Detection of expanded alleles in Huntington patients by polymerase chain reaction

Rajalakshmi K*, Sumitha Danda b and S. Asha Devi c

*Department of Biotechnology, Vels University, Chennai-600 117, India
bDepartment of Clinical Genetics, Christian Medical College and Hospital, Vellore-632 002, India
cDivision of Biomolecules and Genetics, Vellore Institute of Technology, Vellore-632 004, India

*Corresponding author: rajalakshmk06@gmail.com; sdanda@cmcvellore.ac.in; sashadevi@vit.ac.in

Abstract

Huntington disease is an autosomal dominant disorder. It is associated with an expanded CAG trinucleotide repeat, encoding a polyglutamine tract in exon 1 of the IT15 gene on 4p16.3. In normal individual the size of CAG repeats ranged from 9-35. Repeats longer than 35 are considered expanded and no individual with a repeat length <36 triplets has been credibly diagnosed with HD. It is a rare disease, with severe symptoms. The blood samples were collected from the patients who maybe suspected for Huntington disease. Hot start polymerase chain reaction is used to estimate the expanded alleles in Huntingtin gene. Amplified products of PCR Visualized on agarose gel electrophoresis, expanded alleles are compared with normal alleles. The present study focused to standardize a simple diagnostic method to conform Huntington disease. We observed expanded alleles in HD patients and normal allele size in healthy individuals.

Keywords: huntington disease, CAG repeats, hot start PCR, primer, IT15

Introduction

Huntington disease (HD, OMIM #143100) is a slowly progressive adult-onset neurodegenerative disorder presenting an autosomal dominant inheritance. The incidence of disease is 1:10,000. It is completely penetrant, devoid of effective treatment, and universally fatal (John et al., 1993). HD is clinically characterized by involuntary chore form movements, cognitive impairment, and personality changes. Neuronal degeneration is seen in several regions of the central nervous system, but is more evident in the caudate and the putamen of the basal ganglia (Hormozian et al., 2004). The worldwide prevalence of HD is 5-10 cases per 100,000 persons (Sharon et al., 2010). HD is a progressive neurodegenerative disorder characterized by motor disturbance, cognitive loss, and psychiatric manifestations. In 1993, a polymorphic (CAG) n trinucleotide repeat was identified in the 5’ region of a new transcript, HTT gene.

The protein encoded by the HTT gene was named huntingtin. The size of the (CAG) n trinucleotide repeat was analyzed by the polymerase chain reaction (PCR). Diagnosis can be confirmed by direct genetic testing, which is highly sensitive and specific and is now considered definitive. This study focused on some patients presenting with a clinical phenotype showing strong similarity to HD. The gene for HD is located on the short arm of chromosome at 4p16.3 (Wagle et al., 2000).

Gene

Gusella et al. (1993) mapped the HD gene to chromosome 4p16.3. The HD gene was cloned in 1993 (Huntington’s Disease Collaborative Research Group, 1993) and the causal mutation was discovered. Normal
alleles have 9-26 CAG repeats in exon 1, while pathogenic alleles from 36 to more than 120 repeats; alleles with 27-35 copies are termed intermediate alleles (ACMG/ASHG, Statement, 1998). Carriers of intermediate alleles will not become affected with HD, but if they are male, a risk exists that the CAG repeat will expand in meiosis and be transmitted to the offspring with a repeat length in the pathogenic range (the so-called, de novo mutations) (Goldberg, 1993). It contains a sequence of three DNA bases cytosine-adenine-guanine CAG repeated multiple times. (i.e. CAGCAGCAG), known as a trinucleotide repeat (Walker, 2007) CAG is the genetic code for the amino acid glutamine, so a series of them results in the production of a chain of glutamine known as a polyglutamine tract (or polyQ tract), and the repeated part of the gene, the PolyQ region (Katsuno et al., 2008). The mechanism by which the increased trinucleotide repeat length leads to the characteristic clinical symptoms and neuropathology of HD is, as yet, unknown. The CAG repeat of the HD gene is polymorphic in the population, varying between 8 and 36 repeats on normal chromosomes and is expanded with >37 repeats in chromosomes of HD-affected individuals (Tamás Toth, 1997). Triplet expansion is caused by slippage during DNA replication. Due to the repetitive nature of the DNA sequence in these regions, ‘loop out’ structures may form during DNA replication while maintaining complementary base pairing between the parent strand and daughter strand being synthesized. The loop out structure is formed from sequence on the daughter strand; this will result in an increase in the number or repeats.

