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CAG repeat expansions and schizophrenia: association with disease in females and with early age-at-onset

A.G.Morris, E.Gaitonde[1], P.J.McKenna[2], J.D.Mollon[1] and D.M.Hunt*

Department of Molecular Genetics, Institute of Ophthalmology, University College London, Bath St., London, [1]Department of Experimental Psychology, University of Cambridge, Cambridge, and [2]Fulbourn Hospital, Cambridge, UK

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An increase in the severity of schizophrenia through consecutive generations (anticipation) has been found in some studies of families with affected members. Anticipation in five neurological disorders is known to arise from the expansion of CAG repeats between generations of affected individuals. The 'repeat expansion detection' method was used to screen individual genomes for the size of such expansions in a sample of schizophrenic and normal subjects. Comparison of the frequency distribution of CAG expansions observed in schizophrenic patients to that for normal subjects, showed that there are significantly more expansions in patients (p = 0.048). When male and female subjects are considered separately, there is a highly significant difference in the distribution of repeat sizes found between affected and normal females (p = 0.0023) but no significant difference between affected and normal males. Overall there is a 28% excess of expansions observed in affected versus normal females, and their presence confers a relative risk of 4.12 (p <0.005). In contrast, the frequency distribution of age-at-onset with respect to repeat size is nearly the same in male and female patients and, when the sexes are combined, the larger $(CAG)_{69-136}$ expansions are associated with a younger age-at-onset (p = 0.02).

INTRODUCTION

Evidence for the occurrence of anticipation through successive generations in families with mental illness has been known for some time (1,2). More recently, an increase in the risk of schizophrenia over three generations was found in a number of European families (3), and in a Canadian study successive generations of patients appeared to be more severely afflicted and to show an earlier age-at-onset (4). In another study (5), the age-at-onset was lowered by 8 years over a single generation. However, an 11 year reduction in the age-at-onset in the second generation of a number of UK families was attributed to a bias introduced by the lower fertility of patients with earlier onset of disease (6). This may also prove to be a confounding factor in the previous studies of anticipation.

The molecular basis of anticipation has been established in several diseases. Between successive generations, certain trinucleotide repeats in either coding or non-coding regions are found to expand and, the larger the expansion detected in the latter of two generations, the greater the degree of anticipation (7–9). In one type of TRE (trinucleotide repeat expansion), very large repeat expansions are restricted to non-coding regions. For example, 200–2000 repeats of CGG are found in the fragile-X syndrome and 200–4000 repeats of CTG in myotonic dystrophy (10). The other main type of TRE involves CAG repeat expansions situated within the coding regions of particular genes, with an upper limit of less than 150 repeats.

CAG expansions have been associated with several neurodegenerative disorders. In X-linked spinal and bulbar muscular atrophy (SBMA or Kennedy's disease), the repeat is expanded from a normal range of 17–26 repeats to 40–52 in affected patients (11) and codes for an enlarged polyglutamine tract near the N-terminus of the androgen receptor. Although this rare late-onset disorder does not show anticipation, the size of the expansion is correlated with the severity of the disease (12). Huntington's disease (HD) is again associated with an expansion in CAG triplets at the 5′-end of the coding region of the HD gene (13). In this case, the expansion is from a normal range of 11–34 repeats to the common disease range of 37–86 repeats (14). Paternal transmission of the disease leads to an 8–10 year earlier age-at-onset, with some of the progeny even exhibiting juvenile onset (15), compared to maternal transmission which results in no change to the age-at-onset (16,17). This fits with the pattern of $(CAG)_n$ inheritance, where the paternally inherited repeat size frequently doubles, in contrast with maternally transmitted repeats which differ by only a few repeats (18). Other diseases that show a similar pattern of small CAG expansions in the coding regions of disease genes, and a correlation between earlier age-at-onset and increased size of TRE, are dentatorubral-pallidoluysian atrophy (DRPLA) (7), Machado-Joseph's disease (MJD1a) (20) and spinocerebellar ataxia (SCA1) (19,20). DRPLA and SCA1 are similar to HD in showing an increase in anticipation after paternal transmission whereas MJD1a follows the pattern of SBMA where the repeat size appears constant over generations.

