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Single sperm analysis of the CAG repeats in the gene for dentatorubral-pallidoluysian atrophy (DRPLA): the instability of the CAG repeats in the *DRPLA* gene is prominent among the CAG repeat diseases

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Dentatorubral-pallidoluysian atrophy (DRPLA) is known to show the most prominent genetic anticipation among CAG repeat diseases. To investigate the mechanism underlying the meiotic instability of expanded CAG repeats in the gene for DRPLA, we determined the CAG repeat sizes of 427 single sperm from two individuals with DRPLA. The mean variance of the change in the CAG repeat size in sperm from the DRPLA patients (288.0) was larger than any variances of the CAG repeat size in sperm from patients with Machado–Joseph disease (38.5), Huntington's disease (69.0) and spinal and bulbar muscular atrophy (16.3), which is consistent with the clinical observation that the genetic anticipation on the paternal transmission of DRPLA is the most prominent among CAG repeat diseases. The variance of the change in CAG repeat size was significantly different between the two DRPLA patients (F-test, $P < 0.0001$). However, the segregation ratio of single sperm with an expanded allele to ones with a normal allele is not statistically different ($P = 0.161$) from the expected 1:1 segregation ratio, and thus segregation distortion of expanded alleles in meiosis in male patients with DRPLA was not demonstrated.

INTRODUCTION

Dentatorubral-pallidoluysian atrophy (DRPLA) is a rare autosomal dominant neurodegenerative disorder characterized by various combinations of myoclonus, epilepsy, cerebellar ataxia, choreoathetosis, dementia and psychiatric symptoms. Although the first case of DRPLA described by Smith *et al*. was a sporadic one (1), the hereditary form of DRPLA has been reported most commonly in Japan (2–4).

DRPLA belongs to a group of 'CAG repeat diseases', which are caused by the expansion of a CAG repeat in the coding region of the respective genes (5,6). Other neurodegenerative disorders belonging to this group include spinocerebellar ataxia type 1 (SCA1) (7), spinocerebellar ataxia type 2 (SCA2) (8–10), Machado–Joseph disease (MJD/SCA3) (11), spinocerebellar ataxia type 6 (SCA6) (12), spinocerebellar ataxia type 7 (SCA7) (13), Huntington's disease (HD) (14) and spinal and bulbar muscular atrophy (SBMA) (15).

The anticipation in DRPLA, i.e. worsening of clinical symptoms and an earlier age of onset in successive generations, is most prominent among the 'CAG repeat diseases' (16). The molecular basis of the anticipation is mainly the expansion of the CAG repeats on parent-to-offspring transmission. Interestingly, the instability of the CAG repeats in the *DRPLA* gene is more prominent on paternal transmission than on maternal transmission (16). Although this parental bias of CAG repeat expansion is also observed in HD, MJD and SCA1 (16,17), it is most prominent in DRPLA (16).

With regard to the mechanism underlying the instability of CAG repeats in MJD, we found that two factors, paternal transmission and an intragenic CGG/GGG polymorphism at the 3′ end of the CAG repeats in the gene for MJD (*MJD1*) in normal alleles, contribute to the intergenerational instability of the CAG repeats in the *MJD1* gene (inter-allelic interaction) (18). In addition, we found that significant segregation distortion in favor of transmission of mutant alleles in male meiosis (meiotic drive) occurs in DRPLA and MJD (19). These phenomena of inter-allelic interaction and meiotic drive have also been confirmed by single sperm analysis of the CAG repeats in the *MJD1* gene (20).

To investigate the mechanism underlying the intergenerational instability of the CAG repeats in the *DRPLA* gene in male meiosis, we undertook direct analysis of single sperm from DRPLA individuals. It is very difficult to obtain sperm from DRPLA patients with informed consent, because their dementia and psychiatric symptoms progressively worsen. Fortunately, we could obtain the cooperation of two male individuals with DRPLA for this study. We report here that the instability of the CAG repeats in the *DRPLA* gene is prominent among the CAG repeat diseases.

