing arms or may tend to drag leg.
2 = Moderate difficulty, but requires little or no assistance.
3 = Severe disturbance of walking, requiring assistance.
4 = Cannot walk at all, even with assistance.

16. Tremor (Symptomatic complaint of tremor in any part of body.)
0 = Absent.
1 = Slight and infrequently present.
2 = Moderate; bothersome to patient.
3 = Severe; interferes with many activities.
4 = Marked; interferes with most activities.

17. Sensory complaints related to parkinsonism
0 = None.
1 = Occasionally has numbness, tingling, or mild aching.
2 = Frequently has numbness, tingling, or aching; not distressing.
3 = Frequent painful sensations.
4 = Excruciating pain.

III. MOTOR EXAMINATION

18. Speech
0 = Normal.
1 = Slight loss of expression, diction and/or volume.
2 = Monotone, slurred but understandable; moderately impaired.
3 = Marked impairment, difficult to understand.
4 = Unintelligible.

19. Facial Expression
0 = Normal.
1 = Minimal hypomimia, could be normal "Poker Face".
2 = Slight but definitely abnormal diminution of facial expression
3 = Moderate hypomimia; lips parted some of the time.
4 = Masked or fixed facies with severe or complete loss of facial expression; lips parted 1/4 inch or more.

20. Tremor at rest (head, upper and lower extremities)
0 = Absent.
1 = Slight and infrequently present.
2 = Mild in amplitude and persistent. Or moderate in amplitude, but only intermittently present.
3 = Moderate in amplitude and present most of the time.
4 = Marked in amplitude and present most of the time.

21. Action or Postural Tremor of hands
0 = Absent.
1 = Slight; present with action.
2 = Moderate in amplitude, present with action.
3 = Moderate in amplitude with posture holding as well as action.
4 = Marked in amplitude; interferes with feeding.

22. Rigidity (Judged on passive movement of major joints with patient relaxed in sitting position. Cogwheeling to be ignored.)
0 = Absent.
1 = Slight or detectable only when activated by mirror or other movements.
2 = Mild to moderate.
3 = Marked, but full range of motion easily achieved.
4 = Severe, range of motion achieved with difficulty.

23. Finger Taps (Patient taps thumb with index finger in rapid succession.)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

24. Hand Movements (Patient opens and closes hands in rapid succesion.)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

25. Rapid Alternating Movements of Hands (Pronation-supination movements of hands, vertically and horizontally, with as large an amplitude as possible, both hands simultaneously.)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

26. Leg Agility (Patient taps heel on the ground in rapid succession picking up entire leg. Amplitude should be at least 3 inches.)
0 = Normal.
1 = Mild slowing and/or reduction in amplitude.
2 = Moderately impaired. Definite and early fatiguing. May have occasional arrests in movement.
3 = Severely impaired. Frequent hesitation in initiating movements or arrests in ongoing movement.
4 = Can barely perform the task.

27. Arising from Chair (Patient attempts to rise from a straightbacked chair, with arms folded across chest.)
0 = Normal.
1 = Slow; or may need more than one attempt.
2 = Pushes self up from arms of seat.
3 = Tends to fall back and may have to try more than one time, but can get up without help.
4 = Unable to arise without help.

28. Posture
0 = Normal erect.
1 = Not quite erect, slightly stooped posture; could be normal for older person.
2 = Moderately stooped posture, definitely abnormal; can be slightly leaning to one side.
3 = Severely stooped posture with kyphosis; can be moderately leaning to one side.
4 = Marked flexion with extreme abnormality of posture.

29. Gait
0 = Normal.
1 = Walks slowly, may shuffle with short steps, but no festination (hastening steps) or propulsion.
2 = Walks with difficulty, but requires little or no assistance; may have some festination, short steps, or propulsion.
3 = Severe disturbance of gait, requiring assistance.
4 = Cannot walk at all, even with assistance.

30. Postural Stability (Response to sudden, strong posterior displacement produced by pull on shoulders while patient erect with eyes open and feet slightly apart. Patient is prepared.)
0 = Normal.
1 = Retropulsion, but recovers unaided.
2 = Absence of postural response; would fall if not caught by examiner.
3 = Very unstable, tends to lose balance spontaneously.
4 = Unable to stand without assistance.

