Somatic mosaicism in sperm is associated with intergenerational (CAG)$_n$ changes in Huntington disease

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We have analysed the CAG repeat in the Huntington disease (HD) gene in sperm and blood from 20 unrelated HD patients. Although the CAG repeat displayed significant mosaicism in sperm from all individuals, there were marked differences in the degree of repeat instability. Individuals who had either inherited or transmitted an expanded CAG repeat displayed the highest levels of repeat mosaicism, whereas individuals who had inherited or transmitted a contracted repeat had very limited CAG mosaicism in sperm. A strong association between intergenerational change in CAG allele size and the level of sperm repeat mosaicism was determined ($P = 0.019$). In contrast, neither blood CAG size nor repeat mosaicism in blood, were significantly associated with intergenerational CAG changes. These data suggest the presence of a cis-acting factor, separate from CAG size, that strongly influences the intergenerational behaviour of the CAG repeat. Additional studies are needed to determine whether analysis of CAG mosaicism in sperm is useful for assessing an individual's risk for transmitting large expansions or contractions to his offspring.

INTRODUCTION

Huntington disease (HD) is a neurodegenerative disorder inherited as an autosomal dominant trait. Patients typically have onset of psychiatric or neurological symptoms in mid-adult life, and the disease follows an inexorable progression to death over 15–20 years (1,2).

The mutation associated with HD was recently found to be an expanded (CAG)$_n$ trinucleotide repeat in a gene of unknown function (3). Normal chromosomes have less than 30 copies of this repeat, whereas >99% of all chromosomes associated with HD have repeat lengths of 36 or greater (4). The genetic characteristics of this type of mutation are similar to six other neuropsychiatric disorders (5,6) and include anticipation and strong sex of origin effects (7).

Molecular analysis of HD families has provided unique insights into the underlying basis for these effects. Age of onset is inversely correlated with the CAG repeat length, such that a longer repeat in a patient is associated with a younger age of onset (8–15). Analysis of 42 juvenile onset probands revealed that the anticipation observed in these families is closely correlated with an intergenerational expansion of the repeat, from the adult onset parent to the juvenile onset child (16). The largest expansions occurred exclusively via male transmission, providing an explanation for the previously recognised predominance of affected fathers of patients with juvenile onset disease (1,2,17).

The sex of origin effect is even more pronounced in sporadic cases of HD. New mutations are associated with an expansion of an allele of intermediate size (IA: 28–35 CAG repeats) from one of the parents into the HD range in affected offspring (18,19). Analysis of the parental alleles revealed that in 7/7 cases the expanded allele is inherited from the father (18). Since then, additional data from our own and other groups have increased the number of IAs of known descent to 16, all of which are of paternal origin (unpublished data). However, despite the strong sex of origin effect for persons with juvenile disease as well as sporadic cases, only a small proportion of paternal transmissions give rise to large expansions. Indeed, affected fathers transmit contracted repeat lengths in approximately 15% of father-offspring pairs (manuscript in preparation).

The familial aggregation of expansions or reversions in specific families, including the clustering of juvenile and late

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onset in sibships, suggest that specific HD chromosomes are associated with particular levels of CAG repeat instability (16,20). To examine this hypothesis at the molecular level, we have analysed sperm from 20 unrelated persons with CAG expansion in the HD gene. In 13 instances DNA from the parents and/or at least one offspring was available for study. Here, we show that the level of CAG repeat mosaicism in a patient’s sperm closely reflects the change in allele size from the patient’s affected parent, and may predict a similar change in his offspring. These results therefore suggest the presence of cis-acting factors promoting a particular level of repeat instability (contraction, limited expansion or major expansion) over multiple generations and suggests that the degree of CAG mosaicism in sperm of these probands might be a predictor of intergenerational change in CAG length in his offspring.

RESULTS

Blood and sperm were obtained from a total of 20 HD gene carriers. In 20 meioses for 13 persons we could assess the CAG repeat lengths of the affected parent and the offspring who had inherited the mutant HD gene (Table 1).

PCR amplification of the CAG trinucleotide revealed mosaicism of the CAG repeat length in all patients and tissues tested. This was demonstrated by the presence of additional bands, representing cells with larger repeat sizes, above the major size (most common) HD allele. Visual inspection of the amplified alleles suggested that there were large differences in the level of mosaicism (Fig. 1a). For example, sperm appeared consistently more mosaic than blood, but different patients demonstrated different levels of mosaicism in their sperm (Fig. 1a).

