Examination of Huntington's Disease with Atypical Clinical Features in a Bangladeshi Family Tree and Study of Association of miRNAs Expression Level with the Diseased Condition

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A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Bachelor of Science in Microbiology

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DECLARATION BY THE RESEARCHER

This is to declare that the research work aggregating the results reported in this thesis entitled ―Examination of Huntington's Disease with Atypical Clinical Features in a Bangladeshi Family Tree and Study of Association of miRNAs Expression Level with the Diseased Condition" has been carried out by the undersigned under the joint supervision of Dr. Kaiissar Mannoor, Scientist and Head of ideSHi (Institute of Developing Science and Health Initiatives); and Professor Dr. Mahboob Hossain, Coordinator of Microbiology program, Department of Mathematics and Natural Science of BRAC University. It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Bachelors of Science in Microbiology, BRAC University, Dhaka.

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Dedicated To
My Greatest Strength
"My Mother"

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ABSTRACT

Hereditary neurodegenerative diseases are among the most serious and urgent problems of modern neurology. In Bangladesh perspective this is the first study on Huntington disease, mainly due to lack of initiatives, advanced laboratory facilities and funding opportunities. We have identified a family tree in Naogaon where one of two siblings developed a choreatic movement-related disorder almost 125 year ago while the other remained unaffected. The disorder was passed on to some progenies of subsequent generations. At present, 8 members of the affected family are suffering from this disorder. All progenies of the healthy sibling and their subsequent generations remained unaffected. The age of onset of the disorder varies between 20 to 65 years. Notably, the affected people of earlier generations had late onset of the disorder, whereas the affected people of the later generations are showing early onset of the disease. Magnetic Resonance Imaging (MRI) did not show any significant abnormalities in the brain of a seriously affected patient and therefore further investigations were necessary to determine the molecular diagnosis of the disorder. Our DNA- based studies showed that the concerned disorder was Huntington disease. Huntington Disease (HD) is a fatal autosomal dominant neurodegenerative disorder caused by the progressive loss of nerve cells in the brain characterized by motor, cognitive, and psychiatric disturbances. This disease is caused by genetic defect in the Huntingtin gene (HTT) on chromosome 4. Mutation in the HTT gene involves expansion of CAG tri-nucleotide repeat in exon 1. The normal length of CAG repeat stretch varies from 7 to 36 times in case of healthy person. However, in person with Huntington disease, it can be repeated for up to 250 times. Expansion of CAG triplet repeat stretch within the HTT gene results in a misfolded protein leading to gradual apoptosis of brain cells and progressively disordered involuntary movement and ultimately the patients succumb to death within 6-10 years after onset of the disorder. We first used PCR-based method to check the length of the HTT allele. All 4 unaffected subjects tested had typical HD allele length of 81-84bp (27 to 28 CAG repeats), while all the affected individuals were heterozygous, with one normal HD allele and one expanded allele. We used Sanger DNA sequencing to show that the normal allele had 20 CAG repeats, which were within the previously reported normal range of 6 to 35. The expanded allele had either 53 CAG repeats (7 patients) or 70 CAG repeats (1 patient), confirming the diagnosis of Huntington's disease. In addition, we have analyzed the expression patterns of miRNA 125b and miRNA 155 among HD affected patients and healthy controls because these

miRNAs are differentially expressed in other neurodegenerative disorders like Parkinson and Alzheimer's disorder. miRNA 155 increased about three fold in 1 HD patient among 8 patients compared to healthy controls. On the other hand, change of miRNA 125b expression was not significant in HD affected patients compared to unaffected controls. This is the first study on diagnosis of a neurodegenerative disease using a sequencing based approach in Bangladesh, paving the way for expansion of facilities for diagnosis of other genetic disorders.

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List of Abbreviation

HD	Huntington disease
WHO	World Health Organization
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
µL	Microliter
PCR	Polymerase chain reaction
UV light	Ultraviolet light
$MgCl_2$	Magnesium Chloride
TBE	Tris/Borate/EDTA
RNA	Ribonucleic Acid
BMRC	Bangladesh Medical Research Council

CHAPTER 1

Introduction

1.1 Background for the study

Huntington disease (HD) is a hereditary progressive neurodegenerative disorder which is inherited through an autosomal dominant fashion. The global prevalence for this disorder is not well studied; however prevalence of HD is 4-10 cases per 100,000 in Caucasian populations. The disease was named after the scientist Dr. George Huntington who first observed it. Huntington disease occurs because of genetic defect on chromosome 4. Abnormality in the gene that codes for Huntingtin protein is associated with this hereditary disorder. The defective gene encodes a protein which is larger than the normal Huntingtin protein. The change in protein structure occurs due to an increase in CAG repeat numbers in the patients' Huntingtin protein coding gene. Some of our brain cells are sensitive to the larger form of Huntingtin; it undermines their function and eventually destroys them.

There is a common myth about Huntington that it's a disease of elderly people just like Parkinsonism. Although HD symptoms can develop at almost any age, usual onset occurs between 35 and 55 years of age. Early indications of the illness include identity and behavioral changes, lack of concern, displeasure, trouble concentrating and loss of transient memory. Chorea, a type of involuntary movement is the most common symptom in HD and as the disease advances, these movement disorders become more severe and frequent. Late stage symptoms include dementia, difficulty initiating and continuing movements, severe chorea, inability to care for oneself etc. Huntington's disease affects certain nerve cells in the brain and causes brain damage. The part of the brain most affected is a group of nerve cells at the base of the brain collectively known as basal ganglia. The basal ganglia are involved in muscle-driven movement of the body. The cerebral cortex, which is involved in thinking, is another area of brain that also undergoes cellular damage in HD patients. Thus HD patients suffer from involuntary chorea as well as impairment in cognition.

In the present study, participants with movement disorders were enrolled from a family tree. After careful investigation and clinical data analysis they were suspected as suffering from Huntington disorder. Genetic markers were assessed for confirmation of Huntington's

disease. After confirming the disease as HD, expression of several miRNAs in whole blood was investigated.

1.2 Prevalence of Huntington disease

The prevalence of the Huntington's disease varies from one geographical region to another (Table-1.1). It is lowest in Asia (4 per 1 million) and highest in North America (73 per 1 million). The prevalence is 67 per 1 million, 56 per 1 million, 36 per 1 million, and 22 per 1 million in United Kingdom, Oceania, Western Europe, and Central and Eastern Europe respectively {Rawlins, 2016 #63}

Table 1.1 Geographical representation of prevalence of Huntington's disease.

Country	Number of studies	Study years	Average prevalence per 1 million
Asia	7	1957-2013	4
Central and eastern Europe	8	1981-2008	22
North America	6	1950-2012	73
Oceania	8	1981-2008	56
United kingdom	19	1950-2010	67
Western Europe	27	1930-2013	36

1.3 Heredity of Huntington's disease

Abnormal Huntingtin gene that is thought to be associated with Huntington's disease is inherited in autosomal dominant fashion (Figure-1.1). One copy of the abnormal gene either passes from father or mother to offspring will end up inheriting the disease and certain to suffer from all the complications associated with the disease.
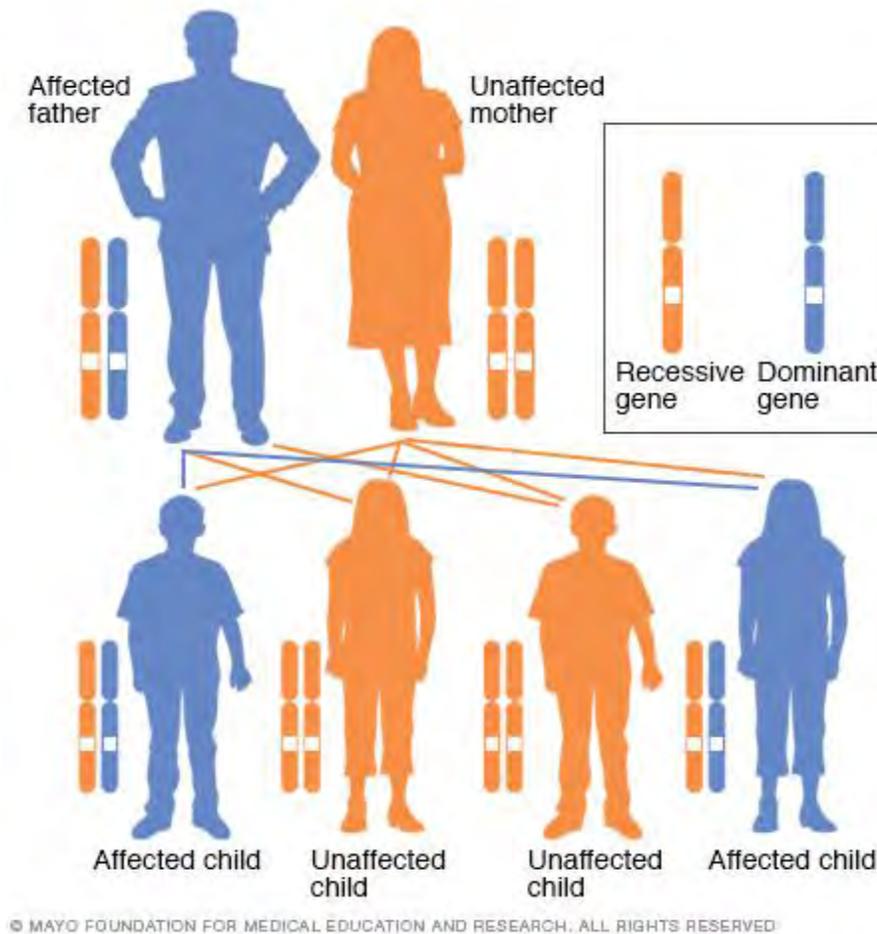
Figure 1.1: Inheritance pattern of Huntington's disease. Yellow colored chromosome in mother harbors the recessive normal gene or allele and blue colored chromosome in father carries the dominant faulty gene. The entire descendants who inherit disease causing dominant gene from father becomes affected with the disease.

1.4 Genes and locus associated with Huntington disease

One of the genetic factors responsible for Huntington's disease (HD) is an expansion of CAG repeats in the HTT gene {Keum, 2016 #3}. In healthy person CAG repeat length varies from 7 to 36 repeats in HTT gene. People with repeat number 37-40 may or may not be affected with Huntington's disease. On the other hand people with CAG repeat numbers more than 40 in HTT gene are certain to develop HD related symptoms and onset of disease has an association with this repeat number. However, in person with Huntington disease, it can be repeated for up to 250 times.