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Table 1. Single sperm typing data for the two DRPLA-affected individuals

	CAG repeat size in leukocytes	No. of sperm analyzed	Mean change	Expanded (%)	Contracted (%)	Range	Variance
Expanded allele 1 (patient 1)	62	101	+21.2	84	14	46–155 (–16 to +93)	524.9
Expanded allele 2 (patient 2)	60	98	+4.6	76	20	42–81 (–18 to +21)	51.0
Normal allele 1 (patient 1)	8	124	0.0	0	0		
Normal allele 2 (patient 2)	16	104	0.0	0	0		

The variance of the change in size of the CAG repeats in single sperm from patient 1 was significantly greater than that in single sperm from patient 2 ($P < 0.0001$).

RESULTS

Mutation frequencies of expanded and normal alleles

We determined the change in the size of the CAG repeats in the *DRPLA* gene from two DRPLA individuals by comparing the size of the CAG repeats from single sperm with that of those from peripheral blood leukocyte DNA of the same individuals, according to the method used previously for single sperm analysis of the CAG repeats in the HD (21) and *MJD1* (20) genes. We refer to the case when the size of the CAG repeats is smaller in sperm than in leukocytes as 'contraction', and the opposite as 'expansion'. Table 1 shows the results of typing of 427 sperm from the two individuals with DRPLA exhibiting two different expanded and two different normal allele sizes. Expanded alleles (60 and 62 repeats) showed a high mutation frequency (96 and 98%). Of the 199 sperm carrying expanded alleles, 97% differed in size from that of the individuals' leukocyte expanded alleles. Of these, 80% showed expansions and 17% contractions.

No mutations in the normal alleles carrying eight and 16 CAG repeats were detected in the remaining 228 sperm (Table 1).

Size distribution of expanded alleles

The expanded CAG repeat size distribution in single sperm is shown in Figure 1. Analysis of expanded allele 1 in sperm from patient 1 showed a mean change of +21.2 repeats, ranging from –16 to +93, while that of expanded allele 2 in sperm from patient 2 showed a mean change of +4.6 repeats, ranging from –18 to +21 (Table 1). The variance of the change in size of the expanded CAG repeats in the *DRPLA* gene of single sperm from patient 1 (524.9) was significantly greater than that in patient 2 (51.0), as shown in Table 1 (F-test, $P < 0.0001$).

We also examined the CAG repeats in the *DRPLA* gene in pooled sperm DNA from the same individuals. Figure 2 shows that the range of expanded CAG repeats in pooled sperm DNA from patient 1 was markedly broader than that in patient 2, which confirms the data in Table 1. When we compared the size distribution of the CAG repeats in pooled sperm DNA carrying expanded alleles with that in leukocyte DNA carrying expanded alleles, we found that the degree of CAG repeat mosaicism in the expanded alleles in the pooled sperm DNA was greater than that in the expanded alleles in the leukocyte DNA from the two DRPLA individuals.