In order to quantitate the different levels of repeat mosaicism in either blood or sperm, we analysed the densitometric tracings of the amplified repeat lengths. For blood, we used the method originally described (21), which is the intensity ratio between the two most common alleles in the HD range (tissue-specific DI, Fig. 1b). This method is appropriate for tissues with limited repeat mosaicism, such as blood and most other somatic tissues. However, it is unsuitable for analysis of sperm, which is frequently more mosaic than can be accurately assessed in this manner. Specifically, the absolute number of alleles of a particular length is not accounted for. For this reason, we quantitated the mosaicism in sperm by relating the intensity of the major allele in blood to the same size allele in sperm (allele-specific DI, Fig. 1c). The relative stability of the HD allele in blood compared to sperm makes the ratio largely dependent on the variation in sperm. Thus, a very weak intensity in sperm, indicative of a highly heterogenous (mosaic) repeat population, will give a large ratio, whereas a more homogenous population will give a lower ratio.

Repeat mosaicism in sperm may be a marker for the likelihood of CAG expansion or contraction in offspring. Quantitative analysis of sperm mosaicism, as well as visual inspection of the amplified alleles, revealed pronounced differ-

<table>
<thead>
<tr>
<th>Category</th>
<th>Proband</th>
<th>Age</th>
<th>Onset age</th>
<th>HD allele CAG Repeat size in blood</th>
<th>Mosaicism of CAG repeat</th>
<th>Intergenerational change in no. of repeats on HD allele</th>
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<tr>
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*Patients are grouped into categories of changes in major allele size from parent to child: I: Inherited or transmitted a smaller (reverted) repeat size. II: Inherited or transmitted an identical repeat size. III: Inherited or transmitted a minor expansion. IV: Patients with extreme heterogeneity of sperm CAG repeats who inherited or transmitted large changes in allele size. V: Patients from whom no information on parental or offspring’s repeat size were available.

*Age at first known symptom of HD. AS = Proband currently asymptomatic.

*DI (densitometric index) is a quantitative assessment of mosaicism. Two different methods were used to estimate DI in blood and sperm (see Methodology for details). The absolute values for each tissue in a patient are therefore not comparable.

*Changes in number of CAG repeats from affected parent to proband, or from proband to his offspring. Negative value = contraction of the repeat; positive value = expansion.

*Inferred from close relatives.
ences between different probands. These differences were clearly associated with intergenerational changes of CAG repeat size.

The sperm CAG mosaicism was very low (DI range 0.94–1.8) in probands where the CAG had contracted, either from the parent to the proband or from the proband to his offspring (Table 1 and Fig. 2, probands 1–4). For example, proband 4 (DI = 0.94) has transmitted the HD allele to six offspring; the first four inherited the same size allele as himself, while the last two offspring had contractions of one and two trinucleotides, respectively (Table 1).

The mosaicism was clearly more prominent (DI range 2.1–3.7) in seven cases, where the CAG had remained unchanged or expanded by 1–2 repeats (Table 1 and Fig. 2, probands 5–11). In two cases, the sperm samples gave only a very faint pattern of alleles similar in size to the blood (DI = 8.5 and 12.1), indicating that a major portion of the alleles were too heterogeneous in size to be detected (Table 1 and Fig. 2, probands 12–13). In these cases, the CAG repeat had expanded by three and four triplets, respectively, from the parent to the proband. In the latter, the repeat had then expanded by a further 74 repeats in the proband’s offspring.

Factors associated with intergenerational CAG changes

The non-parametric Kruskal–Wallis (KW) test was used to examine the significance between the sperm mosaicism (DI) and the likelihood of expansion or contraction of the CAG repeat. We first analysed the association between sperm DI and all intergenerational CAG changes, which revealed a significant association (P = 0.019). Support for this correlation is provided graphically by plotting the changes in allele size against the probands’ levels of sperm mosaicism (Fig. 3).