A CAG repeat coding for the glutamine amino acid longer than the normal range was observed on HD chromosomes in exon 1, from all disease families examined. This type of repeatance is appeared to be located within the coding sequence of a predicted protein of about 348 kD that is widely expressed but unrelated to any known gene. Thus it turned out that the HD mutation involves an unstable DNA segment similar to those that can be found in several other disorders, such as the fragile X syndrome, Kennedy syndrome, and myotonic dystrophy. The fact that the phenotype of HD is completely dominant suggests that the disorder results from a gain-of-function mutation in which either the mRNA product or the protein product of the disease allele has some new property or is expressed inappropriately (Myers et al., 1989).

Symptoms of Huntington's disease commonly become noticeable between the ages of 35 and 44 years, but they can begin at any age from infancy. The most characteristic initial physical symptoms are jerky, random, and uncontrollable movements called chorea (Walker, 2007). Chorea may be initially exhibited as general restlessness, small unintentionally initiated or uncompleted motions, lack of coordination, or slowed saccadic eye movements 1 The clear appearance of symptoms such as rigidity, writhing motions or abnormal posturing appear as the disorder progresses (Walker, 2007). These are signs that the system in the brain that is responsible for movement is affected (Montoya et al., 2006). Psychomotor functions become increasingly impaired, such that any action that requires muscle control is affected. Common consequences are physical instability, abnormal facial expression, and difficulties chewing, swallowing and speaking (Walker, 2007). Eating difficulties commonly cause weight loss and may lead to malnutrition (Aziz, 2008). Sleep disturbances are also associated symptoms (Gagnon et al., 2008). Juvenile HD differs from these symptoms in that it generally progresses faster and chorea
is exhibited briefly, if at all, with rigidity being the dominant symptom. Seizures are also a common symptom of this form of HD (Walker, 2007).

The classic signs of Huntington disease which are progressive chorea (a nervous disorder marked by spasmodic movements of limbs, facial muscles and in coordination), rigidity, and dementia, frequently associated with seizures. The age at onset is highly variable: some showed signs in the first decade and some not until over 60 years of age. The average is between 30 and 40 years old (Chandler et al., 1960). The clinical features develop progressively with severe increase in choreic movements and dementia and the disease terminates in death on average 17 years after manifestation of the first symptoms (Reed, 1959). In juvenile HD patients, rigidity is more common than chorea. Behavioral abnormalities in the form of personality changes, anxiety, irritability and depression usually precede the motor symptoms, but often neglected or not regarded as a disease sign (James et al., 1994).

Suicide is more common in HD patients than in the general population, and is the third most common cause of death (Craufurd, 1994). In the very late stages of the disease, mental activities become slower and patients develop dementia, characterized with poor concentration, inefficient use of memory, and impairment of executive functions (Craufurd, 1996). Molecular diagnosis was done based on Polymerase Chain Reaction (PCR). This method enables diagnosis of Huntington disease (HD) and genetic counseling for newly mutated HD families.

Materials and Methods

Patients selection

Patient selection was made after evaluation by Neurology and Clinical Genetics Department in the outpatients or inpatients of the Christian Medical College and Hospital, Vellore. Blood samples were collected from HD patients in Christian Medical College, Vellore after informal consent.

Diagnostic clues for inclusion

Dementia and choreiform movements (uncontrollable movements), reflexes may be abnormal, the gait is often prancing and wide, speech may be hesistant or enunciation poor.

Collection of Blood sample

The 2 ml of peripheral blood was collected after informed consent and stored at -20°C till DNA was extracted.

DNA isolation

DNA was extracted using commercially available DNA extraction kit (QIAGEN) and stored at 20°C till it was used for the analysis. DNA was isolated from patient and normal person.

HOT start PCR

Hot-start PCR is a modification of conventional PCR that reduces non-specific product.

Analysis of the HTT gene by PCR

The gene was analysed using the HOT start PCR. PCR reagents are added in the PCR tubes & kept it in the PCR thermo cycler.

Fig. 1. PCR conditions for Hot start PCR

![PCR Conditions Diagram]
Calculation of CAG repeats

Formula for calculating the CAG repeats in the HTT allele with the size of PCR product. (PCR product size)-47/3 gives the no of CAG repeat (±3 repeat) in the Exon 1 of HTT allele (e.g.) for 128bp 128\(-47/3\)=27 repeats.

CAG repeat range of some normal control and HD patient.