In addition to an increase in repeat numbers, there are other genetic factors such as SNPs and genetic modifiers which play important role in the disease onset and progression of the HD disease. It has been reported that genetic modifiers in loci 4p16 (LOD 1.93), 6p21–23 (LOD 2.29), and 6q24 –26 (LOD 2.28) has shown association with disease onset and progression of HD {Li, 2003 #62}

1.5 Abnormal Huntingtin protein in Huntington's disease

Abnormality in the gene that codes for Huntingtin protein is associated HD. The defective HTT gene with increased number of CAG repeats encodes for a protein which is larger than the normal Huntingtin protein. Expansion of CAG triplet repeat stretch within the HTT gene results in misfolding of this protein. Misfolded protein interacts with many other proteins expressed in brain cell and undermines their function and eventually destroys them. Abnormal Huntingtin protein causes brain cell damage by formation of inclusion body (protein aggregates), by damaging mitochondria, by activation of caspase pathway followed by apoptosis, by alteration of gene expression in neurons, by inducing proteosomal dysfunction, or by synaptic dysfunction (Figure-1.2).
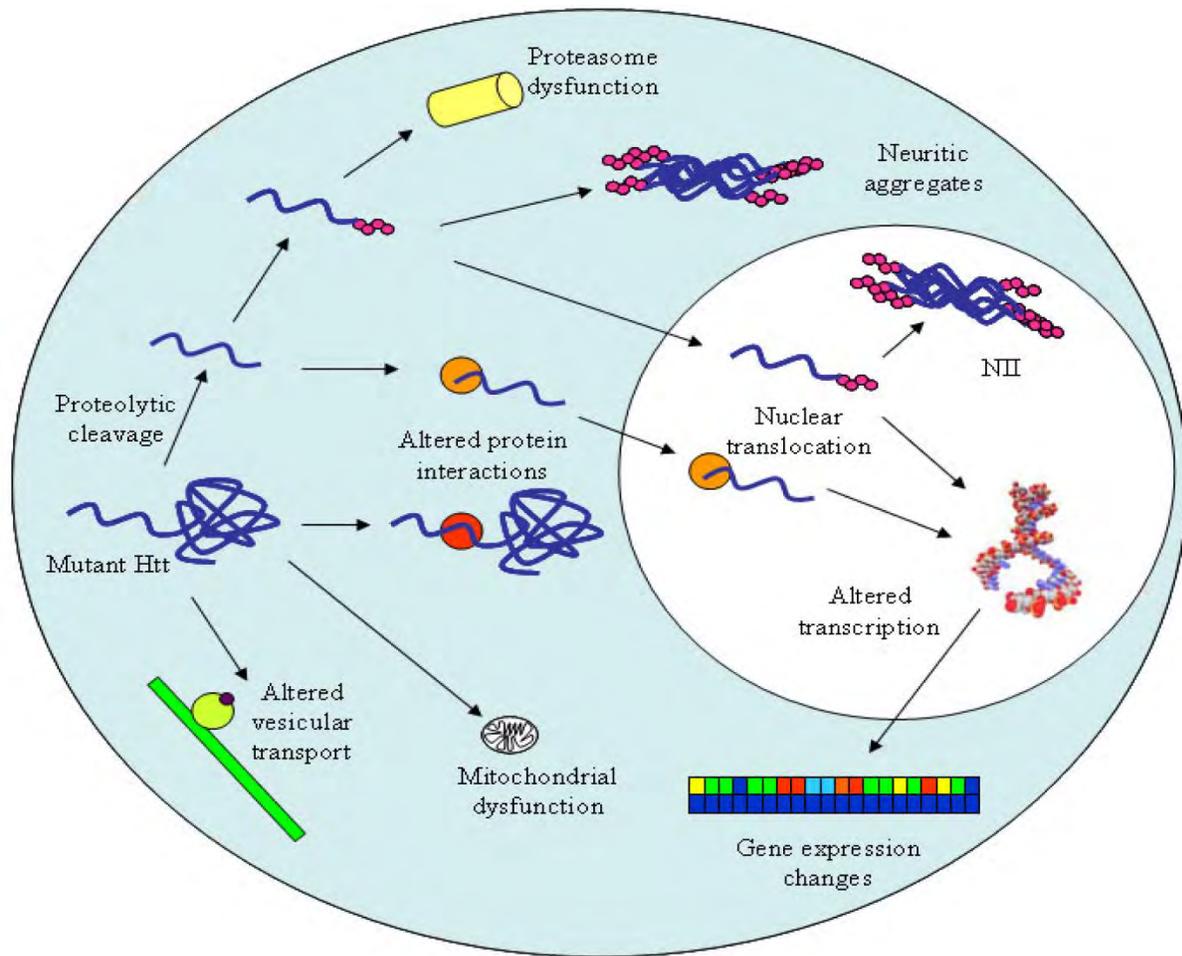
Figure-1.2: Pathogenic mechanism of mutant Huntingtin protein. Mutant Huntingtin protein causes mitochondrial and proteosomal dysfunction; alteration in gene expression and vesicular transportation; change in protein interaction and protein aggregation in neural cells.

1.6 miRNA dysregulation and Huntington's disorders

The microRNAs (miRNAs) are a class of short non-coding RNA molecules about 21-25 nucleotides in length that have attracted tremendous attention from the biological and biomedical research communities over the past few decades. The miRNAs play crucial role in regulation of gene expression. Dysregulation of miRNA expression in cells and tissues has been associated with numerous diseases. For example, miR-15and miR-16 dysregulation in cancer; miR-122 and miR-28 expression change in viral diseases; miR-21and miR-223 abnormal expression in immune system related diseases have been reported. Involvement of miRNA dysregulation with neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington

disease etc. (Table-1.2) is also noteworthy. Association of miR-30b, miR-30c, and miR-26a with Parkinson's disease; miR-29b-1, miR-29a, and miR-9 with Alzheimer's disease; and miR-10b-5p and miR-124 with Huntington disease has been demonstrated. However, Involvement of hyperactive immune system with neurodegenerative disorder has been reported and miRNA 125b and miRNA 155 play an important role in immunity; hence, we focused on the expression levels of miRNA 125b and miRNA 155 in Huntington patients {Li, 2012 #13}.

Table 1.2 Involvement of miRNAs in neurodegenerative diseases

Neurodegenerative diseases	miRNA
Parkinson's disease	miR-30b, miR-30c, miR-26a, miR-133b, miR-184*, let-7
Alzheimer's disease	miR-29b-1, miR-29a, miR-9
Huntington disease	miR-10b-5p, miR-124

1.7 Appearance of Huntington disease

The rate of disease progression and the age of onset vary from person to person. Adult-onset of HD, with its disabling, uncontrolled movements, most often begins in middle age. There are other imbalances of HD that can be differentiated not just by age but also by symptoms. For example, some individuals develop the disease during adulthood, but without chorea. They may appear rigid and move very little, or not at all, a condition called Akinesia. Some individuals develop HD symptoms before the age of 20. This is termed as juvenile HD and affects children and teenagers. A common sign of juvenile HD in younger people is absent mindedness in school performance. Symptoms can also include subtle changes in hand writing, movement problem like slowness, tremor, and rapid muscular twitching called myoclonus (Huntington's Disease: Hope Through Research 2016)

1.8 Signs and symptoms of Huntington disease

HD can affect certain nerve cells in brain in areas of basal ganglia and cerebral cortex which leads to disorders in movement, cognition and behavior. These changes in the brain lead to several symptomatic changes in case of control of movements and also changes in maintenance of normal behavior with surroundings. Some of the early, middle and late symptoms seen in Huntington's disease are listed in Table-1.3 {Warby, 2014 #9}

Table 1.3 Signs and symptoms of Huntington disease

Early symptoms	Middle symptoms	Late symptoms
Clumsiness	Dystonia	Rigidity
Agitation	Involuntary movements	Severe chorea (less common)
Irritability	Slow reaction time	Bradykinesia (difficulty initiating and continuing movements)
Apathy	Weight loss	Inability to walk
Anxiety	Stubbornness	Swallowing problems
Disinhibition	Inability to control speed and force of movement	danger of choking
Delusions	Trouble with balance and walking	severe chorea
Depression	Slow voluntary movements	Inability to walk
Abnormaleye movements	Speech difficulties	Inability to care for oneself

1.9 Huntington's Disease Diagnosis

Medical diagnosis of the onset of HD can be made following the appearance of physical symptoms specific to the disease (Walker, 2007), those symptoms include jerky movement or tremor of the figures or hands and abnormal movement of eye. Brain imaging can also help diagnosis of the disease. Damage in the basal ganglia or cerebral cortex is the indication of the disease onset and disease progression. However, genetic testing can be used to confirm Huntington's disease after initial physical diagnosis of the disease. Even before the onset of symptoms, genetic testing can confirm if an individual or embryo carries an expanded copy of the tri-nucleotide repeats in the HTT gene that causes the disease.

1.10 Treatment of Huntington's disease

There is no treatment to stop or reverse Huntington's disease; however there are some medications that can help keep symptoms under control. Treatment for HD includes the drug tetrabenazine, antipsychotic drugs, antidepressants, and tranquiliz.

1.11 Objectives of the study

1. To diagnose a choreatic movement-related disorder that first appeared in one of the two siblings almost 150 years ago and at present there are 8 patients in the affected family tree.
2. To identify the genetic basis of the disorder.
3. To analyze some miRNA expression patters associated with the disorder.

Chapter 2

Method and materials

2.1 Study participants

One and a half centuries ago common ancestors produced two family trees. One of the family trees was affected, and the other one remains unaffected. Eight patients from the affected family tree, exhibiting choreatic movement disorder and four controls from the unaffected family tree were enrolled into the study and followed up for two years.

2.2 Ethical Clearance

The objectives, benefits and potential effects due to specimen collection procedure were explained in details to the participants before enrollment and a written informed consent was obtained along with clinical information while collecting specimens. Ethical approval was obtained from the Ethical Review Committee (ERC) of Bangladesh Medical Research Council (BMRC), Dhaka, Bangladesh. The study was conducted in accordance with the guidelines of International Huntington Association (1994).

2.3 Study sites

Laboratory analysis of the specimens was conducted in the facilities of Institute for Developing Science & Health Initiatives (ideSHi) at Dhaka in collaboration with the Centre for Medical Biotechnology (CMBT), DGHS, Dhaka, National Institute of Neurosciences & Hospital (NINS), Dhaka, Bangladesh, Broad Institute of MIT and Harvard, USA, and Massachusetts General Hospital, USA. Specimens were collected from patients of the affected family tree and healthy controls of the unaffected family tree.

2.4 Brief outline

Blood samples was collected in EDTA coated vacutainers and in trizol LS. Genomic DNA was isolated using Flexi gene DNA kit (Qiagen,USA). Total RNA was isolated using trizol® LS. The miRNA mini kit (Qiagen,USA) was used for miRNA isolation. Two sets of huntingtin-specific primers were used to amplify the CAG repeat region of exon 1. Finally, genomic DNA sequencing

was done to confirm the CAG repeat length amplification as well as CAG repeat number. The miRNA expression pattern was observed by using miRNA assay kit (Applied Biosystems, USA).

2.5 Primers

Primers were designed to amplify the CAG repeat region of Huntingtin gene (Table-2.1). The following primer pair was used to determine the CAG repeat length of the Huntingtin gene by PCR and Sanger DNA sequencing.

Table 2.1- primers used for molecular diagnosis of HD

Name of the primer	Sequence(5'⟶ 3')
HD-F	5′-CCTTCGAGTCCCTCAAGTCCTT-3′
HD-R	5′-GCGGTGGCGGCTGTTG-3′

2.6 Specimen collection

2 ml of blood samples were collected from each of 12 participants including 8 patients from the affected family tree with choreatic movement disorder and 4 healthy controls from the unaffected family tree. The 8 affected patients and 4 unaffected healthy controls from whom specimens were collected belonged to the fifth generation of the affected and unaffected family trees, respectively. 125 µL of blood specimens were also collected from each participant in 750 µL Trizol LS and made 1ml with nuclease free water. Immediately after collection, specimens were stored at -70ºC.

2.7 Genomic DNA extraction from blood specimens

2.7.1 Principle

For genomic DNA extraction, Flexigene DNA kit was used.The kit consists of alysis buffer which causes lysis of cells and brings DNA out in the solution. Subsequent centrifugation causes the nucleic acid to precipitate at the bottom of the tube. After removal of supernatant, denaturing buffer is added. The presence of protease and chaotropic agents in denaturing buffer causes denaturation of protein. During this step, efficient removal of proteins and other contaminants

occurs. DNA is precipitated by addition of isopropanol. Precipitated DNA is washed with 70% ethanol, dried and resuspended in nuclease free water.

2.7.2 Procedure

Things to do before starting

1. Lyophilized QIAGEN Protease was resuspended in the following volumes of Buffer FG3 (hydration buffer): 0.3 ml when using 50 ml FlexiGene DNA Kit; 1.4 ml when using the 250 ml FlexiGene DNA Kit. Dissolved QIAGEN Protease was stored at 2-8ºC or in aliquots at -20ºC.

2. Buffer FG2/QIAGEN was prepared by mixing QIAGEN protease with buffer FG2 (Ratio should be 1 µl: 100 µl FG2 Buffer), once prepared mixture can't be used after 1 hour.