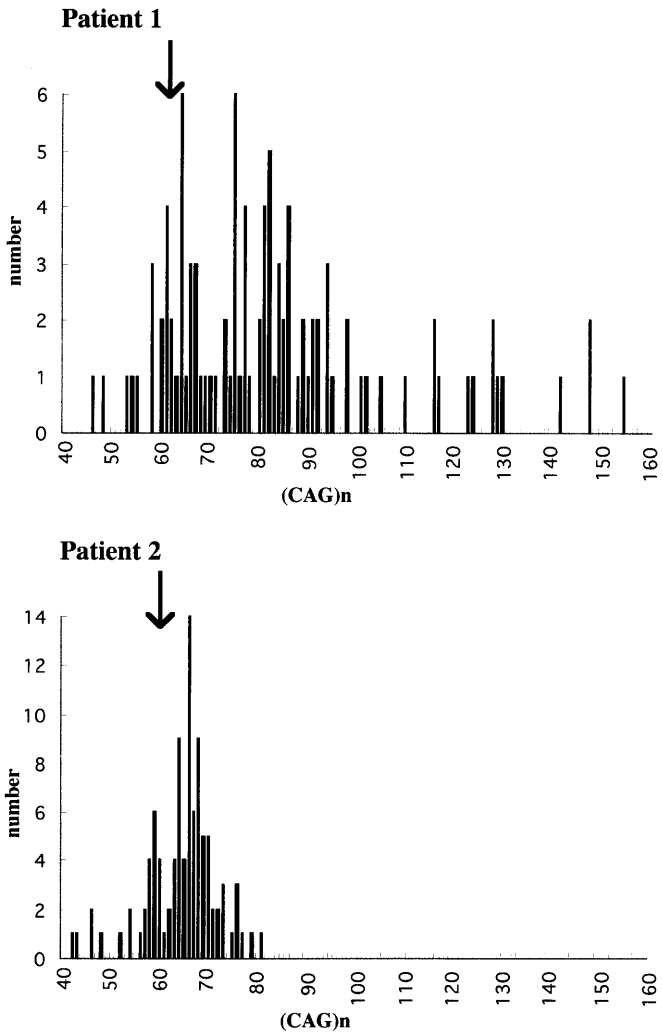

Figure 1. Distribution of single sperm CAG repeat length mutations in two DRPLA individuals. Arrows indicate the lengths of the expanded alleles in somatic cells determined on analysis of lymphocyte DNA.

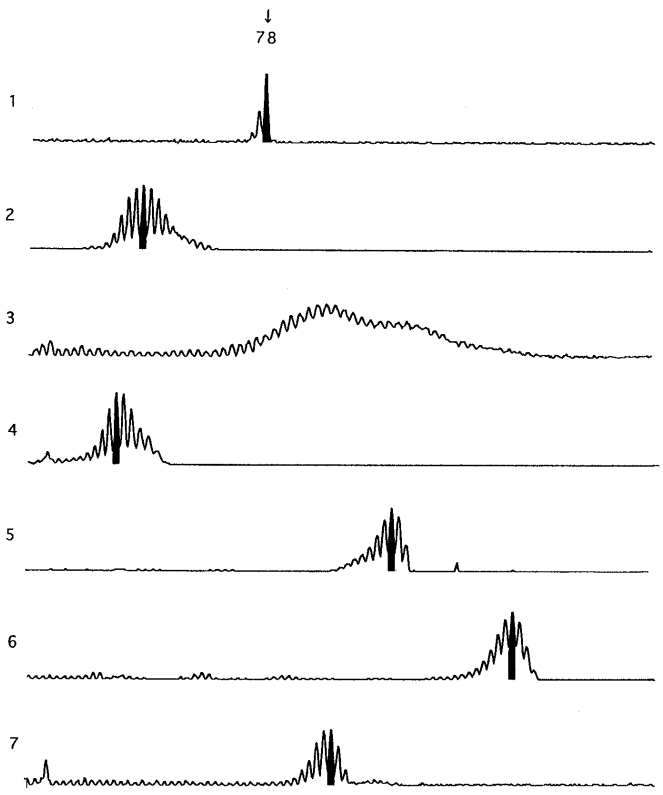

Figure 2. CAG repeat length mosaicism in the *DRPLA* gene in leukocyte (lane 2) and pooled sperm (lane 3) DNA and representative measurements of CAG repeat lengths (lanes 4–7) from patient 1. Forty nanogams each of leukocyte DNA and pooled sperm DNA were amplified. The PCR product derived from plasmid DNA containing 78 CAG repeats was used as a size marker (lane 1).

Meiotic segregation ratio of expanded and normal alleles

In the single sperm from the two individuals with DRPLA, 199 expanded alleles and 228 normal alleles were detected, which is not statistically different (χ^2 =1.97, P = 0.161) from the expected 1:1 segregation ratio. No meiotic segregation distortion of expanded alleles was observed in not only the total but also individual cases.

DISCUSSION

Paternal transmission in CAG repeat diseases has been found to result in more prominent acceleration of age at onset (mean: 25.6, 14.7 and 7.7 years per generation in DRPLA, MJD and HD, respectively) (17,19,22), and a greater intergenerational increase in the number of CAG repeats (mean: 5.8, 3.2 and 4.0 in DRPLA, MJD and HD, respectively) (17,19,22) than maternal transmission does (mean acceleration of age at onset: 14.0, 6.5 and 1.5 in DRPLA, MJD and HD, respectively; mean intergenerational increase: 1.3, 1.2 and 0.2 in DRPLA, MJD and HD, respectively) (17,19,22).

