

Molecular analysis of the (CAG)_n repeat causing huntington's disease in 34 Iranian families

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Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by chorea and progressive dementia. The mutation causing the disease has been identified as an unstable expansion of a trinucleotide (CAG)_n at the 5' end of the IT 15 gene on chromosome 4. We have analyzed the distribution of CAG repeats in 71 Iranian individuals (34 patients and 37 unaffected family members) belonging to 31 unrelated families thought to segregate HD. We found one expanded CAG allele in 22 individuals (65%) belonging to 21 unrelated families. In these HD patients, expanded alleles varied from 40 to 83 CAG units and normal alleles varied from 13 to 36 CAGs. A significant negative correlation between age at onset of symptoms and size of the expanded CAG allele was found ($r = -0.51$; $P = 0.1$). In addition, we genotyped 25 unrelated control individuals (total of 50 alleles) and found normal CAG repeats varying from 10 to 34 units. In conclusion, our results showed that molecular confirmation of the clinical diagnosis in HD should be sought in all suspected patients, making it possible for adequate genetic counseling. This Study is the first report of molecular diagnosis of Huntington disease among Iranian population and ever in Middle East and with regard to high frequency of consanguinity marriage in this region.

Key Words: Huntington Disease, Iranian Families, CAG repeats, Molecular test.

Introduction

Huntington disease (HD) is a progressive adult-onset neurodegenerative disorder presenting an autosomal dominant inheritance.¹⁻³ Onset of symptoms occurs typically in middle ages; however, it may begin at anytime

between childhood and old age. HD is clinically characterized by involuntary choreiform 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.²⁻⁴

The human genome has an abundance of simple sequence repetitions that are unstable and tend to expand in large numbers in some genetic loci. A prime example is the CAG class of triplet repeats whose large expansions occur in genes associated with Huntington's disease and six other neurological disorders.⁵ Such expansions represent a novel form of mutation whose cause is unknown. An attractive possibility under investigation is primer/template slippage during DNA replication or repair of tandemly repeated sequences.⁶⁻¹⁰

HD is associated with a significant expansion of a CAG trinucleotide repeat, which results in a lengthened polyglutamine tract in the single gene product, huntingtin, on human 4p16.3.¹¹ The mutation causing HD was characterized as expansion of an unstable CAG trinucleotide repeat localized in the first exon of the IT15 gene.¹² Studies in a large number of individuals of different ethnic origins have shown that normal alleles carry 6 to 34 CAG units; whereas, HD alleles have more than 40 CAG units.¹³⁻¹⁶

Various investigators generated prevalence data of

ethnic populations worldwide that yielded prevalence rates between 3/100 000 and 7/100 000 people of western European descent.¹⁷ Crucial for the diagnosis was the elucidation of a family history consistent with an autosomal dominant inheritance.

In this study we have genotyped the CAG trinucleotide repeat in the *IT15* gene in Iranian patients and control subjects in order to: (a) confirm the presumptive clinical diagnosis of *HD* in our group of patients, (b) compare the size range of the CAG repeat in the *HD* and control population, and (c) investigate the relationship between the size of the expanded CAG allele and age at onset of the disease.

Materials and Methods

Patients

This study was performed using DNA samples, from 71 family members, including 34 clinically affected individuals belonging to 31 unrelated families. These individuals presented clinically with psychiatric, involuntary movement disorder and received the presumptive clinical diagnosis of *HD*. Besides we determined CAG repeat in 37 at-risk members of affected families. Ages at onset varied from 16 to 57 years, mean of 36 years. All patients showed typical *HD* symptoms like chorea, movement impairment, cognitive decline and personality changes according to their clinical details, which were based on extensive records documenting neurological examination.

For determination of the frequency of the normal alleles we used 50 normal chromosomes identified in 25 unrelated control individuals of Iranian population.

Genetic Analysis: Analyses of DNA were based on genomic DNA isolated from peripheral blood by salting out method.¹⁸ The polymerase chain reaction (PCR) with primers HD1 and HD3 just flanking the CAG repeat, as described previously^{19,20} was used to determine CAG repeat length in the patient's DNA and their families. PCR reactions were performed in a total volume of 25 ml containing 100 ng of genomic DNA; 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP); 20 pmol of each primer; 10% DMSO; 5 mM KCL; 200 mM Tris-HCL (pH 8.4); 1.5 mM MgCl₂; 1.5 unit of Taq DNA polymerase (Roche, Inc.). PCR reaction were carried out in Techne

Thermal Cycle apparatus heated to 96 °C for 5 min as initial denaturation and then the amplification was accomplished in 35 cycles at the following temperatures: denaturing at 94°C for 1 min, annealing at 71 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were separated by electrophoreses through a 8% denaturing polyacrylamide gel loading 5 µl of each PCR products with an equal volume of 95% formamide loading dye. Samples were initially heated, for 9 min at 99° C before loading. The length of triplet repeats were determined by comparing migration relative to a XIII and VIII DNA MW marker (Roche, Inc).

Results

Expansion of CAG repeats in *HD* patients varied from 40 to 83 units (mean = 55.7) and normal alleles was in range of 13 to 36 CAG units with major of 21 (mean = 21.5). Autosomal dominant inheritance could be documented in all but 7 patients who had no or unclear family history of the disease (32%). Transmission of the disease was paternal in 8 cases (36%) and maternal in 7 patients (32%).

We identified 35 individuals, belonging to 21 families, with one expanded CAG allele at the *IT15* gene. Thirteen of these individuals were considered to be unaffected at the time of clinical evaluation, but had affected relatives who confirmed as *HD* patients. Further more, we excluded 34 individuals belonging to 10 families of being affected with Huntington's disease.