3. 75% ethanol was made beforehand as per requirement. [96% molecular grade ethanol is diluted to 75% according to the volume required for the total number of samples being used each time]

$$96\% \text{ ethanol required} = \frac{75}{96} \times \text{ Total volume of 75\% Ethanol be made}$$

Nuclease free water to add to make 75% ethanol = (Total volume of 75% ethanol to be made - 96% ethanol required).

4.   The water bath should be set at 65ºC.

2.7.3 DNA isolation

a)   500 µl Buffer FG1 was pipetted into a 1.5 ml Eppendorf. 200µl whole blood sample was added and mixed by inverting the tube 20 times.
b)   The tubes were centrifuged for 5 minutes at 10,000 x g.
c)   Supernatant was discarded and the tubes were left inverted on a clean tissue paper for 2 minutes, taking care that the pellet remains in the tube.
   [In some cases the pellet may be lost, so the supernatant needs to be poured out slowly.]
d)   100 µl Buffer FG2/QIAGEN Protease was added, mixed and vortexed immediately until the pellet is completely homogenized, centrifuged for 3-5s, and then placed in water bath at 65ºC for 5 min.

e) 100 µl isopropanol (100%) was added and mixed thoroughly by inversion (at least 20 times) until DNA becomes visible as threads or a clump, then centrifuged for 3 minutes at 10,000 xg.

f) The supernatant was discarded and the tube left inverted on a clean tissue paper for 5 minutes, making sure that the pellet is in the tube.

g) 100 µl 75% ethanol was added and vortexed for 5s.

h) The tube was centrifuged for 3 minutes 10,000 xg.

i) The supernatant was discarded and the tube was left inverted on a clean piece of tissue paper for at least 5 minute, taking care that the pellet remains in the tube.

j) The pellet was air dried for 5 minutes until all the liquid had evaporated. Care was taken not to over-dry the DNA pellet since that will make it very difficult to dissolve.

k) 50 µl nuclease free water was added and pipetted (up and down) several times.The DNA was then dissolved by incubating for 1 hour at 65ºC in a water bath. If the DNA was not completely dissolved, the solution was incubated overnight at room temperature.

2.7.4 DNA quantification

The quantity and quality of DNA was checked spectrophotometrically by using EON ELISA reader (BioTek, USA). The absorbance of the solution at 260nm represents the DNA concentration, while absorbance at 280nm represents protein concentration and absorbance at 230 nm for dissolved salts. A 260/280 ratio close to 1.8 represents DNA with little or no Protein contamination.

2.8 PCR amplification of CAG Repeats at HTT Locus:

2.8.1 Principle

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. In molecular biology, polymerase chain reaction (PCR) is a technique used for polymerization of nucleotides in a nucleic acid sequence and for amplification of a single copy or a few copies of a piece of DNA across several orders of magnitude, generating to millions of copies of a particular DNA sequence of interest (Figure 2.1). The nucleic acid sequence of interest is amplified using specific primers, which can later be used for analysis and for drawing a conclusion. By using PCR, it is possible to produce 2^{36} copies of desired DNA fragments from

a single template DNA copy after 36 cycles. Thus, it is possible to amplify DNA copy numbers from a very small amount of specimen.
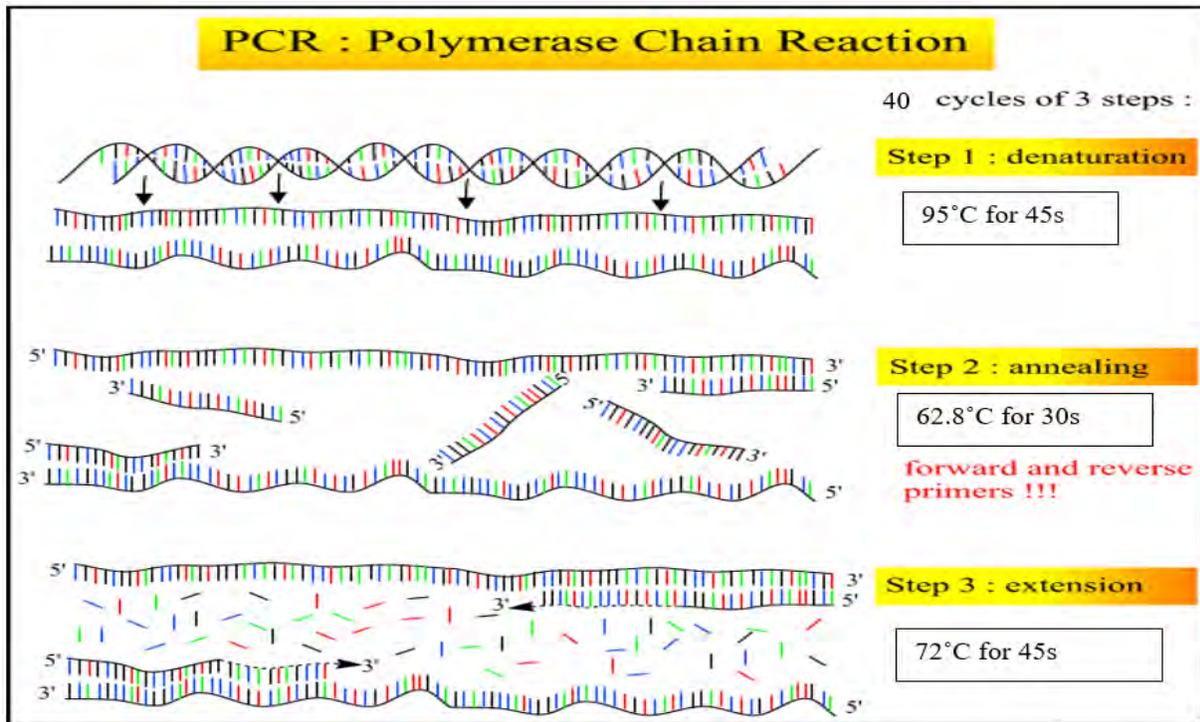
Figure 2.1-polymerase chain reaction (PCR)

2.8.2 Equipment and Supplies for polymerase chain reaction

2.8.2.1 Equipment

PCR machine

Electrophoresis machine

Micropipettes

Micro-centrifuge tubes

PCR cabinet

Refrigerators (-20° C, +4° C)

2.8.2.2 Reagents

The reagents that were used for polymerase chain reaction are given along with their storage temperature in the table-2.2 below

Table 2.2-Reagents used for PCR and their storage temperature

Reagents	Temperature
10X Buffer (MgCl$_2$)	-20°C
25 mM MgCl$_2$	-20°C
2.5 mMdNTPs	-20°C
Primer (Forward and reverse)	-20°C
Taq polymerase (Takara Taq and Hot start Taq)	-20°C
Q-solution (provided with Hot start Taq polymerase)	-20°C
Template DNA	-20°C
Nuclease-free water	Room temperature

2.8.3 PCR amplification and sequencing of PCR product

DNA was amplified in a final reaction volume of 20 μL by using HD-F primer (5′-CCTTCGAGTCCCTCAAGTCCTT-3') and HD-R primer (5′-GCGGTGGCGGCTGTTG-3′). Each reaction mixture contained 0.25 μmol/L of each primer, 0.4 mmol/L of each deoxynucleoside triphosphate, 0.75 U of DNA polymerase (Takara), 10% DMSO, 1× of the supplied PCR buffer, and 100 ng genomic DNA. For PCR, an initial denaturation step at 95 °C for 5 min was followed by 35 cycles at 95 °C for 45s, 62.8 °C for 30s, and 72 °C for 45s and final extension at 72 °C for 10 minutes. The PCR products were characterized by high resolution agarose gel (2%) electrophoresis and visualized under ultraviolet trans-illuminator (BioRad, USA). Amplified PCR products were purified from agarose gels using gel extraction kit (Qiagen,USA) according to the manufacturer's protocol and used for sequencing purpose.

2.9 Agarose Gel Electrophoresis

2.9.1 Principle

Gel electrophoresis involves the electric charge and size of PCR products or DNA fragments to distinguish or detect fragments of different length. During electrophoresis, an electrical field is applied so that the negatively charged DNA fragments move towards the positively charged electrode (anode) through the pores in the gel. The velocity of movement is inversely proportional to the molecular weight of the DNA molecule. Therefore, the largest molecules will have most difficulty passing through the pores, whereas the smallest molecules are relatively unhindered, moving the fastest. Usually, 1% or 2% (w/v) gel is prepared for visualization of DNA fragments.

2.9.2 Materials

a) Ultrapure agarose (Invitrogen, CA, USA)

b) TBE buffer

c) Gel loading dye

d) Gel red

2.9.3 TBE buffer (10X stock solution)

a) 108 g Tris Base
b) 40mL of 0.5 M EDTA
c) 55 g Boric Acid

Take above mentioned components in a 1 L bottle and add 700 mL filtered deionized water. Put the bottle in a magnetic stirrer and run the machine and wait till all components get dissolved. Then adjust the P^H to 8.3 by HCl and finally make the volume 1 L adding required amount of filtered deionized water.

2.9.4 Preparation of 1X TBE buffer

For preparation of 1000 ml of 1X TBE buffer 100 mL of 10X stock is added with 900 mL of filtered deionized water.

2.9.5 Preparation of 2.0% Agarose Gel

To prepare 100ml of 2.0% agarose gel, 2.0g of agarose powder was weighed and transferred in a conical flask. 100 ml 1X TAE buffer was poured into flask containing agarose and then melted in microwave oven at 60^0C for 2-3 minutes. 1.0 µL of Gel Red was added, and the flask was swirled to mix. The melted agarose gel was poured carefully into the gel tray that had a comb fixed in place near one end so that loading wells could be formed in the gel. Then the gel was allowed to solidified by placing it at the room temperature for at least about 15-20 mins. After the gel had solidified, the comb was removed gently, and the gel tray was placed within the electrophoresis tank.

2.9.6 Electrophoresis of PCR product

Electrophoresis was carried out on a horizontal slab gel apparatus (Bio-Rad, USA) fill with 1X TBE buffer. PCR products were loaded in wells of gel mixing with 6X loading dye (Invitrogen, CA, USA) and the electrophoresis was carried out at 150 volts. After completion of electrophoresis, the PCR products in agarose gel were visualized under ultraviolet trans-illuminator (BioRad, USA).

2.10 Expression analysis of miRNAs

TaqMan® MicroRNA Assays were used for quantification of miRNAs in Huntington (HD) patients and non-HD controls. Stem-looped primers were used for reverse transcription and a sequence specific TaqMan® assay was employed to accurately detect mature miRNAs. Procedural overviews are as follows:

Whole blood of HD patients and non-HD controls in Trizol® LS

↓

Isolation of total RNA

↓

Reverse transcription of target and internal control miRNAs using sequence specific primers

↓

Real time qPCR for miR expression analysis using miRNAs sequence specific primers and probes

↓

Analysis of real time qPCR data to get the cycles of threshold for samples and controls

↓

Calculation of change in the relative miRNA expression in HD patient compared to non-HD controls

2.10.1 RNA isolation

2.10.1.1 Principle for total RNA isolation

Mixing of homogenized sample in Trizol® LS with chloroform and subsequent centrifugation leads to separation of three phases. The upper aqueous layer contains RNA; the interphase contains a small amount of DNA and proteins, and the lower organic phase contains the majority of DNA, proteins, lipids and other cellular debris. Application of upper aqueous layer in a column embedded with nitrocellulose membrane causes RNA to bind to the membrane. Bound

total RNA then can be eluted into a collection tube employing nuclease free water into column and subsequent centrifugation.