Our sperm data are consistent with those of a clinical and genetic study that showed the genetic anticipation on paternal transmission in DRPLA is most prominent among the CAG repeat diseases. As shown in Table 2, the variance of the change in size of the expanded CAG repeats in the *DRPLA* gene, that is the most appropriate index of the instability of CAG repeats, is the greatest among the CAG repeat diseases. In addition, the mean change in size of the expanded CAG repeats of single sperm from DRPLA patients is prominent among the CAG repeat diseases.

Table 2. Comparison of the instability of CAG repeats in the causative genes in single sperm among DRPLA, MJD, HD and SBMA

	DRPLA	MJD	HD	SBMA
Number	2	6	3	1
$(CAG)_n$	60–62	74–80	38–51	47
Mean variance	288.0	38.5	69.0	16.3
Mean change	+12.9	–2.5	+12.1	+1.0
Mutation frequency (%)				
Total	97	92	96	81
Expansion	80	32	93	66
Contraction	17	60	3	15
EAs:NAs[a]	1:1	6:4	1:1	

[a]EAs, sperm with expanded alleles; NAs, sperm with normal alleles.

Of particular interest is the significant difference in the variance of the change in size of the expanded CAG repeats between patients 1 and 2. Since the ages of the two patients and the CAG repeat sizes of their leukocyte DNA were very close, these factors may not have contributed to the difference in the variance. In single sperm from six MJD patients, we found the effect of a CGG/GGG polymorphism, which is located at the 3′ end of the CAG repeat in the *MJD1* gene, on the instability of the expanded CAG repeats (20). With regard to the *cis*-acting elements which contribute to the CAG tract instability, La Spada *et al*. reported that *cis*-acting instability elements might be present within the 70 kb segment of the androgen receptor locus (23). In SCA1 and SCA2, the loss of CAT or CAA interruptions of the CAG repeats can affect the stability of the CAG tract (24,25). However, we could not find any difference in the sequences flanking the first-round PCR primers including the 3′ end of the CAG repeat. Since the variance of the change in size of the expanded CAG repeats in patient 1 was extremely large, some *cis*- and/or *trans*-acting elements might have contributed to the instability of the CAG repeats in the *DRPLA* gene. Further examination is required to elucidate the mechanism underlying the instability of the CAG repeats in the *DRPLA* gene of patient 1.

Clinical and genetic observation of male meiosis in DRPLA revealed that significant distortion in favor of transmission of mutant alleles was transmitted to 62% of all offspring (78 affected individuals versus 47 unaffected in 125 paternal transmissions) (19). Our single sperm data, however, failed to confirm the occurrence of meiotic segregation distortion of expanded alleles in contrast to MJD (20). One reason might be that the total numbers of DRPLA patients and sperm analyzed were smaller than those of MJD patients and sperm. In MJD, meiotic segregation distortion of expanded alleles in sperm was not observed in two of six patients (20).

As shown in Table 2, single sperm analysis of the CAG repeats in the causative genes of the CAG repeat diseases has to date been performed for only very small numbers of patients: three for HD (21), one for SBMA (26), six for MJD (20) and two for DRPLA

in this study. With regard to the mutation frequency of the CAG repeats in the causative genes of the CAG repeat diseases, expansions are more frequent than contractions in sperm in DRPLA, HD and SBMA. In contrast to these diseases, contractions are more frequent than expansions in the *MJD1* gene in MJD sperm. The differences in the frequencies of contraction and expansion mutations in the causative genes between DRPLA, HD and SBMA sperm, and MJD sperm might be explained by differences in the CAG repeat size. The CAG repeats of the expanded alleles in MJD (74–80 repeats) are larger than those in DRPLA (60 and 62 repeats), HD (38, 49 and 51 repeats) and SBMA (47 repeats). In order to compare CAG repeat instabilities at different disease loci, comparison of alleles with similar repeat size is more appropriate since several studies revealed that an increase in CAG repeat instability is correlated with an increase in the repeat number. Further study is required to determine whether a common mechanism underlies the instability of the CAG repeats in the CAG repeat diseases.