A correlation coefficient of -0.51 was obtained ($r^2 = 0.26$; $P=0.1$), assuming a linear relationship between age at onset and repeat length in *HD* alleles.

In two cases we found Juvenile onset (symptomatic before age of 20) at age of 16 and 18. In one case transmission was maternally.

In our study population we found two individuals with *HD* expanded alleles who remained unaffected till age of 56 and 55.

In the 25 individuals of the control group (total of 50 alleles) we found normal CAG repeats varying from 10 to 34 CAG units (mean = 20.9) and with 23 most frequent allele. No expanded alleles were found in the control population.

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, several spinocerebellar ataxias, fragile X syndrome, and Friedreich's ataxia are caused by the expansions of CTG.CAG, CGG.CCG, or GAA.TTC repeats. The mechanisms of the expansion process have been investigated intensely in *E. coli*, yeast, transgenic mice, mammalian cell culture, and in human clinical cases. Whereas studies from 1994-1999 have implicated DNA replication and repair at the paused synthesis sites due to the unusual conformations of the triplet repeat sequences, recent work has shown that homologous recombination (gene conversion) is a powerful mechanism for generating massive expansions, in addition to, or in concert with, replication and repair²¹.

Huntington's disease (HD) is caused by CAG repeat expansion in exon 1 of a large gene, IT15, possessing 67 exons.

The hypothesis that there is a relationship between psychiatric and CAG repeats was tested by seeking direct correlations between psychiatric systems and CAG repeats, and also by correcting the correlation by the number of years above or below the estimated age of onset in Huntington's disease. Scores for irritability and cognitive failures were high in the sample. There was no correlation between any psychiatric variable and CAG repeats.²²

The range of CAG repeats in the normal and HD alleles in our population is similar to those reported elsewhere.^{12-16,23} 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.²⁴

Study of tri-nucleotides repeats in HD patients in Brazilian by Raskin et al (2000), showed a range from 7 to 33 repeats in normal subjects and 39 to 88 repeats in affected subjects. A trend between early age at onset of first symptoms and increasing number of repeats was seen.²⁵

Differences in the stability of the CAG tract according to sex of transmitting parent have been reported,^{13-16,26}

with male transmissions being more unstable and with a tendency for further expansions of the abnormal CAG tract,^{13,27} Kovtun et al.²⁷ 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. We could document one transmission of the expanded CAG in this study, which shows differences in the distribution of CAG alleles according to gender of transmitting parent. (41 units increase to 58 units in offspring due to paternal transmission). There was no significant difference in the size of expanded alleles through maternal transmission ($r = 0.97$, $P=0.1$).

Laccione et al.²⁸ (2000) hypothesize that large expansions also occur in the female germline and that a negative selection of oocytes with long repeats might explain the different instability behavior of the male and the female germlines.

But Roth et al. 1999 found statistically significant reversed correlation between CAG repeats and the age at the onset of HD ($P<0.0001$, $r -0.6$). The earlier onset of HD in patients with the paternal transmission compared to the maternal one was found statistically significant ($P<0.05$). 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. Measurement of the flow from disease alleles provides a minimum estimate of the flow in the whole population and implies that the new mutation rate for HD in each generation is $>$ or $=$ 10% of currently known cases (95% confidence limits 6%-14%). Analysis of the pattern of flow demonstrates systematic under ascertainment for repeat lengths <44 . Ascertainment falls to $<50\%$ for individuals with 40 repeats and to $<5\%$ for individuals with 36-38 repeats. Clinicians should not assume that HD is rare outside known pedigrees or that most cases have onset at age <50 years.³⁰

There are several well documented *de novo* mutations in sporadic HD patients which have been reported in the literature.^{13,31-33} We identified 7 patients with no family

history of the disease who were found to have the *HD* mutation. However, in all 7 families clinical information on the parents could not be accurately obtained, since they either died at a very young age or were not available for examination. Therefore, it seems more likely that in these families autosomal dominant transmission was not documented due to missing information. However, the possibility that in these patients the CAG repeat in the *IT15* gene could have undergone a new mutation event cannot be completely excluded.

We found a significant correlation between age at onset of the disease and length of the expanded CAG tract. This indicates a tendency for age at onset to decrease as the CAG repeat length increases.

In our study we could document 2 cases with CAG repeats of 40 and 41 who had not manifest HD symptoms until age of 51 and 56. According to reported articles this founding clearly shows that reduced penetrance for HD may occur only for a CAG repeat length <42 units. there were no individual with a CAG repeat length more than 42 who remains asymptomatic till age of 56. This indicated that clinical manifestation of disease was fully penetrate with in a normal life span for this CAG repeat range.

In conclusion, our results showed that not all patients with the "HD" phenotype carried the expansion at the *IT15* gene and that autosomal dominant inheritance may not be clearly documented in all *HD* families. Therefore, molecular confirmation of the clinical diagnosis should be sought in all patients with suspected *HD*, even in apparently isolated cases. Molecular analysis of the *IT15* 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.³⁴⁻³⁸

Analysis of the CAG repeat in the *IT15* gene in Iranian families confirmed the presence of the expanded CAG repeat in 65% of patients with the presumptive diagnosis. The presymptomatic diagnosis of 36 family members at risk for HD disclosed that 13 subjects carried the affected alleles.

This Study is the first report of molecular diagnosis of Huntington's disease among Iranian population and maybe in Middle East and with regard to high frequency of consanguinity marriage in this region, we thought the

frequency of this disease will be more than expected amount for this geographical region.

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