2.10.1.2 Equipment and reagents for RNA extraction:

2.10.1.2.1 Equipment

a) Micropipettes

b) Sterile, RNase-free pipette tips

c) 1.5 mL and 2.0 mL micro-centrifuge tubes

d) Bio-safety hood

e) Vortex mixture

f) Water bath

g) Centrifuge machine

2.10.1.2.2 Reagents

a) Trizol$^®$ LS

b) Chloroform

c) Isopropanol

d) Ethanol

Table 2.3 Reagents and their storage temperature

Storage temperature	Reagent
4°C	Trizol$^®$ LS reagent
Room temperature	Chloroform
Room temperature	100% Isopropanol
Room temperature	100% Ethanol
Room temperature	RNase-free water

2.10.1.3 Procedure

2.10.1.3.1 Sample preparation

125 µL of whole blood was thoroughly mixed with 750 µL of Trizol® LS (Invitrogen, USA) and 125 µL of nuclease free water and incubated at room temperature for 5 minutes. Prepared mixture of whole blood was then used for extraction of total RNA.

2.10.1.3.2 Phase separation:

100 µL Chloroform was added to the homogenized mixture and then incubated for 15 minutes at room temperature. After completion of incubation the mixture was centrifuged at 14000 × g for 15 minutes at 4°c. Following completion of centrifugation the upper aqueous phase was aspirated and transferred into another micro-centrifuge tube without drawing the inter phase or organic phase.

2.10.1.3.3 Total RNA isolation using QIAzol reagent

a) 5 volumes of QIAzol reagent (Qiagen, Germany) was added to the separated upper aqueous phase and mixed by vortexing.

b) The tube containing the Qiazol mixture was placed on benchtop at room temperature for 5 minutes.

c) Following incubation, 125 µL of chloroform was added to mixture and vortexed for 15 s.

d) The tube was then incubated at room temperature for 2–3 mins.

e) After completion of incubation the chloroform-Qiazol mixture was centrifuged for 15 min at 12,000 x g at 4°C.

f) After centrifugation the upper aqueous phase was transferred to a new collection tube. 1.5 volumes of 100% ethanol were added and mixed thoroughly by pipetting up and down several times.

g) 700µl of the mixture was pipetted, including any precipitate that may had formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). The lid was gently closed and centrifuged at 8000 x g (10,000 rpm) for 15 s at room temperature. The flow-through was discarded.

h) Step-g was repeated using the remainder of the sample. The flow through was discarded.

h) Buffer RWT (700 μl) was added to the RNeasy MinElute spin column. Centrifugation was done for 15 s at 8000 x *g* (10,000 rpm) to wash the column. The flow-through was discarded.

i) Following step-i, Buffer RPE 500 μl was added into the RNeasy MinElute spin column. Centrifugation was done for 15 s at 8000 x *g* (10,000 rpm) to wash the column. The flow-through was discarded.

j) 500 μl of 80% ethanol was pipetted onto the RNeasy MinElute spin column. The lid was closed gently and centrifugation was done for 2 min at 8000 x *g* (10,000 rpm) to wash the spin column membrane. The collection tube containing the flow-through was discareded.

k) RNeasy MinElute spin column was placed into a new 2 ml collection tube. Centrifugation was done at full speed for 5 min to dry the membrane. The collection tube with the flow-through was discarded.

l) The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube. For elution of the RNA, 20μl of RNase-free water was added directly to the center of the spin column membrane, the lid was closed gently, and centrifuged for 1 min at full speed.

m) The column was discarded and the RNA in flow through was stored at -80^0c or used immediately after extraction.

2.10.1.3.4 Precaution

Since RNase is a ubiquitous enzyme, the hood was cleaned before starting the process and special care was taken to ensure an RNase-free environment at all time during the process.

2.10.2 Reverse transcription

2.10.2.1 Input quantity

For each sample 10 ng of total RNA was used per 15 μL RT-mixture.

2.10.2.2 Preparation of the RT reaction master mix

a) The reverse transcription (RT) kit components were allowed to thaw on ice.

b) The RT master mix was prepared in a 0.2 mL polypropylene tube on ice by scaling the volumes listed below (Table2.4) for each sample.

Table 2.4- reagents used for RT master mix

Component	Master mix volume per15-µL reaction
10 mM dNTPs (with dTTP)	1.0 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.0 µL
10✕ Reverse Transcription Buffer	1.0 µL
5✕ RT primer	3.0 µL
RNase Inhibitor, 20 U/µL	0.2 µL
RNA	10 ng
Nuclease-free water	Upto15 µL

c) The tube was capped properly and the solution mixed thoroughly by inverting and then spun briefly.

d) The tube was incubated on ice for 5 minutes and kept on ice until ready to load on the thermal cycler.

2.10.2.3 Performing reverse transcription

a) The following program (Table-2.5) was set in the thermal cycler:

Table 2.5 Thermal cycling profile of reverse transcription

Step	Time	Temperature
	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	12°C

b) The reaction volume was set to 15.0 µL.

c) The reaction tubes were loaded into the thermal cycler and the PCR reaction was run.

2.10.3 Performing the real time qPCR

2.10.3.1 Thawing and mixing of the reagents

a) The following reagents were thawed on ice, resuspnded completely by gentle vortexing, and then centrifuging briefly:

- o TaqMan® Assay (20✕)

- o Complementary DNA (cDNA) samples

b) The master mix reagent was mixed by gently swirling the bottle.

2.10.3.2 Preparation of the qPCR reaction mix

To prepare the qPCR reaction mix following components were (Table 2.6) mixed together:

Table 2.6- reagents used for qPCR

Component	Volume per 10 µL Reaction	
	Single reaction	**Three replicates**
TaqMan® Small RNA Assay (20✕)	0.5 µL	1.5 µL
Product from RT reaction*	1.0 µL	3.00 µL
TaqMan® Universal PCR Master Mix II (2✕), no UNG‡	5.0 µL	15.0 µL
Nuclease-free water	3.5 µL	10.5 µL
Total volume	10.0 µL	30.00 µL

2.10.3.3 Preparation of the PCR reaction plate

a) For each sample 10 µL of qPCR reaction mix (including assay and RT product) was transferred into three wells on a 96-well plate.

b) The plate was sealed with the appropriate cover.

c) Centrifuged briefly.

d) The plate was then loaded into the instrument.

2.10.3.4 Setting up the experiment or plate document and run the plate

a) In the real-time PCR system software, an experiment or plate document was created using the following parameters:

   I.   Run Mode: Standard

   II.   Sample Volume: 10 µL

   III.   Thermal Cycling Conditions: as provided in Table 2.7

Table 2.7- thermal cycle for qPCR

Step	Enzyme Activation	PCR	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/extend
Temperature	95 °C	95 °C	60 °C
Time	10 minutes	15 seconds	60 seconds

b) After completion of real time PCR machine set up the reaction was run.

2.10.3.5 Data analysis

The general process for analyzing the data from gene expression assays involves the following procedures:

a) Viewing the amplification plots.

b) Setting the baseline and threshold values (C_q) for samples and controls.

c) Relative quantitation using the following equation. miRNA16 was used as an internal control:

$2^{-\Delta\Delta C_t} = 2^{-\{(\text{target miR} - \text{miR16}) \text{ expression of HD patients} - (\text{target miR} - \text{miR16}) \text{ expression of non-HD controls}\}}$.

3.1 Pedigree analysis

In pedigree analysis, family history was considered to be HD suspect when one parent had an ambiguous history or had died or when the family history disclosed a movement disorder. If information was not available, the family history was considered to be unknown. Age of onset was termed as the age at which the patients started showing symptoms like involuntary movements, behavioral disorders, or characteristic changes {Siesling, 2000 #62}.

■: Male patient
□: Healthy male
●: Female patient
○: Healthy female
/: dead patient
? HD positive/negative not
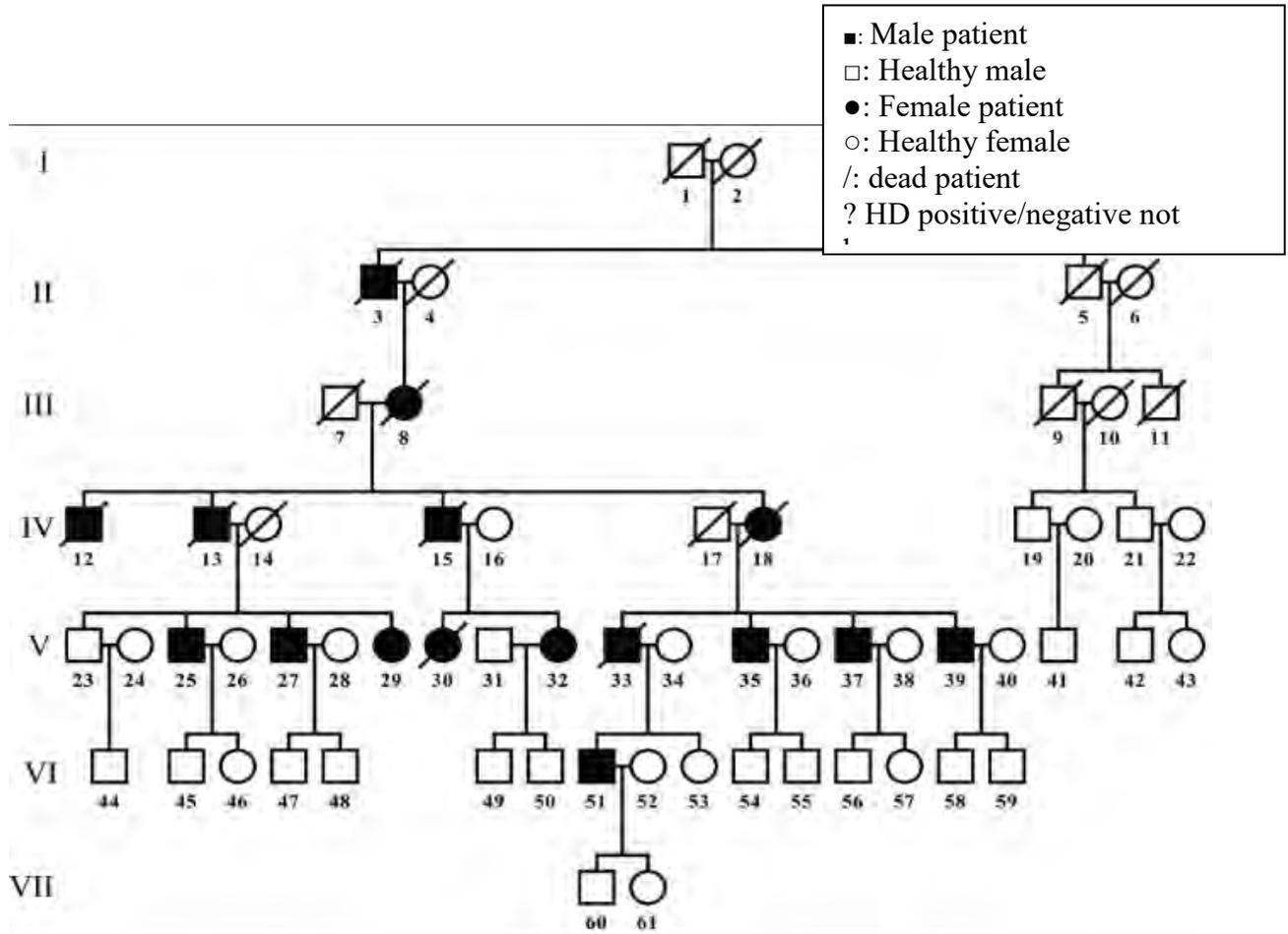
Figure 3.1: Pedigree of family tree affected with genetic disorder and distantly related unaffected family tree. Pedigree analysis was performed using free pedigree drawing tool Haplo Painter (Version: 1.043). Squares indicate males and circles indicate females. Individuals affected with genetic disorders are presented as black symbols while white symbols indicate

unaffected individuals. The Roman Numerals indicate generations, whereas individual IDs beneath each symbol are represented by Arabic numerals.

1st Generation: 1 and 2 was parent of HD suspect 3 and normal 5. Information is not available on whether one of the parents (1 or 2) of 1st generation was affected or not with the genetic disorder.