MATERIALS AND METHODS

DNA preparation and determination of the size of CAG repeats in genomic DNA

Samples of peripheral blood and semen were obtained with informed consent from two DRPLA patients aged 51 and 52 years. DNA from peripheral blood leukocytes was extracted using standard DNA extraction protocols (27). Sperm DNA was obtained by the method of Jeffreys *et al.* (28). The primer sequences used for PCR analysis of genomic DNA and the PCR conditions were as described by Koide *et al.* (5). The PCR-produced fragment was electrophoresed in a capillary filled with Performance Optimized Polymer 4 on an automated ABI PRISM 310 genetic analyzer; analysis was performed with the GeneScan analysis software, version 2.0 (ABI-Perkin Elmer, Urayasu, Japan). The CAG repeat sizes were determined by using, as size markers, the PCR products derived from plasmid DNAs containing CAG repeats of various sizes (8, 16 and 78 repeat units) and GeneScan-500 TAMRA. The allele sizes were defined as the signal peak with the greatest area.

Single sperm isolation and preparation

Single sperm cells were micromanipulated using a Trancell TS-008 (JEOL Trading, Tokyo, Japan) (29). A single sperm was placed in a 0.2 ml tube containing 2.5 µl of alkaline lysis buffer. Prior to PCR, the tubes were left at room temperature for 15 min and then heated at 65°C for 10 min, followed by neutralization with 2.5 µl of a solution comprising 300 mM KCl and 900 mM Tris–HCl, pH 8.5 (30).

First-round PCR

Two rounds of PCR were performed to amplify the *DRPLA* gene CAG repeat region using the nested PCR strategy. The first-round PCR was performed in a total volume of 25 µl containing the neutralized sample, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0 mM $MgCl_2$, 10% dimethylsulfoxide (DMSO), 200 µM each of dATP, dGTP, dCTP and TTP, 0.8 µM of each primer and 2.5 U *Taq* polymerase (Takara, Kyoto, Japan). The sequences of the primers used for the first-round PCR were: DRPLA-1593, 5′-TAGCCAACAGCAATGCCCATCCAG-3′; and DRPLA-1817, 5′-TCAGAGACCCCAGGGAGGGAGACAT-3′. After initial denaturation at 95°C for 2 min, PCR was carried out for 35 cycles consisting of 1 min at 95°C, 1 min at 62°C and 2 min at 72°C, followed by final extension at 72°C for 5 min.

Second-round PCR and analysis of the CAG repeats of a single sperm

The second-round PCR was performed in a total volume of 20 µl containing 1 µl of the first PCR product, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.5 mM $MgCl_2$, 2.5 M betaine, 200 µM each of dATP, dGTP, dCTP and dTTP, 0.8 µM of each primer and 5 U *Taq* polymerase. The sequences of the primers used for the second-round PCR were: DRPLA-1659, 5′-FAM-CACCAGTCTCAACACATCACCATC-3′; and DRPLA-1784, 5′-CCTCCAGTGGGTGGGGAAATGCTC-3′. After initial denaturation at 95°C, PCR was carried out for 40 cycles consisting of 1 min at 95°C, 1 min at 62°C and 1 min at 72°C, followed by final extension at 72°C for 7 min. The PCR products were then electrophoresed in a capillary filled with Performance Optimized Polymer 4 on an automated ABI PRISM 310 genetic analyzer, and the numbers of CAG repeats of the *DRPLA* gene in single sperm were determined as described under DNA preparation and determination of the size of CAG repeats in genomic DNA.

If *DRPLA* PCR products were detected in any one of three no-sperm controls, the data set was discarded. Thus, the possibility of contamination from an exogenous source during the isolation of single sperm and PCR amplification was excluded. This occurred only rarely.

The sample size being tested was 503 single sperm for patient 1 and 314 for patient 2, and thus PCR amplification efficiencies were 45 and 68% for patients 1 and 2, respectively.

Statistical analysis

Differences in the segregation ratio of a single sperm with an expanded allele and one with a normal allele were determined using the χ^2 test. Differences in the variance of the change in size of the expanded CAG repeats of the *DRPLA* gene between sperm from the two individuals were analyzed using the F-test.

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