All these CAG-based TRE diseases commonly show chorea, ataxia, dementia and sometimes psychosis (10). They all involve neurodegeneration that presumably arises from the

*To whom correspondence should be addressed

presence of polyglutamine tracts, encoded by exonic CAG repeats, in an expressed protein and the dominant mode of action of these tracts indicates a toxic gain of function of the altered protein (21,22).

A method called repeat expansion detection (RED) has been used to look for TREs in normal unrelated individuals. In this method, oligomers of a given trinucleotide repeat are ligated together when they bind to an equivalently long stretch of complementary template in the genome of an individual, thereby generating a range of detectable products, of which the maximum size corresponds to that present in the genome. The commonest expansions detected by the RED method were the CAG/CTG repeats (23). Since CAG repeats are more likely to form expansions and because they have, as described, already been implicated in neurological diseases exhibiting anticipation, they would seem to be the best candidates for TREs in schizophrenia. The RED method was therefore used to screen the genomes for repeats of this type in normal and schizophrenic subjects, all of whom were unrelated, and who were drawn from the Cambridge area of England. A preliminary account of this work was presented at the Vth International Congress on Schizophrenia Research held in April 1995 (24).

RESULTS

Association of CAG repeat size with schizophrenia

In our sample of 70 normals from the Cambridge area, RED showed the presence of a CAG TRE in 26% of individuals. This is almost identical to the frequency observed in 167 normal and unrelated individuals (23), indicating that the frequency in our Cambridge sample may be representative of the population frequency. In contrast, of 84 schizophrenics collected from the Cambridge area, 37% had CAG expansions, an excess therefore of 11%. The frequency distribution of expansions in both normal and schizophrenic Cambridge groups (Fig. 1A) shows a mode in the $(CAG)_{52-68}$ category. In this category, the percentage of normals exceeds that of schizophrenics, whereas there is an excess of schizophrenics found in all other size categories, especially the larger ones.

The difference in the frequencies of repeat sizes observed in our schizophrenic and normal samples is statistically significant ($U = 1.67$, $p = 0.048$). However, when males and females are considered separately (Fig. 1B,C), the frequency distributions of expansions in schizophrenic subjects exhibit marked differences. There is a highly significant difference in the distribution of repeat sizes found between affected and normal females ($p = 0.0023$) but no significant difference between affected and normal males. Overall there is a 28% excess of expansions observed in affected versus normal females, and their presence confers a relative risk of 4.12 ($p < 0.005$).

Association of $(CAG)_{69-119}$ repeats with earlier age-at-onset of schizophrenia

Schizophrenic subjects were assigned to one of three CAG size classes: non-expanded, intermediate expansions in the range of 35–68 repeats and large expansions in the range of 69–136 repeats; the corresponding distributions of age-at-onset frequencies are shown in Figure 2. Within each CAG size class, the frequency distribution of age-at-onset in males was compared to that in females. No significant difference in the age-at-onset distributions was found between the sexes in

