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Employing Real Time PCR for the Diagnosis of Huntington Disease

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Abstract

***Background*:** Huntington disease (HD) is a dominantly inherited, neurodegenerative disease characterized by choreiform movement disturbances and dementia. The onset age of this disease is varied but usually is between the ages 40-50. Huntington's disease is caused by a triplet-repeat expansion in the IT15 gene (also known as huntingtin or HD) which is located on chromosome 4p3.1. Since many clinical picture of HD are indistinguishable from other distinct genetic disorders molecular test such as PCR is the only way to confirm the disease. The aim of this study was to introduce a new and fast technique for the diagnosis of Huntington disease.

***Materials and Methods*:** Blood specimens were collected from individuals suspected for Huntington disease and also people with no symptoms and family history of this disease. DNAs were extracted according to standard protocol. Using conventional PCR, patient positive for Huntington disease were diagnosed. Then employing real time PCR on the basis of difference between melting temperature (Tm) a new and fast diagnostic method was introduced.

***Results*:** Among 29 patients suspected to be HD only 8 HD patients were confirmed using PCR and real time PCR. The numbers of CAG repeat were between 42-50 and melting temperatures were between 89-92.

***Conclusion*:** The concept of using melting temperature in real time PCR protocol presented in here could be employed for the rapid diagnosis of the diseases caused by the increased in triple repeat sequences. It is fast, robust and has the potential use for the prenatal diagnosis.

Introduction

Huntington's disease (HD) is a progressive neurological degenerative disease. Autosomal dominant inheritance pattern has been identified [1]. Scientists in 1993, succeeded in separating unstable CAG repeats in the first exon of the IT15 gene located on chromosome 4 [2]. Studies showed that increase in the number of CAG repeat cause HD [3, 4]. The alleles containing more than 36 repeats cause HD. In people with more than 36 repeats there is a strong negative relationship between symptoms and the number of repeats. It has been shown that in many people with more than 60 repeats the symptoms of HD appear even before puberty (before age 20) [Juvenile HD: JHD] [3]. HD typically occurs in the fourth and fifth decades of life and death occurs 15 to 20 years after symptoms [4]. Young patients with early onset age of the disease (JHD) usually inherit the disease from their parents, while older patients who have symptoms probably have genes from their mother. Prognosis of the disease is very different in these two groups [5]. Diagnosis of HD based on the number of (CAG) repeat by using techniques such as PCR is now possible. Based on the studies carried out in recent years, the number of alleles with a 7-29 repeat represents healthy and allele with 29-36 repeats are very unstable in the transition to the next generation. This number of repeat does not usually cause HD in the carriers but may cause HD in the next generation due to the further expansion of the repeat.

Real time PCR is a technique in which the amount of PCR products can be measured at each cycle. In this method, a fluorescent reporter molecule is present in the reaction mixture, which is connected to the products in each cycle. Due to increase in product level at the end of the each cycle, the number of bound fluorescence reporter will be more. Since in real time PCR electrophoresis gel is not used, the specific melting temperature (Tm) of each product is used to ensure the specificity of the product. When there is a single peak in melting curve it represents the specificity of the product.

Due to the ability of real time PCR to differentiate each PCR product via its specific Tm, so we hypothesized that the increase in the number of CAG repeats should increase the Tm which further could be used as a diagnosis of HD.

Materials and Methods

Sampling: Individuals suspected of being HD and normal individuals without symptoms and with no history

of Huntington's disease in the family (as controls) were enrolled in this study. Patients had similar symptoms, such as choreiform movement disturbances and dementia, drooping eyelids, difficulty in eating, limb weakness, speech disorder, tremor and involuntary movements of the hands. Peripheral blood (5 ml) was collected in tubes containing anticoagulant ethylenediaminetetraacetic acid (EDTA) (20 mM).

DNA extraction and PCR: DNA extracted from peripheral blood using standard Salting Out method (DNA extraction kit Tadbir Fan Azma Co.). PCR method is commonly used to estimate the number of repeats (CAG). In many PCR based methods chemical additives, such as DMSO, formamide or glycerol is added. The reason is that the proliferation of CG-rich sequences by conventional methods (standard PCR) is difficult or impossible. Therefore, these reagents are added to facilitate the PCR.