Results

Interpretations of the PCR products on agarose gel electrophoresis

In the present investigation, PCR products on gel were analyzed for the results whether the patients are having the disease, with range of PCR bands. The range of normal (CAG) alleles of the HTT gene, were calculated from normal and Huntington patients. One normal and one expanded bands were found in all HD patients. In normal subject only one normal band was seen. Out of 11 samples suspected to be affected with HD five of them were found to have expanded allele in diseased range. Size of the alleles ranged from 40-52 repeats in the positive patient. The comparison of the size variation of triplet repeats in the HTT gene between some normal and HD patient are shown in the table 3. The normal range varied from 9 to 36 repeats. The HD range is from 40 to 52. The expanded alleles resolute well on agarose gel electrophoresis and native PAGE. HD ranges are well separated and the two distributions do not overlap. Size of the PCR product was possible by gel doc. System which is shown in the table 3. Sizing was done by the formula as mentioned in the methodology.

Pedigree analysis

Autosomal dominant inheritance could be documented in all, except in those patients who had no family history of the disease. Transmission of the disease was paternal in some cases and maternal in some patients. It was confirmed that all affected subjects with HD show expansion of the CAG repeat. Out of 11 patients 2 were having family history consistent with Autosomal Dominant inheritance, other three families failed to give a positive family history. Figure 2 Proband is 43 yrs old female with age of onset at the age of 34. Her grandmother and some siblings are also affected with same disease. She came for diagnosis with the symptoms fall of objects, Difficulty in remembering, can walk but can’t cooking for past 6 months. The transmission of CAG repeats in two generation was seen in this family.

Fig. 2. Pedigree of the family effected with Huntington Disease

Specificity of PCR

The specificity of PCR product was checked with Mutational analysis (2, 4, 6, 8, 10, 15, 20, 25 and 30%) by
mixing normal DNA sample and Huntington Patient sample. Amplified products showed bands in all these percentages, indicating the robustness of PCR. Therefore, this method has a high sensitivity and specificity. PCR products results were also checked with Native PAGE. Resolution was better than agarose gel electrophoresis. But some non specific Bands found with actual bands.

**Fig. 3.** Analysis of PCR products. Genomic DNA was amplified using primers HD1 and HD3 PCR products were analyzed using Agarose gel electrophoresis. Band analysis was done to check the size of the HD allele. All the bands are analyzed with molecular weight which is corresponding to marker. In diagram 3, lane A and B was analyzed, the results of those bands are showed in the diagram.

Lane: A-100 base pair marker, Lane: B-Normal, Lane: C-Patient 1--ve HD Lane: D-Patient 2 HD, Lane: E-Patient 3-ve HD, Lane: F-Patient 4 HD, Lane: G-Patient 5 HD, Lane: H Patient 6- -ve HD Lane: I Patient 7- -ve HD, Lane: J Patient 8--ve HD, Lane: K-Patient 9 HD, Lane: L-Patient 10 HD 1. Expanded allele, 2. Normal allele. Patient 2, 4, 5 and 9 showing an expanded band in the agarose, they conformed as HD

**Fig. 4.** Molecular Analysis of PCR product. Lane 1-50 bp marker. Lane 2 to Lane 12-PCR Product of HD patient in agarose gel electrophoresis.
Fig. 5. Peak of Lane 2

Note: peaks for Lane 2 (band A and B). Molecular analysis was done for all the samples. The molecular weights of the bands are listed in the table.

Table 1. Base pair size and CAG repeat range in some normal subject and Huntington patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Normal allele size</th>
<th>no of repeats</th>
<th>Expanded allele size</th>
<th>No of repeats</th>
<th>Presence of disease for HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>128</td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 2</td>
<td>114</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 3</td>
<td>107</td>
<td>20</td>
<td>205</td>
<td>52</td>
<td>+ve</td>
</tr>
<tr>
<td>Patient 4</td>
<td>103</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 5</td>
<td>110</td>
<td>21</td>
<td>93</td>
<td>48</td>
<td>+ve</td>
</tr>
<tr>
<td>Patient 6</td>
<td>111</td>
<td>21</td>
<td>200</td>
<td>51</td>
<td>+ve</td>
</tr>
<tr>
<td>Patient 7</td>
<td>103</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 8</td>
<td>114</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 9</td>
<td>115</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-ve</td>
</tr>
<tr>
<td>Patient 10</td>
<td>124</td>
<td>25</td>
<td>219</td>
<td>57</td>
<td>+ve</td>
</tr>
<tr>
<td>Patient 11</td>
<td>110</td>
<td>21</td>
<td>220</td>
<td>56</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Discussion