2nd Generation: It has been known that patient designated as 3 faced choreiform movements at the age of ~66 years and succumbed to the disorder at ~81 years of age. However, patient designated as 5 had died a normal death without showing any HD or HD-like movement disorders and subsequent generations of 5, designated as 9, 11, 19, 21, 41, 42, and 43 had no HD or HD-like positive history.

3rd Generation: The patient designated as 8 had been known to be affected by movement disorder at the age of ~58 and died ~13 years following onset of the disease.

4th Generation: The patient designated as 8 had three sons & one daughter, and they all died of movement disorders: The movement disorders started appearing at the age of 42, 45, 44 & 43 respectively in offspring of patient 8, designated as 12, 13, 15 & 18.

5th Generation: The patients designated as 30 & 33 died due to the same movement disorder as 5th generation, and age of disease onset for the former was 32 years & that of latter one was 36 years. No consanguineous marriage has been known among the family members. However, pedigree analysis, pattern of genetic inheritance and family history were unable to confirm whether the study participants with neurodegenerative movement disorders were HD or HD-like disease (Figure 3.1). In addition, clinical features of some patients resembled that of Wilson's disease which have been described later, further highlighting the importance of differential diagnosis. Pedigree analysis shows that the patterns of inheritance were independent of gender bias and there is no skipping of inheritance, indicating that the disorder might be of Autosomal dominant type.

3.2 Background information on deceased and living patients of the affected family

According to pedigree, 8 people of different generations had died due to inherited disorder. All patients succumbed to motor disorders and it has been known that these patients were

unable to move and completely bed ridden three to six months before death. There was a gradual decrease in age of onset of disease symptoms with each successive generations followed by deaths between 14 to 19 years after onset of illness (Table 3.1). Both males (n=4) and females (n=4) were affected with the disorder. Transmission traits were of both paternal and maternal origins. The mean age between disease onset and death of these diseased people was 16 years. However, these patients died without any knowledge of the genetic etiology of the disorder. On the other hand a further decrease in age of onset was observed for the subsequent generations of living people affected with movement disorders compared to their deceased counterpart (Table 3.2). That is, like the deceased people, the affected people of any generation had earlier onset of the disease than that of any previous generations. Both maternal and paternal origins of transmissions were observed.

Table 3.1: Patterns showing age at onset and death, duration of illness, motor status, and transmission traits for deceased people of the affected family. All information gathered was dependent on family records of the ancestors.

Pedigree ID	Age at Onset	Age at Death	Duration of Illness	Sex	Choreatic Movement	Transmission	Familial History of inherited disorder
3	~66	85	19	Male	+	Not available	Not available
8	~58	76	18	Female	+	Paternal	Yes
12	42	58	16	Male	+	Maternal	Yes
13	45	59	14	Male	+	Maternal	Yes
15	44	60	16	Male	+	Maternal	Yes
18	43	57	14	Female	+	Maternal	Yes
30	32	47	15	Female	+	Paternal	Yes
33	27	45	18	Male	+	Maternal	Yes

Table 3.2: Patient's background information. The study participants of the affected family members designated as 25, 27, 29, 32, 35, 37, 39, and 51 indicate patients with movement disorder, whereas the study participant designated as 44 indicate a control of the affected family member without showing any clinical signs and symptoms including movement disorder. On the other hand, the study participants designated as 19, 41, and 42 of the unaffected family tree indicate healthy controls.

Participant ID	Age at Onset (Year)	Present Age (Year)	Duration of Illness (Year)	Sex	Suspect HD Familial History (Pedigree Analysis)	Inheritance (Pedigree Analysis)
25	32	36	4	Male	+	Paternal
27	31	32	1	Male	+	Paternal
29	33	39	6	Female	+	Paternal
32	34	35	1	Female	+	Paternal
35	35	40	5	Male	+	Maternal
37	31	33	1	Male	+	Maternal
39	30	31	1	Male	+	Maternal
44	-	25	-	Male	+	Paternal (Grandpa)
51	20	25	5	Male	+	Paternal
41*	-	26	-	Male	-	Not found
42*	-	25	-	Male	-	Not found
19*	-	75	-	Male	-	Not found

3.3 Clinical characteristics of eight living patients of affected family tree

Clinical information was obtained for all 8 patients suspected of having neurodegenerative disease(s) (Table 3.3). All of the patients (100%) exhibited mild jerky movement of fingers and

slow movement of eyes as the first detectable clinical symptoms. Four of the patients (50%) exhibited moderate to severe chorea, whereas the remaining 4 cases (50%) did not

exhibit any choreatic movement disorder except mild jerky movement of fingers and slow movement of eyes. All 8 patients (100%) seemed normal in terms of cognitive function because there was no significant dementia as manifested by mini mentalist test (Table 3.3). In addition, 100% (all 8 cases) did not show any psychiatric disturbances. Although 3 cases (37.5%) showed characteristic features of anxiety, there was no tendency of committing suicide among the suspected study participants. Notably, suicidal tendency has not been observed in any suspected living or dead members of the affected family tree irrespective of generations.

Table 3.3: Clinical evaluations of eight living patients of affected family tree. Clinical signs and symptoms were determined by observation of movement disorders including motor symptoms as well as examination of mini metal status manifested by dementia and psychiatric disturbances. In addition, all the parameters for clinical evaluations were investigated for healthy controls from both affected and unaffected family.

Sample ID	1st Clinical Symptom	Prior Clinical Diagnosis	Motor Symptoms	Dementia	Psychiatric Disturbances
25	a. mild involuntary movement of fingers b. mild movement of eye	Chorea of unknown cause	+	-	Depression
27	a. mild involuntary movement of fingers b. mild movement of eye	Normal	-	-	-
29	a. mild involuntary movement of fingers b. mild movement of eye	Chorea of unknown cause	+	-	-
32	a. mild involuntary movement of fingers	Normal	-	-	-

	b. mild movement of eye				
35	a. mild involuntary movement of fingers b. mild movement of eye	Chorea of unknown cause	+	-	-
37	a. mild involuntary movement of fingers b. sudden feelings of agitation at right hand	Normal	-	-	-
39	a. mild involuntary movement of fingers	Normal	-	-	-
51	a. mild involuntary movement of fingers b. mild movement of eye	Chorea of unknown cause	+	Slight dementia	-
44	No visible symptom	Normal	-	-	-
41	No visible symptom	Normal	-	-	-
42	No visible symptom	Normal	-	-	-
19	No visible symptom	Normal	-	-	-

It is mentionable here that the patient designated as 35 had severe chorea characterized by balance trouble, clumsiness, tremor, significant weight loss, fidgeting, facial grimaces, increased appetite, less control over hand-writing, rigidity, speech difficulties, gruntingand abnormal speech patterns, inability to control speed and force of movement, general weakness and impairment of superficial sensation and it was suspected that the patient had been suffering from Wilson's disease. However, analysis of blood parameters like T. bilirubin, ALT and ceruloplasmin well as Kayser-Fleischer (K-F) Ring test did not show any abnormal results (data not shown), excluding the possibility of Wilson's disease. Also, MRI examination of the patient's brain did not reveal any pathology typical of Huntington's disease (Figure 3.2).
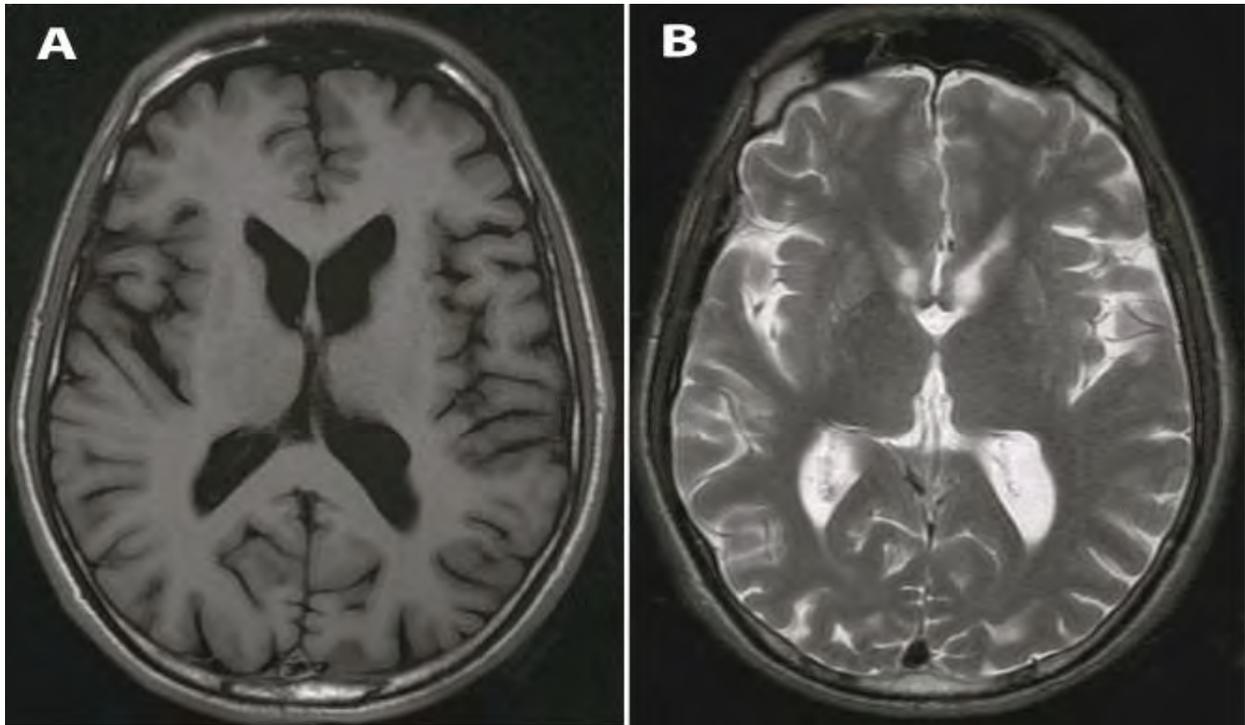
Figure 3.2: **A representative brain MRI examination of patient with severe choreatic movement disorder.** Bio-images of brain were obtained using the 1.5 T MRI systems (GE Healthcare, UK), with transverse T1- and T2-weighted scans at the level of basal ganglia. Fig. 1A indicates T1-weighted scan, whereas Fig. 1B indicates T2-weighted scan.

Putamen and Caudate are the primary sites to be affected for the structural changes in Huntington's disease. It is thought that putamen involvement affects some motor functions and caudate involvement affects cognitive functions. Changes have not been observed in the caudate nucleus and striatum regions like putamen and globus pallidus. However, mild cerebellar atrophy, otherwise unremarkable MRI findings were observed.