PCR reaction contained 1 unit of Fast Start DNA polymerase (Roche), 200 mM dNTP, 10 microliters of DMSO, 10 picomol of each primer, 100 ng DNA templates. In figure 1 and table 1 the sequence of each primers and PCR product are shown. The PCR reaction condition was as follows: The first denaturation step at 95°C for 10 min, following denaturing of 95°C for 1 min, primer annealing at 61°C for 1 min, and extension at 72°C for 50 s, for 32 cycles and finally 10 min at 72°C.

The PCR products were electrophoreses on an 8% acrylamid gel. Product length varies depending on the number of repeats. The PCR product length is determined using the logaritmic table. After deduction of non-overlapping parts of the primers the length of each fragment is divided by 3, and so the number of repeat is obtained (Table 1).

Real-time RT-PCR: Real-time PCR was performed in a lightcycler^{TM} system (Roche Applied Sciences) using Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). All reactions were carried out in a total volume of 10 µL in capillary tubes. Each reaction mix contained 1.5 pmol of each primer, 1.2 mM $MgCl_2$ and 1 µL of Fast Start Master solution and 50 ng of DNA were added. The capillary tubes were capped and placed in the carousel under reduced light conditions.

Thermal cycling consisted of an initial denaturation step 95°C for 6 min followed by an amplification program (primer annealing, amplification and quantification) repeated for 50 cycles with temperature ramp rate of 20°C/sec. The amplification program was 95°C for 0 sec, 68°C for 27 sec and 72°C for 15 sec with a single fluorescence acquisition at the end of the elongation step. The third segment consisted of a melting curve program at 95°C for 0 sec, 60°C for 10 sec and 95°C for 0 sec with a liner temperature transition rate of 0.05°C/sec with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40°C.

Results

In the present study of 27 individuals clinically suspected as HD only 8 individuals conformed to be HD (Fig. 2, Table 2). Subsequently, a method was developed based on the Tm differentiation in real time PCR technique for rapid and inexpensive diagnosis of HD.

Real time PCR products with increased number of repeats showed higher Tm (Fig. 3-5). However, because this repeats are CG-rich bases sometimes smaller Tm curves peak could be seen which negligible (Fig. 4).

Discussion

In this study by employing real time PCR technique and Tm we could diagnosed 8 positive HD patients among 29 suspected ones. The number of CAG repeat was 44-50 and the range of Tm was 89-92°C.

The diagnosis of this disease has been possible in many countries including in Iran. Hormozian et al. used PCR technique in 35 Iranian suspected patients and 22 patients was finally diagnosed molecularly as HD [8]. It is noteworthy to mention that the diagnosis PCR method has its own problems. CAG repeats itself is very CG rich which makes PCR difficult. In addition, a variety of the polymorphic CCG repeat has also attached to the 3' of the gene. In addition, to determine the number of repeat polyacrylamide gel electrophoresis of PCR products is used. In most cases, the amounts of product (PCR product yields) are low. Furthermore the presence of many shadow bands makes it difficult to identify the main band [6] (Fig. 2). In our study only 8 patients from a collection of 29 suspected HD was confirmed as having this disease.

This suggests that the clinical symptoms of this disease are very similar to other neurodegenerative disease. Thus the need for molecular diagnosis of this disease is very important. All HD patients were heterozygous meaning having one allele with CAG repeat within normal range and one allele with increased CAG repeats. This is expected since individuals with 2 expanded allele cannot survive. The shortest CAG repeat in our patients was 42 and the range of the repeats was between 42-50. In real time PCR the electrophoresis gel is not used, so Tm is used to ensure the specificity of the PCR products. By increasing the PCR length, Tm increases. Thus, increasing the number of CAG repeat should increase the Tm as it has been proven in this study. All patients in this study had two distinct Tm peaks, indicating that the patients were heterozygous for this allele. In some control cases only one peak (which may be indicative of homozygosity for this allele) and in some two peaks were present. Real time PCR results showed that all patients with the normal range in CAG repeats (8-17) had Tm between 79-86 while increased in CAG repeat caused higher Tm. For example, in the patient with 48 repeat the Tm was 91 and in the patient with 50 repeat the Tm was 92 (Fig. 3-5). Therefore, higher Tm could be strong indication of increase in CAG repeat. This method has also been used to determine the number of repeat in androgen receptor gene by Chatzikyriakidou et al. [9]. This gen has also CAG repeat. These researchers believe that this method is quite reliable and reproducible [9]. In another study done by Teo et al. the range of Tm for 40-99 repeats was 88-90.