In order to separate the two generational events (parent → proband and proband → offspring), we next performed the analysis including only the CAG changes occurring from the parent to the proband. A significant association was determined between the nature of intergenerational CAG changes from the parent and the level of mosaicism in the proband’s sperm (P = 0.04). Although limited by the low number of cases in

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Figure 2. The expanded HD allele (upper panel) and normal allele (lower panel) in blood and sperm from 13 probands. (For details of individual probands, refer to Table 1.) The probands are grouped into four categories, based on the intergenerational change: I: Inherited or transmitted a smaller CAG repeat; II: Inherited or transmitted an identical CAG repeat size; III: Inherited or transmitted a small CAG expansion (+1 or +2); IV: Inherited or transmitted large changes in allele size (>3). From visual inspection it is apparent that probands in group I (reversions) have very limited sperm mosaicism, whereas groups with larger CAG expansion have increasing levels of sperm mosaicism. Quantitative analyses support this data (sperm DI values). The lower alleles of the same gels are shown in the bottom panel for comparison.
each category (Table 1), this analysis indicated that the factor promoting instability in the previous generation is inherited with the CAG mutation and reflected in the level of mosaicism in sperm. The association between sperm DI and CAG changes in the following generation (probands $\rightarrow$ offspring) could not be examined by this test due to the limited number of cases in each category. However, all available data support the general trend that a low sperm DI value is associated with CAG contraction, whereas a high sperm DI value is associated with an increased risk for CAG expansion (Table 1, Fig. 2, Fig. 3).

We next examined whether blood CAG size, or the level of mosaicism in blood (blood DI), were associated with intergenerational changes in CAG allele size. The repeat size in blood was not significantly associated with the categories of CAG changes ($P = 0.22$), nor was the blood DI ($P = 0.15$). Thus, in this analysis, only sperm mosaicism was significantly associated with the change in intergenerational CAG size.

In addition to the non-parametric KW-test, we also analysed the data by linear regression analysis. In the total cohort, the sperm CAG mosaicism was highly associated with allele size changes ($P = 0.002; r^2 = 0.43; n = 19$). Without the extreme outlier (+74 CAG repeats from proband 13), the association was even stronger ($P = 10^{-6}; r^2 = 0.76; n = 18$). Similarly, we also examined whether blood CAG repeat size, or blood DI, could be used to predict intergenerational CAG changes. Neither factor showed any significant correlation to intergenerational changes in CAG length ($P = 0.12$ and $P = 0.10$, respectively).

In summary, using either parametric or non-parametric analysis, sperm CAG mosaicism was the only factor significantly associated with intergenerational CAG repeat size changes in this group of patients.

Because of the demonstrated relationship between sperm mosaicism and intergenerational CAG allele size changes, we
also analysed which factors are associated with different levels of sperm DI. Both blood CAG size and mosaicism in blood are significantly associated with sperm mosaicism ($P = 0.03, r^2 = 0.25, n = 20$) and $P = 0.03, r^2 = 0.23, n = 20$, respectively).

In one case, an additional band was seen above the lower allele in sperm (proband 10, Fig. 2). The appearance of the extra band was highly reproducible and could be due to specific contamination, at a low level, of this particular sample. However, when we amplified the sperm DNA with another highly polymorphic marker, only two alleles were present, identical in size to the blood sample (not shown), suggesting that this result was not an artifact and more in keeping with occasional mosaicism in sperm even with CAG repeat sizes seen in normal persons.

Instability of CAG repeat on new mutation chromosomes

We also analysed the level of repeat mosaicism in sperm from one individual with an upper CAG repeat size of 34 in his blood (Fig. 4). This individual is an unaffected brother of a new mutation for HD, who has inherited the same HD chromosome as his affected brother. Although expansion of the CAG repeat length into the HD range had not occurred in his case, his sperm showed clear evidence of mosaicism, indicative of CAG instability (Fig. 4). However, the DI of 1.1 would indicate that the overwhelming majority of his sperm have a CAG size of 34 repeats.

DISCUSSION

We and others have shown previously that sperm cells from HD patients are mosaic with regard to the number of CAG repeats and furthermore that differences in levels of mosaicism in sperm exist between patients (9,21–23). However, it is not known which factor/s determine the stability of the CAG repeat at the HD locus. In this study, we found a clear difference in the level of mosaicism in sperm between those individuals who have either inherited or passed on a larger repeat, compared to those individuals who inherited or transmitted on a smaller repeat.

These data suggest that cis-acting factors are promoting either instability or (relative) stability on HD chromosomes. Intergenerational CAG expansion is associated with considerable repeat instability in sperm of the offspring, while intergenerational CAG contraction is associated with a relatively stable repeat length in sperm of the offspring. The close association between CAG changes across generations is consistent with a cis-acting influence on CAG stability. Furthermore, patients with a stable repeat pattern in sperm appear to have a higher likelihood of passing on a reduced repeat size to their offspring, compared to patients with an obvious pattern of multiple CAG repeats in sperm. This suggests that sperm analysis may be useful to predict an individual’s risk of transmitting either an expanded or contracted CAG repeat.