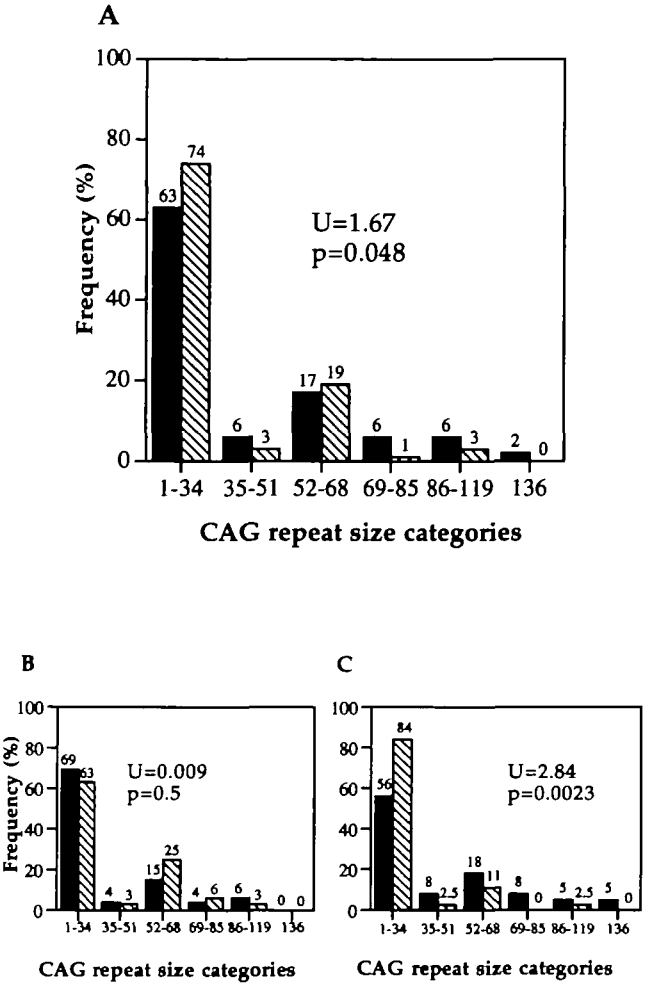

Figure 1. Frequency (%) of $(CAG)_n$ size categories in normal and schizophrenic subjects. (A) Percentage of schizophrenic subjects ($n = 84$) compared to normal subjects ($n = 70$) with the robust rank order estimate of a significant difference between these distributions, using test statistic U. (B) Male subjects (45 patients and 32 normals). (C) Female subjects (39 patients and 38 normals). Actual percentage values are shown above each bar.

any of the CAG size categories. Therefore the age-at-onset frequencies of males and females were pooled for further analysis. Each pooled size class (Fig. 2) exhibits a non-normal distribution where there is a strong association between the large expansion group and an early age-at-onset. For example, in the large expansion class, 92% of patients developed schizophrenia before 25 years of age, whereas in the non-expanded class only 61% had developed schizophrenia by this age. Statistical comparisons of the age-at-onset distribution of this large expansion group to those of the non-expanded and the intermediate expansion groups, both yielded significant robust rank order values of $U = 2.0$ ($p = 0.02$).

DISCUSSION

In this study, we have shown a significant association between the presence of CAG TREs and schizophrenia in females. The presence of larger expansions is also associated with early age-at-onset of the disease in a sample of pooled male and

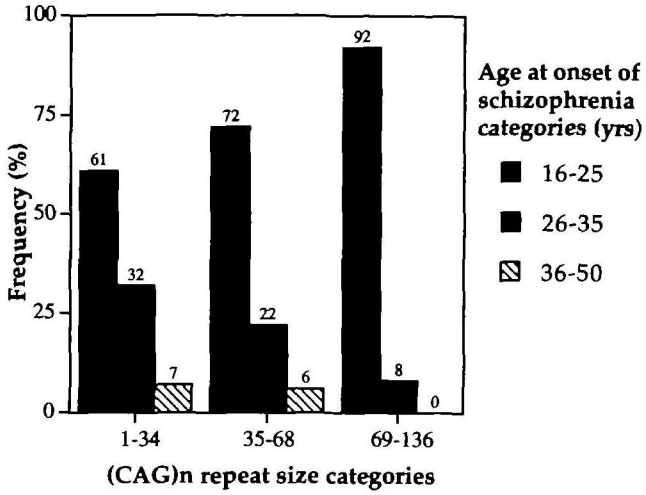

Figure 2. Earlier age-at-onset of schizophrenia with increase in size of $(CAG)_n$ repeats for combined sexes. A separate distribution of age-at-onset is shown for each of the $(CAG)_{1-34}$, $(CAG)_{35-68}$ and $(CAG)_{69-136}$ categories, for which the sample sizes are $n = 54$, $n = 18$ and $n = 12$ respectively. Actual percentage values are shown above each bar.