The expansion of triplet repeat sequences is an initial step in the disease etiology of a number of hereditary neurological disorders in humans. Diseases such as Myotonic dystrophy, Huntington’s disease, several spinocerebellar ataxias (SCA), Fragile X syndrome, and Friedreich’s ataxia are caused by the expansions of CTG.CAG, CGG.CCG, or GAA.TTC repeats. Huntington’s disease (HD) is caused by CAG repeat expansion in exon 1 of a large gene, HTT, possessing 67 exons (Jakupciak et al., 2000). HD is very rare disease during my project time 5 Huntington patient sample were collected from Christian Medical College, Vellore & done molecular diagnosis with the blood sample. Normal chromosomes possess 6-35 CAG repeats inherited in a Mendelian fashion; HD chromosomes bear 36-250 repeat units that are inherited in a non-Mendelbian manner, because of repeat instability upon transmission. Moreover, rare alleles with 36-39 repeats were found in the unaffected elderly relatives of sporadic HD cases (Rubinsztein et al., 1996). The hypothesis that there is a relationship between psychiatric and CAG repeats. There was no correlation between any psychiatric variable and CAG repeats (Berrios et al., 2001). The range of CAG repeats in the normal and
HD alleles 9-36 and 41-60. An accurate sizing can only be obtained with sequencing. For allele sizes in the intermediate range (37-40), sequencing should be carried out to confirm the carrier status of a patient.

The CAG repeat region in the HTT gene is amplified with PCR, subjected to electrophoresis and the repeat numbers are calculated. It is important for Diagnostic confirmation in a symptomatic individual & Presymptomatic testing for adults with a family history of HD. Differences in the stability of the CAG tract according to sex of transmitting parent have been reported with male transmissions being more unstable and with a tendency for further expansions of the abnormal CAG tract. Results raise the possibility that there are X- or Y-encoded factors that influence repair or replication of DNA in the embryo. Gender dependence in the embryo may explain why expansion in HD from premutation to disease primarily occurs through the paternal line (Kovtun et al., 2000). The earlier onset of HD in patients with the paternal transmission compared to the maternal one was found significant. This phenomenon was not related to the larger number of CAG triplets in patients with the paternal transmission. No differences either of the age at the onset of HD or numbers of CAG repeats were found between subgroups of HD patients starting with motor or psychiatric symptoms.29 The disease is 100% penetrant in individuals with > or = 42 repeats (Roth et al., 1999).

Molecular confirmation of the clinical diagnosis should be required in all patients with suspected HD, even in apparently isolated cases. Molecular analysis of the HTT gene is extremely important in sporadic cases of Huntington’s disease, providing correct diagnosis of the disorder and facilitating genetic counseling to the family members. Analysis of the CAG repeat in the HTT gene in confirmed the presence of the expanded CAG repeat in patients with the presumptive diagnosis (Falush et al., 2001).

Conclusion

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor disturbance, cognitive loss, and psychiatric manifestations. In 1993, a polymorphic (CAG) n trinucleotide repeat was identified in the 5' region of a new transcript, HTT gene. The protein encoded by the HTT gene was named huntingtin. The size of the (CAG) n trinucleotide repeat was analyzed by the polymerase chain reaction (PCR). Diagnosis can be confirmed by direct genetic testing, which is highly sensitive and specific and is now considered definitive. This study focused on some patients presenting with a clinical phenotype showing strong similarity to HD. Higher amounts of CAG repeats are associated with earlier disease onset; however, it is not possible to predict the specific age at onset, severity, type of symptoms, and rate of disease progression from CAG repeat length. Most individuals with HD have an affected parent; apparent de novo cases may be explained by death of a parent before symptom onset, unrecognized diagnosis in family intermediate, reduced penetrance allele resulting in absent or late-onset symptoms in a parent, or non-paternity. Allele sizes may increase during paternal transmission, and anticipation (earlier age of onset in successive generations) is often observed in families. The genomic primer sequences used in our PCR assay for the (CAG) n repeat were HD-1: 5' ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC 3', and HD-3: 5' GGC GGT GGC GGC TGT TGC TGC TGC TGC 3'. Huntington's patient expanded alleles varied from 40 to 85, with 40-52 repeats being the most common whereas, in
normal person \( HTT \) alleles ranged from 9 to 32 with 11 (CAG), repeats being the most frequent allele. In 2 patients with the number of (CAG), repeats were greater than 50. There was a clear inverse correlation between the number of repeats and the age of onset of the disease. With the discovery of the mutation that causes HD, definitive molecular diagnosis of HD is now possible. Laboratories performing HD testing, and clinicians interpreting the results of testing, must be aware of potential know. This method can be applicable for the diagnosis of HD.

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**References**