Blood CAG size was not a useful predictor of the likelihood of intergenerational CAG expansion or contraction in any individual patient. In contrast, the level of sperm CAG mosaicism was significantly associated with intergenerational CAG repeat changes. This is illustrated by four probands with exactly the same size CAG repeat lengths in blood (44 repeats) (Table 1). In one offspring, the CAG had decreased in size from the parent (proband 2), in two cases there was either no change or moderate expansion (+2 CAG) (proband 6 and 7) and in one offspring the CAG repeat size had expanded by 3 repeats on transmission from the parent (proband 12). The likelihood of expansion or contraction, however, was clearly discernible when considering their level of sperm mosaicism either visually or quantitatively (Fig. 2). Proband 2 inherited a contracted CAG repeat (−1 CAG) and had a relatively low sperm DI of 1.8. In contrast, proband 12 had inherited a clearly expanded repeat (+3 CAG) and had a sperm DI of 8.5. Probands 6 and 7 with sperm DIs of 2.8 and 3.7 transmitted or inherited repeats with no or little expansion (0 and +1 CAG). The level of sperm mosaicism thus readily differentiates between the four individuals with identical CAG repeat lengths.

The method used to quantitate the level of mosaicism in sperm relies on a comparison of the density (in effect, copy number) of a particular allele between blood and sperm (allele-specific DI, Fig. 1c). Because mosaicism in sperm is much greater than in blood, it is largely the sperm density value (the denominator), that influences the ratio. However, the ratio assumes equal amplification efficiency and sample loading volume of the two separate PCRs (blood and sperm). In order to correct for any such differences, it is in most cases necessary to introduce a correction factor into the ratio. The correction factor is obtained by comparing the densities of blood and sperm from the lower (normal) CAG allele, which is not subject to repeat instability. For example, if the density ratio between blood and sperm is 1.2 at the lower allele, the blood density value of the expanded allele is divided by the same factor (1.2) prior to comparing its value to the density of the same allele in sperm. We have found the allele-specific DI to be a reliable, rapid and convenient method to quantitate the CAG mosaicism in sperm.

Richards and Sutherland originally used the term ‘dynamic mutation’ to describe the correlation between repeat instability and CAG repeat length with longer CAG repeats associated with a higher probability of further instability (25). Although this association is valid overall, our data suggest that, at the level of individual HD chromosomes, factors other than CAG repeat size have a stronger influence on repeat instability for the ranges of CAG repeats assessed (39–52 repeats). Haplotype data lends additional support to the presence of cis-acting factors on HD chromosomes promoting CAG instability (20). For example, a CCG of 7 repeats adjacent to the CAG repeat in the HD gene and the absence of GAG in the coding region of the gene approximately 150 kb from the CAG repeat are associated with higher CAG repeat lengths on normal chromosomes and are over-represented on HD chromosomes (27,28). Furthermore, in a recent report, it was demonstrated that minisatellite mutations can be ‘polar’, occurring at one end of the repeat (29). One explanation for the polarity is that sequences located close to the site of the mutation influence the probability of mutation. Strong support for this type of mechanism has been provided by recent reports of sequence changes occurring in the trinucleotide repeats of spinocerebellar ataxia type 1 and fragile X syndrome. In either gene, it has been shown that stable chromosomes are associated with a repeat interrupted by one or more different trinucleotides, and that instability is associated with loss of one or more of these
interspersed sequences (30–32). It is not known if similar mechanisms are operative in HD.

Although we have shown here that PCR of total sperm is able to detect different levels of mosaicism, analysis of single sperm cells from HD patients will provide a further representative profile of the CAG repeat length distribution. In particular, smaller alleles can be detected with this analysis and may be further used to test the association between repeat length distribution in sperm cells and the intergenerational CAG change. In a recent report (33), single sperm analysis from normal controls was used to study the mutation rate at the human androgen receptor CAG repeat, associated with spinal bulbar muscular atrophy (SBMA) (34), which revealed that the mutation rate in alleles of normal length (20–22 repeats) was very similar to that expected from segregation analysis in families. However, for larger alleles in the unaffected range (30–33 repeats), the rate of mutation was higher, with contractions much more common than expansions, thus preventing the CAG repeat size in the normal range from further expansion (33). This suggests that a factor promoting repeat stability on normal chromosomes may be different, or perhaps absent, on chromosomes with repeat lengths in the disease range, since analysis of families with diseases caused by expansions of a trinucleotide repeat, such as HD or SBMA, reveals that intergenerational CAG expansions are much more frequent than contractions (6,7,35).