Table 1. Primer sequences and expected PCR products

Forward primer Sequence: FIT-15(HD)	Reverse primer Sequence: RIT-15(HD)	PCR Product Sequence	Size of PCR Product with normal CAG repeat
5′ cgagtccctcaag tccttccagca 3′	5′ gtggcggctgttg ctgct 3′	CGAGTCCCTCAAGTCCTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC AGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAACAGCCGCCAC	95 bp: 21 repeat

Table 2. Characteristics of patients with Huntington's disease diagnosed using molecular methods

Family No.	Gender	Age	Repeat No.	Children No.	Patient Children
Family1	Female	41	44 & 14	3 sons	Two son with 42 & 44 repeats
Family2	Female With 11 years disease history	39	46 &1 6	?	
Family3	Male	37	49 & 17	1 daughter	A 6 year old daughter with 49 & 17 repeats
Family4	Male	43	50 & 17	?	
Family5	Female	45	46& 16	?	

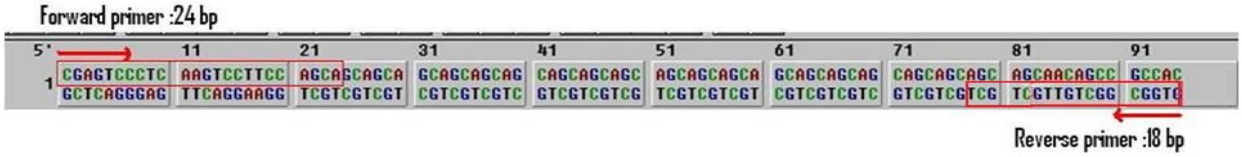

Figure 1. Sequence of the exon 1 of the Huntington's gene (Genbank Accession # _002111) and the location of the primers

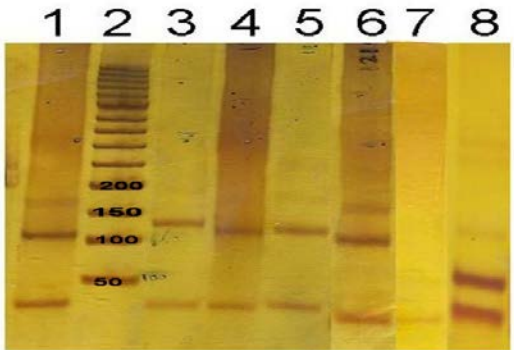

Figure 2. Resulting PCR products were analyzed on acrylamide gels. The arrows point to the shadow bands (explained further in the discussion section).
Line1: Familly 1, the son with 14 and 44 repeat, Line 2: DNA weight marker 50 bp, Line 3: Family 3, the patient with 17 and 49 repeat, Line 4: Family 1, the mother with 14 and 44 repeat, Line 5: Family 2; the patient with 16 and 46 repeat, Line 6: Family 1, the second patient with 12 and 42 repeat, Line 7: Negative control sample, Line 8: A healthy subject

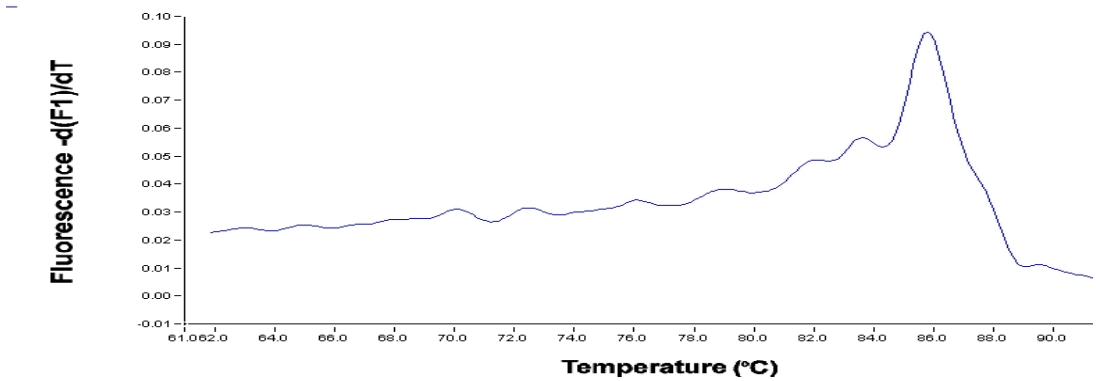

Figure 3. Tm curve of a healthy individual (Tm=85.7)