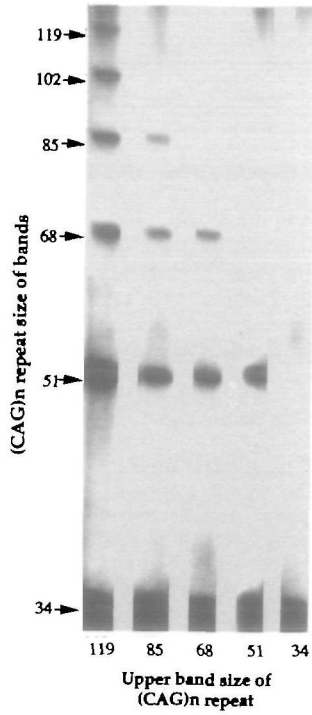

Figure 3. Montage of autoradiographs showing the sizes of ligation products generated by the RED method. The highest repeat size observed in a given lane corresponds to the uppermost $(CAG)_n$ repeat size detected in an individual's genome. The far right lane shows a single ligation product of $(CAG)_{34}$ in a genome without expansions; the other lanes all show expansions of different sizes.

female schizophrenics. The presence of a TRE may therefore be one of the components that contributes to the genetic risk of schizophrenia.

In interpreting this result, two factors should be taken into consideration. Firstly, the RED method employed in this study is not gene-specific but will detect a TRE anywhere in the genome (23). This accounts for the relatively high frequency of 'background' CAG expansions seen in normal individuals, since expansions that are not disease-related will also be identified. Secondly, where TREs have been implicated in disease, the important feature is not the presence but the size of the TRE, and increases in the size of the expansion over generations have been associated with anticipation (7–9,15,20,29–31). The association of large expansions with an early age-at-onset amongst our schizophrenic patients is therefore particularly interesting. Repeat size, however, may be only one of a number of factors that influences the age-at-onset in schizophrenia, since even in an apparently monogenic disease like HD, repeat size accounts for only 50% of the age-at-onset variance (32).

A number of different mechanisms may underlie the association of TREs with schizophrenia in females. The simplest explanation is that the excess of CAG expansions in affected females is conspicuous only because of the lower background of genomic CAG expansions detected in normal females. The background frequency distribution in normal females is significantly lower than that seen in normal males ($p < 0.02$). This effect does not seem to be due to a selection bias applied to normal females since, as noted in the methods, only one male and one female were excluded from the sample. So the higher frequency of expansions observed in the normal male sample may be masking a disease-associated expansion and an obvious candidate site for such male-specific expansions may be the Y chromosome. If this is the case, then the risk of schizophrenia from CAG expansions occurring at one or more loci in the genome may be alike in males and females. This is consistent both with the absence of a significant difference in the age-at-onset distributions that is found between the sexes in all three of the CAG size categories, and with the absence of a significant difference in the frequency distribution of repeats found between schizophrenic males and females.

Finally, the increased risk of schizophrenia in females with CAG expansions could have a physiological basis, for example through an interaction with the female sex hormones or through differences in the development of the brain in females. The presence of a TRE in the androgen receptor gene (11) and its effect on plasma testosterone and FSH levels (33) demonstrates that the presence of CAG expansions can have effects on normal hormonal function.

For the future, we intend to increase our sample of male and female normals so as to verify the sex difference reported here. There are a number of other trinucleotides that can undergo expansion and it will be interesting to establish whether any of these are also associated with schizophrenia. Finally, it will be important to identify the genetic localization of these repeat expansions and to determine whether CAG expansions are correlated with anticipation in families.

MATERIALS AND METHODS

Patient and normal subject selection

Eighty-four unrelated schizophrenic subjects, 45 males and 39 females, were recruited from the rehabilitation and acute services at Fulbourn Hospital,

Cambridge. The majority of subjects attended the clozapine clinic. All probands were unrelated. Informed consent was obtained and EDTA anticoagulated venous blood samples of 10–20 ml were drawn. Subjects were interviewed using the Present State Examination (25) and diagnoses made according to DSMIIIR crtiteria to assess recent or chronic psychopathology. Data were gathered on personal and family psychiatric as well as medical history, age, gender, age-at-onset of psychotic symptoms, and outcome of illness.

Seventy unrelated control subjects, 32 males and 38 females, were recruited from oral surgery and ophthalmology clinics and wards at Addenbrooke's Hospital, Cambridge. A semi-structured interview was administered and subjects with any history of major mental illness either in themselves or in a first degree relative were excluded. Only one male and female subject were excluded from the normal sample on this basis.