In contrast to the female, the male germline undergoes an increasing number of mitotic events with age. It is unknown, however, if the HD CAG repeat gradually expands with each successive germline mitosis and whether, therefore, older HD males are more likely to transmit larger expansions than younger males. There is some prior evidence for an age-dependent effect on sperm CAG repeat length in fathers of new mutations who in general are of an advanced age (18). Furthermore, in a large study carried out prior to the identification of the HD mutation, it was suggested that anticipation of >10 years occurred more frequently in affected fathers of more advanced age (36). The correlation between paternal age and anticipation in the offspring was particularly notable when the affected grandparent was a father. In contrast, maternal age did not influence anticipation (36). It has been shown subsequently that anticipation is closely correlated with an expansion of the CAG repeat, and in cases of very large expansions results in juvenile onset (16). Taken together, these data would appear to suggest that older fathers are more likely to transmit further expanded CAG repeats, with anticipation in the affected offspring.

In the present study, increased CAG mosaicism in sperm was not evident in older probands (Table 1). However, there is clearly a bias of ascertainment in this cohort. Only those persons with less advanced disease were able to provide sperm for analysis. The older persons who had a more severe disease are likely to have had earlier onset associated with higher CAG repeat size, but those samples were not available for study. Conclusive evidence for an ongoing mutational process in the male gonads will therefore require sperm samples from specific probands to be analysed over a number of years to identify changes in the level of CAG mosaicism.

New mutations for HD arise from intermediate alleles (28–35 CAG), which expand to >36 repeats on transmission through the male germline (18,19). The frequency of IAs in the general population has been estimated to be 0.75% (4). A major concern for these persons as well as persons with IAs from families with sporadic HD is whether they are likely to produce offspring with CAG sizes in the affected range. The level of CAG repeat instability in their sperm may be a useful marker to assess the likelihood of intergenerational CAG expansion. A sibling of a sporadic case of HD (Fig. 4) has a CAG repeat size of 34 and is therefore unlikely ever to develop signs or symptoms of HD. However, his sperm displays some signs of CAG repeat instability with a low but distinct level of mosaicism (DI = 1.1). However, the vast majority of larger sized CAG alleles have 34 repeats with only a minority of sperm with allele sizes in the HD range. The prediction, based on data presented in this manuscript, would be that this man's offspring would have a low risk of inheriting a CAG repeat size in the affected range. However, additional studies of larger numbers of patients are needed to determine whether the model proposed in this manuscript is correct and whether this has any utility in a clinical setting. In particular, analysis of sperm from individuals with intermediate sized alleles would be particularly important.

In conclusion, our results show an important association between the level of repeat instability in sperm and the propensity towards either expansion or contraction of the CAG repeat. The extent of CAG mosaicism in sperm of a proband reflects the intergenerational CAG size change from the proband's parent, and also predicts the change in his affected offspring. These data are consistent with cis-acting factors in close association with the CAG repeat on HD chromosomes influencing intergenerational CAG change.

**MATERIALS AND METHODS**

**Tissue preparation**

Samples of blood and semen were collected at clinic or sent by overnight courier from patients' residences. DNA was extracted as reported (21) upon arrival in the laboratory.

**DNA analysis**

Amplification of the HD CAG trinucleotide was carried out as described (16) and alleles separated on 6% denaturing acrylamide gels. Allele sizes were determined against an M13 sequencing ladder.

**Measurement of densitometric indices**

Densitometric tracings were obtained from autoradiographs and the peak values recorded for each expanded allele in blood and sperm, without overexposing the film (21). Most PCRs were repeated at least once. When comparing the intensity in blood and sperm (the allele-specific method used to quantify sperm CAG mosaicism), we used the lower (normal) allele to correct for the different amounts of PCR products in the two lanes.

**Statistical analysis**

Due to the small numbers involved and the non-normal distribution of dependent variables, with one clearly outlying value, the non-parametric Kruskal–Wallis test was used to compare grouped variables. Only when the association between two variables was examined for the whole sample of subjects was simple correlational analysis employed.

**ACKNOWLEDGMENTS**

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