3.4 Molecular diagnosis

Next we wanted to investigate whether the patients were suffering from HD or HD-like disorders. DNA-based assays including PCR indicated that the patients were not suffering from HD-like disorders. Surprisingly, HD primer-specific PCR and DNA sequencing results confirmed that every member of the affected people with the movement disorder had an

abnormally expanded CAG repeat unit in the mutant IT15 allele (~200-250bp) and a normal HD allele showing CAG repeat length in the range of ~80-85bp (Figure 3.3). PCR and DNA sequencing data indicated that 7 specimens (87.5%) from affected family tree contained 53 CAG repeats and 1 specimen had 70 CAG repeat units in mutant HTT allele, and 20 CAG repeats in normal HD allele (Table 3.4).
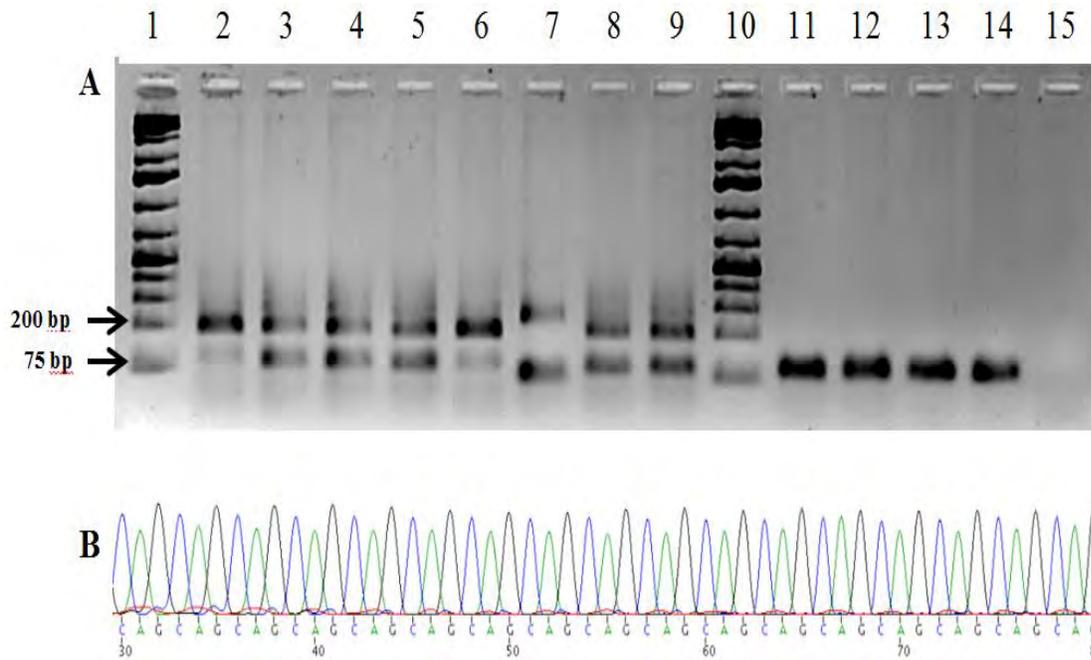
Figure 3.3: (A) Agarose gel (2.0%) electrophoresis of human HTT gene polymerase chain reaction of 8 patient's specimens and 4 healthy controls, stained with GelRed™ (Biotium Inc., Hayward, CA, USA) and photographed under UV light. Lane 1 & 10 , denotes 1 kb[+] Ladder (Thermo Fisher Scientific (Fermentas)) ; Lanes 2-9, denotes patient's specimen, Lanes 11-14, denotes healthy control's specimen and Lane 15, denotes template negative control. **(B)** A representative partial sequencing chromatogram for a HD suspected patient. Gel extracted PCR product for the patient designated as 29 (in pedigree analysis) were used for Sanger DNA sequencing.

Table 3.4: Calculation of CAG repeat units based on HTT gene PCR gel photograph and Sanger DNA sequencing of the purified PCR products. Molecular diagnosis was made based on HTT mutant allele-specific expanded PCR amplicons followed by confirmation of CAG repeat units by DNA sequencing. (* indicates healthy controls).

Sample ID	CAG Repeat Length (Mutant Allele)	CAG Repeat Length (Normal Allele)	Final Diagnosis
25	53	20	HD
27	53	20	HD
29	53	20	HD
32	53	20	HD
35	53	20	HD
37	53	20	HD
39	53	20	HD
51	70	12	Juvenile HD
44*	-	20	Normal
41*	-	20	Normal
42*	-	20	Normal
19*	-	20	Normal

3.5 Observation of anticipation for the genetic disorders diagnosed as Huntington's disease

Anticipation is most often seen with Huntington's disease. So we wanted to investigate whether the data of the present study could support such view. The results in (Figure 3.4) demonstrate that the age of onset of the disorder tended to decrease with each successive generation. For the affected people of each successive generation had developed the disease 11±1 years earlier on average than their affected parents, supporting the anticipation hypothesis in Huntington's disease.
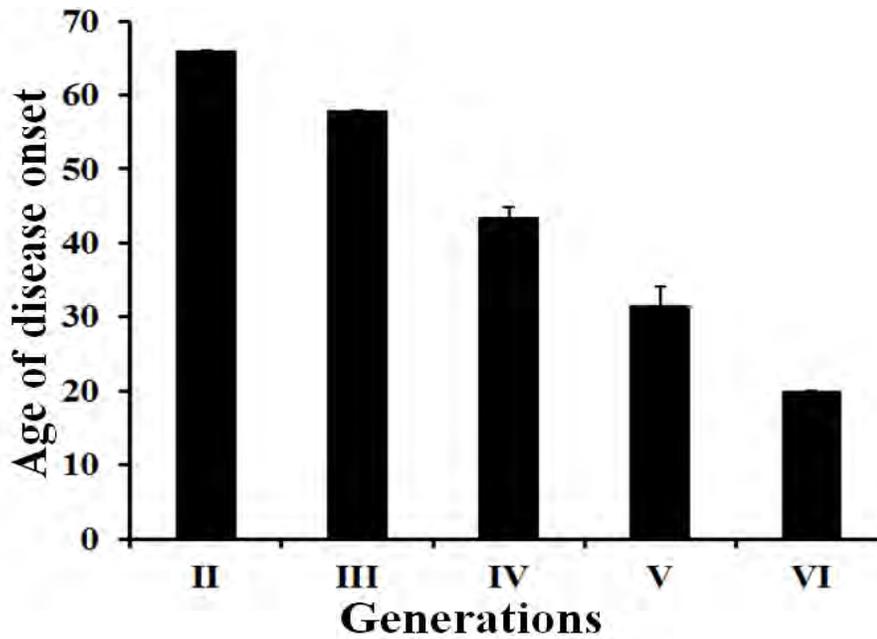
Figure 3.4: Observation of anticipation following diagnosis of Huntington's disease. All living and dead patients were included to calculate differences of mean age between 2 consecutive generations, namely generations 4 and 5. Sample sizes were not enough to calculate mean ages for generations 2, 3, and 6. A $p < 0.05$ was considered significant.

3.6 Analysis of changes of miRNAs expression in Huntington participants

$2^{-\Delta\Delta C_t}$ method was used for analysis of fold changes of miR155 and miR125b in participants with Huntington disease compared to non-diseased controls. The miR-16 was used as internal control for observation of change in relative expression of miR155 and miR125b in Huntington patients.

3.7 miR-155 expression analysis

It was hypothesized that miR-155 expression will change in whole blood of HD patients. The multifunctional miR-155 has significant role in immunity. As there have been reports on involvement of immunity in HD brain damage, so we wanted to check miR-155 expression in HD patient and non-HD controls. Real time PCR result is presented in Figure-3.5. The Table-3.4 and the Figure-3.6 present information on changes of miR155 expression pattern in HD participants compared to controls. The miR-155 expression was more or less similar in Huntington patients and controls except one patient, designated as 29 who was also suffering from Thalassemia. MiR-155 increased by three folds (2-4 folds compared to controls) in participant-29 compared to non-HD controls. Thus, form our study we report that miR-155 expression does not change in whole blood of HD patient, but combination of thalassemia and HD or thalassemia alone can cause change in the expression of miR-155 in patients.

Figure-3.5: Demonstration of miRNA155 expression in Huntington patients and controls. The fluorescence curves showing early appearance (10-15 cycles) of Ct (cycle of threshold) values indicate expression of miR-16. On the other hand, curves with Ct values

Table 3.5 Expression of miR155 in HD patients.

Pedigree ID	Fold change ($2^{-\Delta\Delta C}t$)				Mean fold changes	Standard deviation of fold changes
	Control-1	Control-2	Control-3	Control-4		
25	1.26	1.45	1.62	0.8	1.28	0.35
27	1.16	1.33	1.49	0.73	1.18	0.32
29 HD+Thalassemia	3.11	3.58	4.01	1.97	3.17	0.87
32	0.91	1.04	1.16	0.57	0.92	0.25
35	0.93	1.07	1.19	0.59	0.94	0.26
37	1.22	1.4	1.56	0.77	1.24	0.34
39	1.07	0.93	0.83	1.69	1.13	0.38
51 Juvenile HD	0.65	0.75	0.84	0.41	0.66	0.18

- Participants designated as 25,27, 29, 32, 35, 37, 39, and 51 denote 8 Huntington's disease patients. Four unaffected persons from the the same family tree were used as controls, namely, control-1, control-2, control-3, and control-4).Participants 25,27, 32, 35, 37, 39 and 51 had HD and participant 29 had comorbid condition of HD and thalassemia
- $\Delta\Delta Ct$ = (miR155 – miR16) expression of HD patients - (miR155 – miR16) expression of non-HD patients
- Fold change in miR-155 in HD patients compared to control = $2^{-\Delta\Delta C}t$

Figure 3.6- Relative changes in the expression of miR-155 in HD patients compared to controls. Eight patients are designated as 25, 27, 29, 32, 35, 37, 39, and 51. Relative fold changes (mean ± SD) in miR155 expression levels in HD patients are shown using different colored bars with respect to four controls.