The age-at-onset of illness was determined from the case notes as well as from direct questioning of the patients, available relatives and any staff who knew the patients at or before the onset of symptoms. Age-at-onset was normally defined as the first documented occurrence of schizophrenic symptoms; where this could not be determined, the age at first admission was used. Although the method of rating was consistent, a degree of uncertainty remained for some subjects with little insight and a long history of vague behavioural disturbance before admission. Any inaccuracy would tend to be in the direction of over-estimation of age-at-onset. The determination of the age-at-onset was ascertained blind to the measurement of CAG repeat sizes.

Extraction of genomic DNA and oligonucleotide preparation

DNA was extracted from 10 ml of whole blood using the Nucleon III™ and dissolved in 0.1 ml of sterile distilled water. Oligomers of $(CTG)_{17}$ were purified by polyacrylamide gel electrophoresis prior to 5'-phosphorylation using polynucleotide kinase, followed by washing with Tris–EDTA buffer pH 8.0 in a NAP-5 column (Pharmacia™).

Repeat Expansion Detection (RED) method

The RED method (23) was modified as follows. The ligation mixture consisted of 50 ng of 5'-phosphorylated $(CTG)_{17}$, 5 U *Pfu* ligase and 2–5 μg of genomic DNA in 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM ATP, 1 mM DTT and 0.1% NP40 made up to a final volume of 35 μl. Thermal cycling of this mixture was carried out in a Perkin Elmer GeneAmp PCR System 9600 at an annealing-ligation temperature of 77°C for 60 s and a denaturing temperature of 98°C for 10 s over 198 cycles. Each ligation mix was then replenished with 4 U *Pfu* ligase, 2–5 μg of the respective genomic DNA and subjected to a further 198 thermal cycles. The use of a slightly lower annealing temperature than previously reported and the replenishment of enzyme and template after the first 198 cycles, generated consistently good yields of the triplet repeat bands. The reaction products were separated, electroblotted and visualized as previously described (23).

Assessment and scoring of $(CAG)_n$ ligation products

The presence of TREs in the whole genome of an individual is detected as a series of multiples of a $(CTG)_{17}$ oligomer. A single ligation product generates a $(CTG)_{34}$ band; if this is the only product detected in a given individual, this represents a size limit of less than 35 repeats of CAG present within that genome. Since this is the upper size limit that has not previously been associated with disease, it is considered to represent the unexpanded range of $(CAG)_n$ repeats. All reactions produced a single ligation product of this size. Expansions greater than 34 repeats give a ladder of products (Fig. 3) and the uppermost band is taken to indicate the largest repeat size present. All experiments included a reference reaction with DNA from a patient with myotonic dystrophy (DM) to generate a repeat ladder with a larger expansion size than seen with DNA from any of the schizophrenic patients or normal controls. Each DNA sample was tested at least twice. In general, the single ligation product was found to be more intense than the expansion ladder, presumably because it is derived from many loci scattered throughout the genome. Finally, all subjects were assigned to one category of repeat size, corresponding to the largest multiple of $(CTG)_{17}$ oligomer detected, starting with the non-expanded 1–34 repeat product, expansion categories of 35–51, 52–68, 69–85, 86–119 and finally the highest observed product of 136 repeats.

Statistical analysis

The robust rank order method (test statistic *U*) was used to examine the statistical significance of both the differences in expansion size in cases versus controls as detected by RED and the differences in age-at-onset between patients with large and small expansions This test makes use of the size ranking of the expansions but makes no assumptions about the normal distribution of the samples or about inter-sample distribution differences (26,27). This statistic tests for the likelihood that the repeat size of schizophrenic (s) and normal (n) samples arise from the same population, so that the medians are the same ($\theta_s = \theta_n$). The suspected alternative, based on prior associations of $(CAG)_n > 34$ with neurological disorders and on the trend observed in the frequency distributions noted in this study, is that the repeat size of the schizophrenic population is larger than that in the normal population ($\theta_s > \theta_n$). Since non-expanded repeats have never been associated with disease, this hypothesis ($\theta_s < \theta_n$) is not included in the test. The relative risk was estimated using the odds ratio (28).

The data for age-at-onset of schizophrenia were ranked according to year and then subdivided into three $(CAG)_n$ classes (see below). The ranked data from one class were compared to those from the other two classes using the robust rank order, which again is suited to the non-normal distribution of the data.

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