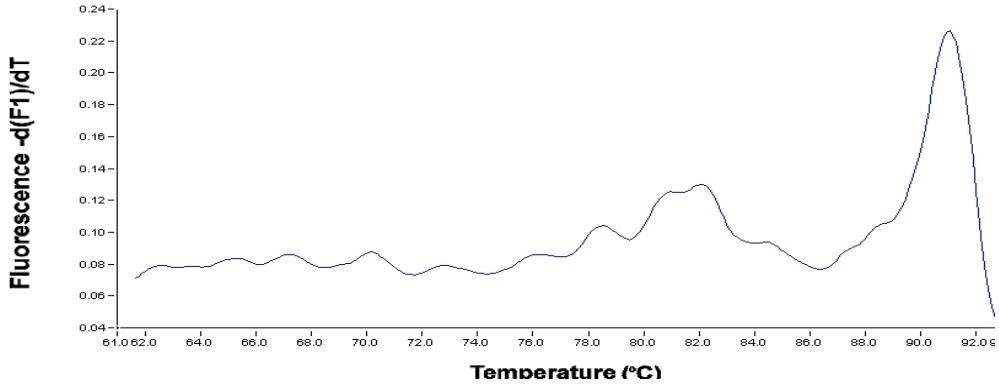

Figure 4. Tm curve of a HD patient with 16 and 48 CAG repeats and Tm 81.81 and 91.90 respectively

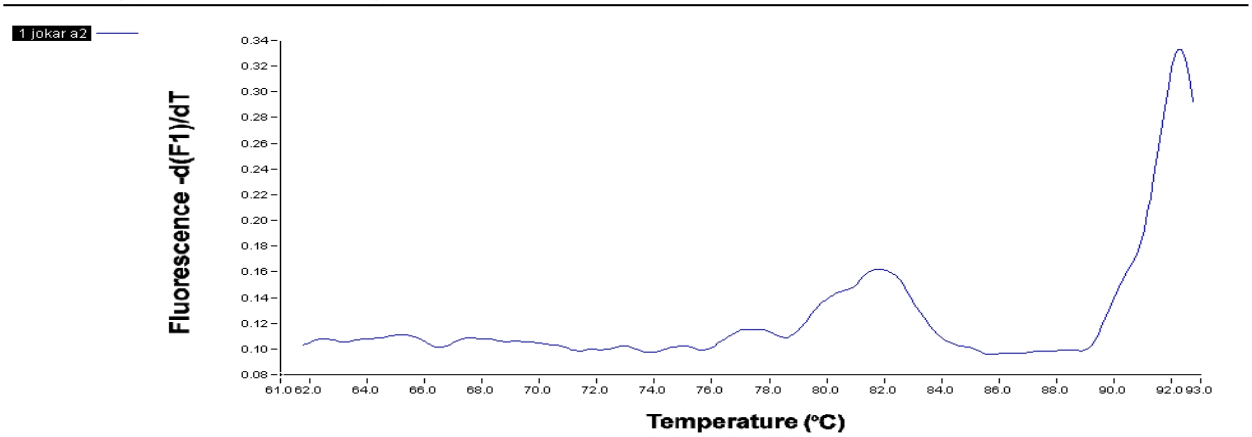

Figure 5. Tm curve of a HD patient with 17 and 50 CAG repeat and Tm 82.65 and 92.27 respectively

It means that 99 repeats had Tm 90 while in our data 50 repeats had Tm 92. This means that in each laboratory the range of Tm for the normal range has to be set up before applying the technique for the diagnosis of the triple repeat related diseases.

In summary, we could introduce an advanced, quick, reproducible real time PCR based method for the diagnosis of Huntington disease which is quite useful for prenatal diagnosis. The time require to do the test is less than 2 hours. To our best of knowledge, this is the first time real time technique is employed for the diagnosis of Huntington disease in Iran. Use of Syber green makes the technique very cheap. Although, the technique is specific enough to differentiate healthy from sick individuals, the sensitivity to determine the exact number of repeats is not sufficient. Therefore, in order to determine the exact number of repeats, real time high resolution melting technique is recommended.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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