3.8 miR125b expression analysis

The miR-125b plays a role in immunity and also controls cognitive and behavioral modes. We compared miR-125b expression levels between HD and non-HD participants to see whether this miR was associated with cognitive and behavioral changes that occurred in HD patients. Real time PCR results for miR-125b expression in HD and non-HD participants have been presented in Figure-3.7. A decrease in miR-125b level was observed in two patients designated as participant-25 and participant-44 (Table 3.6 and Figure 3.8). On the other hand, miR-125b expression was higher in participant-35. Lower levels of expression of miR-125b could be an inducer of NF-κB which is known to be associated with anxiety, depression, and dementia etc. κB-Ras2 is an inhibitor of NF-κB, which is positively induced by miR-125b. A decrease in expression of miR-125b would result in enhancement of NF-κB expression {Murphy, 2010 #143}. So depression and dementia in those two patients could be attributed to decreased expression of miR-125b. On the other hand, participant-35 had higher expression of miR125b and this patient did not show any signs and symptoms of dementia or depression.
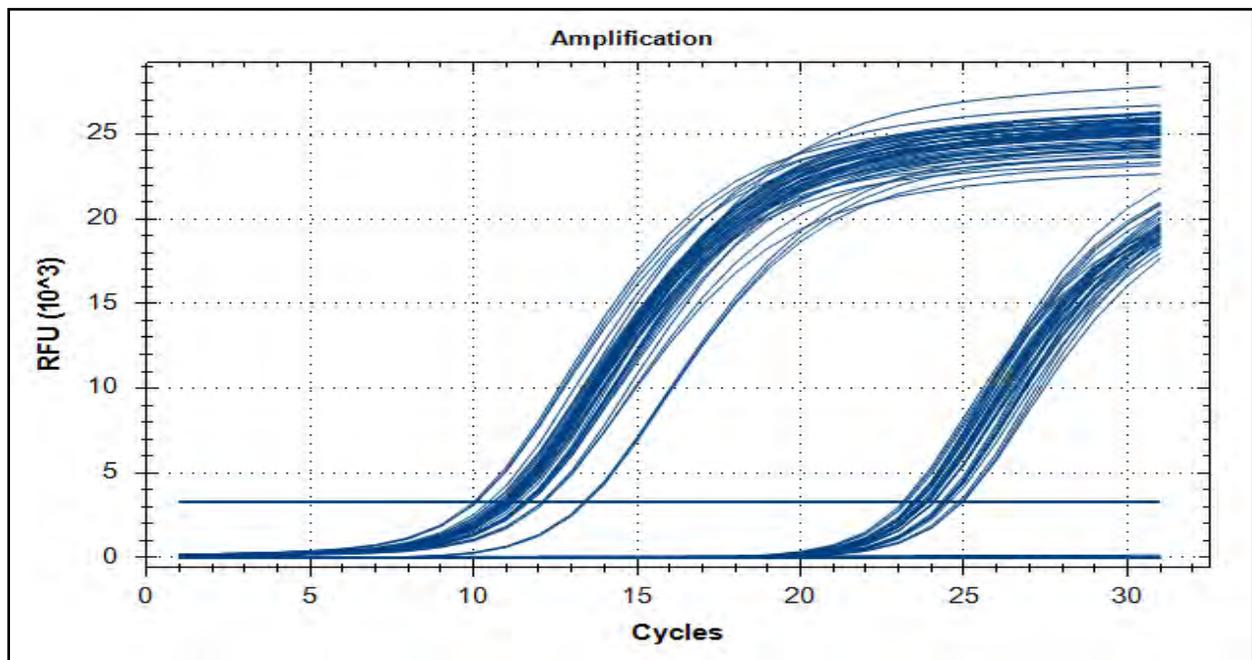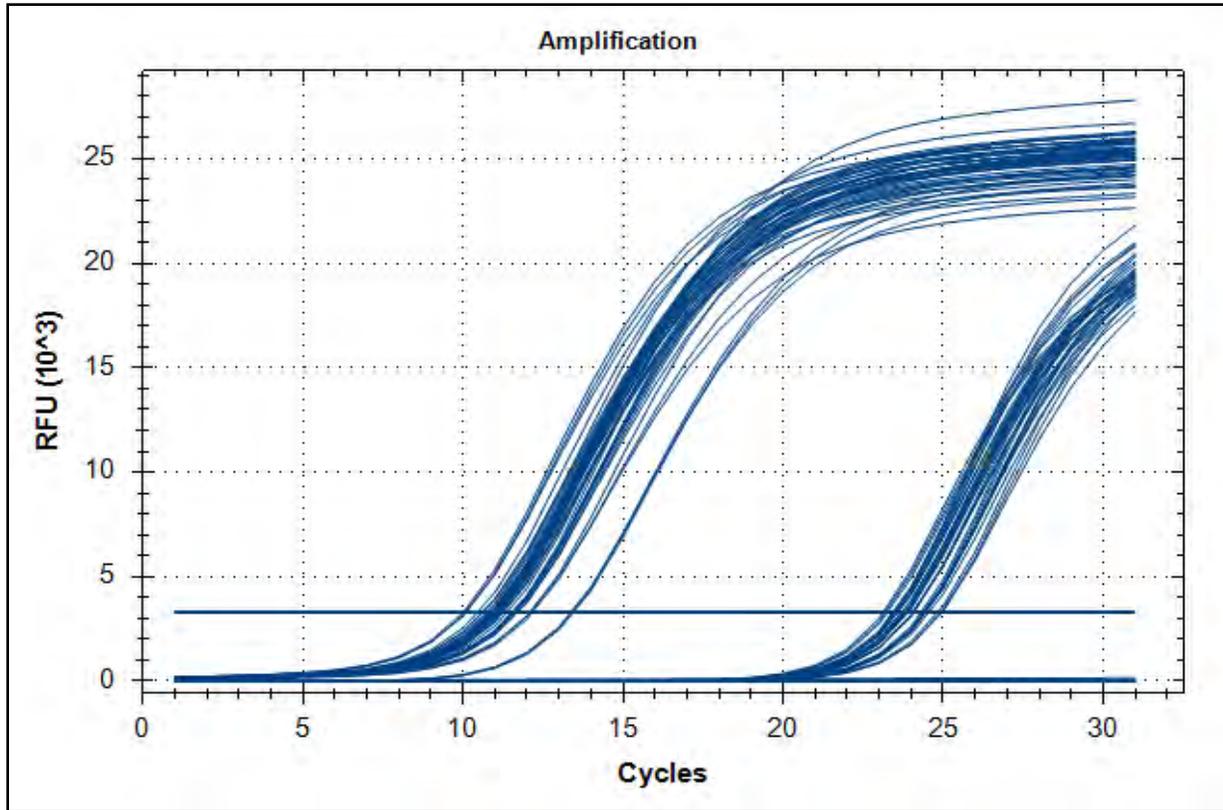
Figure 3.7: Demonstration of miRNA125b expression in Huntington patients and controls. The fluorescence curves showing early appearance (10-15 cycles) of Ct (cycle of threshold) values indicate expression of miR-16. On the other hand, curves with Ct values in between 20-25 cycles correspond to miR125b expression.

Table 3.6: Expression of miR125b in HD patients

Pedigree ID	Fold change ($2^{-\Delta\Delta Ct}$)			Mean fold changes	Standard deviation of fold changes
	Control-1	Control-2	Control-3		
25 (Depression)	0.467	0.943	0.377	0.595	0.304
27	0.79	1.596	0.638	1.008	0.514
29	1.137	2.297	0.919	1.451	0.740
32	1.046	2.114	0.845	1.335	0.682
35	2.578	5.207	2.082	3.289	1.679
37	1.397	2.823	1.129	1.783	0.910
39	1.616	3.263	1.305	2.061	1.052
51 (Slight dementia)	0.382	0.773	0.309	0.488	0.249

- Participants 25, 27, 29, 32, 35, 37, 39, 51denote 8 Huntington patients and three non-affected persons (control-1, control-2, and control-3) from same family tree were used as controls.
- $\Delta\Delta$Ct = (miR125b – miR16) expression of HD patients - (miR125b – miR16) expression of non-HD patients.
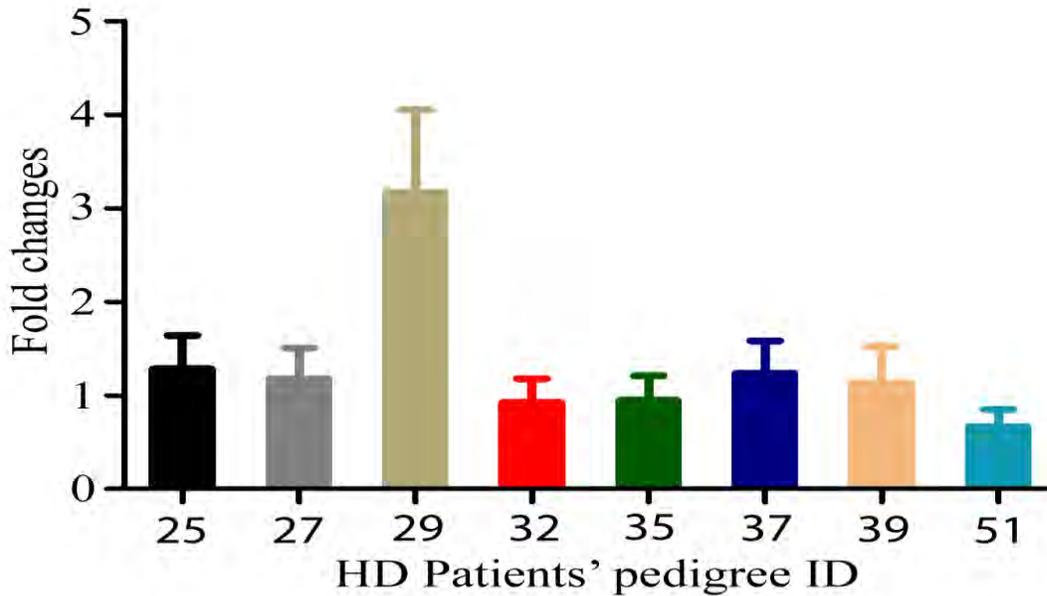- Fold change in miR-125b in HD patients compared to control = $2^{-\Delta\Delta Ct}$.
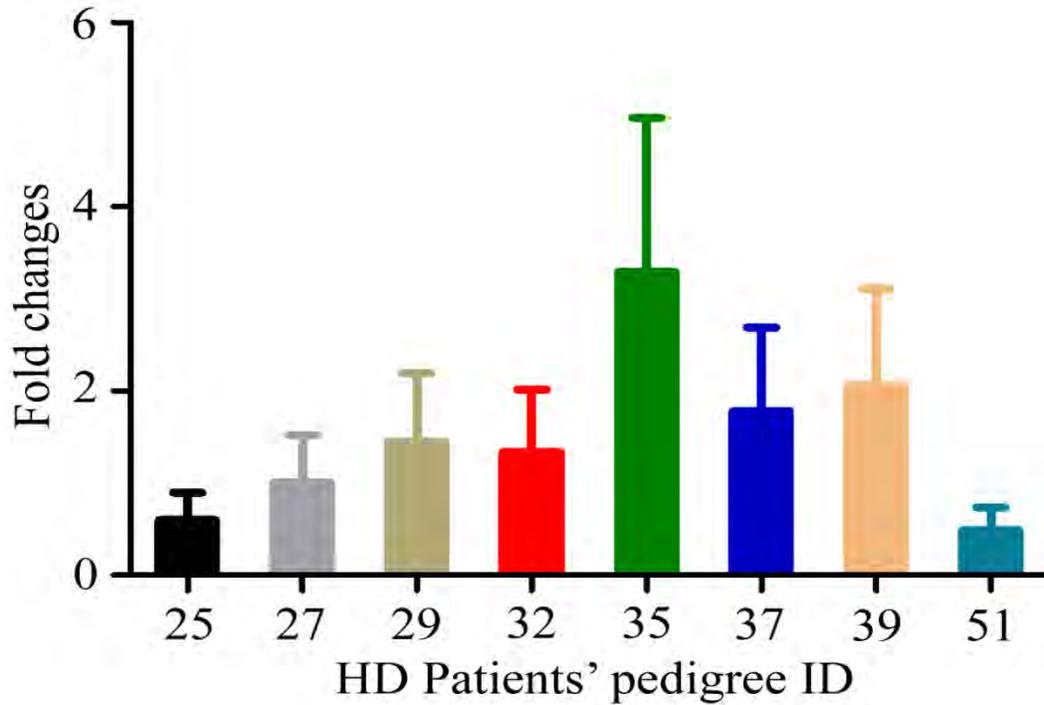
Figure 3.8 - Relative changes in the expression of miR-125b in HD patients compared to controls. Eight patients are denoted as 25, 27, 29, 32, 35, 37, 39, and 51; and relative fold changes (mean ± SD) in miR125b expression levels in HD patients are shown with respect to three controls.

Chapter 4

Discussion

It is estimated that 1%-7% of cases with a HD-like syndrome do not have HD (Wild et al., 2008) and frequently misdiagnosed with HD-resembling genetic disorders like Huntington disease-like 1 (HDL1), Huntington disease-like 2 (HDL 2), denatatorubral-pallidoluysian atrophy (DRPLA), benign hereditary chorea, Fahr disease, hereditary Creutzfeldt–Jakob disease, spinocerebellar ataxia (SCA) 17, Wilson disease, Parkinson's disease, neuroacanthocytosis, ceroid neuronal lipofuscinoses and Pantothenate kinase-associated neurodegeneration, and non-hereditary disorders such as Tardive dyskinesia, Sydenham chorea, Systemic lupus erythematosus, hyperthyroidism and Polycythemia vera (Gambardella et al., 2001; Holmes et al., 2001; Margolis & Ross, 2003; Martino, Stamelou, & Bhatia, 2013; Moore et al., 2001). Hence, genetic testing for HD suspects is recommended more frequently for accurate diagnosis. However, HD falls into the differential diagnosis of chorea, dementia, and psychiatric disturbances. In our study clinical features and primary medical diagnosis proved unsuccessful to determine HD as most of the patients lacked typical HD symptoms (Novak & Tabrizi, 2010) like motor symptoms (50.0%), dementia (12.5 %), and psychiatric disorders (0.0%), although (Paulsen et al., 2008; Tabrizi et al., 2009) numerous reports have demonstrated that majority of patients developed cognitive or psychiatric symptoms (or both) during the prodromal period, often many years before any motor signs appeared. Four out of eight cases (50%) exhibited voluntary motor impairment and the findings are consistent with the studies conducted by Rosenblatt et al. in 2012 and 2006. Cognitive impairments and neuropsychiatric problems are worth mentioning features in patients with HD, the former one characterized by gradual declining of attention, mental flexibility, planning, and emotion recognition, (Reilmann et al., 2014; Smith et al., 2014; Stout et al., 2011) and the latter one by depression, anxiety, irritability and apathy(Thompson et al., 2012; van Duijn et al., 2014).Molecular diagnosis using DNA-based assays offers more reliable approach to determine the prevalence of HD compared to diagnostic approach using clinical features and pedigree analysis, suggesting pre-molecular prevalence estimates were likely to have excluded numerous cases that are now genetically proven to represent at least 5–8% of diagnosed patients (Almqvist, Elterman, MacLeod, & Hayden, 2001; Ramos-Arroyo, Moreno, & Valiente, 2005). In particular, the molecular diagnosis has facilitated ascertainment of late onset Huntington disease

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in the elderly population, for which family history is often missing and neurological tests can be more challenging owing to the higher rates of dementia and other neurodegenerative disorders in this population(Evans et al., 2013; Koutsis, Karadima, Kladi, & Panas, 2014; Ramos-Arroyo et al., 2005) In recent decades a consistent increase was noticed in prevalence of HD due to wide spread availability of the genetic testing.