31. Body Bradykinesia and Hypokinesia (Combining slowness, hesitancy, decreased armswing, small amplitude, and poverty of movement in general.)
0 = None.
1 = Minimal slowness, giving movement a deliberate character; could be normal for some persons. Possibly reduced amplitude.
2 = Mild degree of slowness and poverty of movement which is definitely abnormal. Alternatively, some reduced amplitude.
3 = Moderate slowness, poverty or small amplitude of movement.
4 = Marked slowness, poverty or small amplitude of movement.

IV. COMPLICATIONS OF THERAPY (In the past week)

A. DYSKINESIAS

32. Duration: What proportion of the waking day are dyskinesias present? (Historical information.)
0 = None
1 = 1-25% of day.
2 = 26-50% of day.
3 = 51-75% of day.
4 = 76-100% of day.

33. Disability: How disabling are the dyskinesias? (Historical information; may be modified by office examination.)
0 = Not disabling.
1 = Mildly disabling.
2 = Moderately disabling.
3 = Severely disabling.
4 = Completely disabled.

34. Painful Dyskinesias: How painful are the dyskinesias?
0 = No painful dyskinesias.
1 = Slight.
2 = Moderate.
3 = Severe.
4 = Marked.

35. Presence of Early Morning Dystonia (Historical information.)
0 = No
1 = Yes

B. CLINICAL FLUCTUATIONS

36. Are "off" periods predictable?
0 = No
1 = Yes

37. Are "off" periods unpredictable?
0 = No
1 = Yes

38. Do "off" periods come on suddenly, within a few seconds?
0 = No
1 = Yes

39. What proportion of the waking day is the patient "off" on average?
0 = None
1 = 1-25% of day.
2 = 26-50% of day.
3 = 51-75% of day.
4 = 76-100% of day.

C. OTHER COMPLICATIONS

40. Does the patient have anorexia, nausea, or vomiting?
0 = No
1 = Yes

41. Any sleep disturbances, such as insomnia or hypersomnolence?
0 = No
1 = Yes

42. Does the patient have symptomatic orthostasis?
(Record the patient's blood pressure, height and weight on the scoring form)
0 = No
1 = Yes

V. MODIFIED HOEHN AND YAHR STAGING

STAGE 0 = No signs of disease.
STAGE 1 = Unilateral disease.
STAGE 1.5 = Unilateral plus axial involvement.
STAGE 2 = Bilateral disease, without impairment of balance.
STAGE 2.5 = Mild bilateral disease, with recovery on pull test.
STAGE 3 = Mild to moderate bilateral disease; some postural instability; physically independent.
STAGE 4 = Severe disability; still able to walk or stand unassisted.
STAGE 5 = Wheelchair bound or bedridden unless aided.

VI. SCHWAB AND ENGLAND ACTIVITIES OF DAILY LIVING SCALE

100% = Completely independent. Able to do all chores without slowness, difficulty or impairment. Essentially normal. Unaware of any difficulty.
90% = Completely independent. Able to do all chores with some degree of slowness, difficulty and impairment. Might take twice as long. Beginning to be aware of difficulty.
80% = Completely independent in most chores. Takes twice as long. Conscious of difficulty and slowness.
70% = Not completely independent. More difficulty with some chores. Three to four times as long in some. Must spend a large part of the day with chores.
60% = Some dependency. Can do most chores, but exceedingly slowly and with much effort. Errors; some impossible.
50% = More dependent. Help with half, slower, etc. Difficulty with everything.
40% = Very dependent. Can assist with all chores, but few alone.
30% = With effort, now and then does a few chores alone or begins alone. Much help needed.
20% = Nothing alone. Can be a slight help with some chores. Severe invalid.
10% = Totally dependent, helpless. Complete invalid.
0% = Vegetative functions such as swallowing, bladder and bowel functions are not functioning. Bedridden.

Special Thanks to

Dr. Wajid

Dr. Rashda Abassi

Dr. Kehkashan Mazhar

Mrs Sadia Rehman

Mr. Amjab sb

Mr. Husnain sb.

Special Thanks to

Dr. Umair Qamar Usmani

Anwarullah

Sadia Sapara

Maleeha Ghafar

Madiha Sadiq