To our knowledge, this is the first study reporting DNA-based testing of HD patients in the Bangladeshi population. In line with the clinical investigation the first visible clinical symptoms appearing on HD onset were slow jerky involuntary movement of fingers and eyes and it was reported in 100% of HD suspected cases in this study (**Table 3.3**). Confirmed diagnosis of HD requires DNA-based testing to determine the number of CAG repeats in exon 1 of HTT gene (Craufurd et al., 2015). Simple PCR based method was applied for genetic testing of HD suspect subjects and then Sanger DNA sequencing method was used to validate the PCR results. The PCR results showed two types of DNA band for patients: (i) Normal HTT Allele (~80-85bp) and (ii) Expanded (mutant) HTT Allele (~200-250bp), whereas only one band of normal HD Allele (~80-85bp) was observed for normal subjects (**Figure 3.3**). The presence of the expanded and normal alleles in HD patients (heterozygous) in the study was supported by the literature (Barron et al., 1993; De Rooij et al., 1993; MacDonald et al., 1993; Novelletto et al., 1994; Zühike et al., 1993) indicating the accuracy of our molecular diagnosis. We determined the length of CAG repeat from the PCR products with an error of ±1 triplicate (Margolis & Ross, 2003; Nance et al., 1998; Potter, Spector, & Prior, 2004; Raskin et al., 2000) which were estimated to be about 20 for normal HTT allele and 53-70 for expanded HTT allele. Since calculation of CAG repeat length often suffers from slightly altered electrophoretic mobility relative to standard size markers, less reliability for longer expansions and variability in determining repeat length due to presence of multiple bands on gels for expanded alleles (Margolis & Ross, 2003; Nance et al., 1998). DNA sequencing approach was used to precisely determine the number of CAG repeats. The DNA sequencing results demonstrated consistency in computing CAG repeat expansion in the PCR products. Out of eight HD suspects, the mutant HTT allele of seven patients contained 53 CAG repeats and that of another one contained 70 CAG repeats, whereas negative control group had only 20 CAG repeats. All HD confirmed cases had heterozygous type of mutations (**Figure 3.3**) and no consanguineous marriages had been known for the study participants. That

is, all the diseased participants that had been clinically suspected as patients exhibiting HD or HD-like syndrome or Wilson's disease or other unknown neurodegenerative disease(s) with movement disorders were finally confirmed as HD patients and all eight patients had one mutant HD allele with CAG repeat >50 **(Table 3.4)**. On the other hand, all individuals of negative control group had normal HTT alleles with CAG repeat length of 20 which falls within the normal range of CAG repeat (6–35).Our findings are consistent with previous reports demonstrating that a person having CAG repeat more than 40 will have full-penetrance leading to development of HD with great certainty and normal population having CAG repeats in the range of 6–35 units will not develop HD (Nance et al., 1998; Rubinsztein et al., 1996; Wheeler et al., 2007). Previous report has also demonstrated that Juvenile-onset HD occurs in 5% of HD patients and is genetically characterized by larger stretch of CAG repeats and a predominance of paternal inheritance (Potter et al., 2004). In our study, one patient in 6th generation (VI 51**)** with pattern of parental transmission was finally diagnosed as juvenile HD (JHD) because the patient had 70 CAG repeats, supporting the previous report that approximately 50% of JHD cases contain   a CAG repeat stretch of ~60 or more (Quarrell, Brewer, & Squitieri, 2009). The patient diagnosed as JHD had an early onset of symptoms at age of 20 which is consistent with findings of Nance &Myers (2001) (Nance & Myers, 2001)and Gonzalez-Alegre & Afifi (2006) (Gonzalez-Alegre & Afifi, 2006), supporting that highly expanded CAG sequences cause disease onset at a younger age (Roos, 2010) and the age of onset is inversely correlated with length of the pathogenic CAG stretch in the HTT gene (Wexler, 2004). The age of disease onset for the remaining 7 patients varied between 30-35 years and the patients had CAG repeat units of 53. Although there are reports demonstrating correlation between CAG repeat size and age of onset (Evans et al., 2013; A Rosenblatt et al., 2006), it is widely acknowledged that the repeat size is a poor predictor of age of onset (Myers, 2004). On average, the CAG repeat size accounts for up to 70% of the variability affecting age of onset, with an estimated 10%-20% of the residual variability being accounted for by heritable factors (Gonzalez-Alegre & Afifi, 2006; Wexler, 2004)

Documentation of autosomal dominant inheritance for all subjects including living and dead persons indicated that transmission of the disease was of paternal origin for 6 cases (excluding JHD) and maternal origin for 8 patients. There were no significant differences in the size of the

expanded CAG repeat between patients with paternal (mean = 51.25 CAG units) (excluding JHD) and maternal transmission (mean = 51.33 CAGs). However, the CAG repeat size of paternally inherited expanded allele in JHD (VI 51) was 70. Reports have demonstrated maternally inherited alleles are estimated to be of similar repeat length, whereas paternally inherited ones may have a higher chance of an increase in CAG repeat length (Ridley, Frith, Crow, & Conneally, 1988; Walker, 2007). The sibs of the HD affected parents are at probable risk of suffering from this disease as offsprings of HD affected parents have a 50% chance of inheriting the genetic abnormality, and males and females are affected equally (Novak & Tabrizi, 2010), supporting the findings of our study.

The pedigree analysis and current results of HD mutation analysis in the patients of 5th and 6th generation family tree suggests that 8 probands with suspected HD in 5th, 4th, 3rd and 2nd generations whose DNA specimens were unavailable due to deaths were actually affected by HD. Our study provides a good of example for explaining the phenomenon of anticipation occurring in HD in which age of onset tends to decrease in successive generations (Margolis & Ross, 2003; McInnis, 1996).

In addition, we have analyzed the expression pattern of miRNA 125b and miRNA 155 in HD affected patients and healthy controls. The miRNAs are highly conserved, small non-coding RNA molecules about length of 21-25 nucleotides, complementary to one or more messenger RNA molecules. It is assumed that the miRNAs account for 1-5% of the human genome and regulate at least 30% of protein coding genes. The function of miRNAs appears to be in gene regulation. In animals miRNAs inhibit protein translation of the target mRNAs mostly by mRNA degradation. Dysregulation of miRNA expression in cells and tissues has been associated with numerous diseases. There are miRNAs which become dysregulated in neurodegenerative diseases. For example, miRNA 30b, miRNA 30c, miRNA 26a expression undergoes changes in parkinson's disease; miRNA 125b, miRNA 29a and miRNA 9 expression alters in Alzheimer disease: and miR-10b-5p and miR-124 expression customizes in Huntington disease (Hoss et al., 2015; Li & Kowdley, 2012; Liu, Im, Mook-Jung, & Kim, 2015). As there have been reports which showed association between Huntington disease and immunity so we wanted to observe

changes in the expression levels of two miRNAs (miR-125b and miR-155) which play important role in immunity (Rodriguez et al., 2007; Zhao et al., 2014).

The miR155 has association with immunity and apoptosis (Faraoni, Antonetti, Cardone, & Bonmassar, 2009; Palma et al., 2014). Mutant huntingtin (htt) protein causes a number of changes in immune system such as activation of the innate immune system in the striatum. Mutant htt also affects adaptive immune system responses and compromises migration of immune cells to chemotactic stimuli in periphery, emphasizing the importance of the immune response study in HD patients (Ellrichmann, Reick, Saft, & Linker, 2013). The miR-155 expression did not differ significantly in seven Huntington patients, which might be due to typical nature of the HD patients. Interestingly, there was an increase in miR-155 level in one HD participant who also had thalassemia. The findings suggest comorbid condition of HD and thalassemia or thalassemia alone may influence miR155 expression in peripheral blood. There have been reports demonstrating association between myeloid cell differentiation and miR155 level. Elevation of miR155 inhibits differentiation of myeloid progenitor cells to erythroblasts (O'Connell, Zhao, & Rao, 2011). Further study in thalassemic patient would reveal whether thalassemia alone causes a change in expression level of miR155 or HD and thalassemia together influence the expression of miR-155 in whole blood.

Similar to miR-155, miR125b play an important role in immunity and can influence cognitive and behavioral modes (Banzhaf-Strathmann et al., 2014). NF-kB is a protein complex that controls transcription of RNA, cytokine production, and cell survival. Also, this protein complex has a role in cognition and behavioral modes. Changes in the expression of miR125b levels in central nervous system of HD patients can induce changes in expression of NF-kB which can play an important role in cognitive and behavioral changes that occur in HD patients. In the present study, two HD patients had decreased level of miR-125b. One of them had dementia and other person was suffering from depression in his daily life. As decreased expression of miR-125b causes a reduction in κB-Ras2 expression. κB-Ras2 is an inhibitor of NF-kB expression. As a result NF-kB expression becomes up-regulated in diseased person. Thus dementia and depression in those two patients could be attributed to lower expression of miR-125b. on the other hand, one patient had ~3 folds increase in miR-125b compared to control but didn't have any adverse effect on his cognitive and behavioral modes. In conclusion, it can be deduced that

lower expression of miR-125b in whole blood of HD patients can be correlated with cognitive and behavioral modes but higher expression of miR-125b does not affect cognition and behavior.

Molecular diagnosis for genetic disorders in Bangladesh still remains at preliminary stage and almost all cases of rare genetic disorders have remained unknown. It is true that there is no specific cure of HD, symptoms-specific intervention are available. For example, tetrabenazine (U.S. FDA approved) controls the chorea and associated involuntary sporadic movements of face and the extremities by decreasing dopamine uptake by inhibiting vesicular monoamine transporter 2 (VMAT2). (Hayden et al., 2009; Wang et al., 2010). Some antipsychotic medications, like haloperidol, chlorpromazine also decrease dopamine levels (Jankovic, 2009; Phillips et al., 2008). But, before medication, proper diagnosis of genetic disorders is most important. Highly specific and sensitive diagnosis is prerequisite for providing individuals and families with information on the nature, inheritance, and implications of HD to help them make informed medical and personal decisions through genetic counseling. This study will pave the way for testing of the disease-causing mutation in the absence of definitive symptoms of the disease called predictive testing or prenatal testing in order to make personal decisions regarding reproduction, financial matters, family planning, and career issues. There is no established database of genetic disorder in Bangladesh. So, we are unaware of the real scenario of the diseases including their regional and environmental predisposition, if any. So, this can be the commencement of baseline study to estimate prevalence of HD in Bangladesh which will be highly important for proper planning to take preventive measures by policy makers, clinicians and public health experts.

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Appendices:

Appendix-I

Instruments:

The important equipment used through the study are listed below:

Instruments	Manufacturer
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	Fisherbrand®
Spectrophotometer	Eon™ BioTek®
96-Well Plate	Nunc™ 96F Microwell Plate
Centrifuge Machine	Thermo SCIENTIFIC
T 100 ™ thermal cycler	Infinigen
Gel documentation machine	Bio-Rad
Take 3 plate	Bio-Tek
Real-time PCR	BioRad,USA