French Machado–Joseph disease patients do not exhibit gametic segregation distortion: a sperm typing analysis

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Segregation distortion has been reported to occur in a number of the trinucleotide repeat disorders. On the basis of a sperm typing study performed in patients of Japanese descent with Machado–Joseph disease (MJD), it was reported that disease alleles are preferentially transmitted during meiosis. We performed a sperm typing study of five MJD patients of French descent and analysis of the pooled data shows a ratio of mutant to normal alleles of 379:436 (46.5:53.5%), which does not support meiotic segregation distortion. To confirm these results, sperm typing analysis was also performed using a polymorphic marker, D14S1050, closely linked to the MJD1 gene. Among 910 sperm analyzed, the allele linked to the disease chromosome was detected in 50.3% of the samples and the allele linked to the normal chromosome was found in 49.6% of the sperm. The difference in frequency of these two alleles is not significant (P = 0.8423). Likelihood-based analysis of segregation distortion in the single sperm data using the SPERMSEG program also showed no support for segregation distortion at the gamete level in this patient population. The previous report on the Japanese patients also suggested that disease allele stability may be influenced by a trans effect of an intragenic polymorphism (987 G/C) found in the 3'-end of the wild-type allele of the gene (3,4). It was reported that those MJD patients who carried the 987G/C genotype showed a significantly greater CAG repeat transmission instability when compared with patients who carried the 987C/C genotype. It has been proposed that an interallelic interaction due to a 'trans' effect occurs in which the genotype of the wild-type allele influences the transmission stability of the expanded allele in MJD heterozygotes (4). Furthermore, in comparing the transmission of disease and normal MJD1 alleles it has been noted that the disease allele is preferentially transmitted to the offspring from their affected father. Initially, this observation was based upon the clinical data of 80 transmissions in seven Japanese MJD pedigrees but not confirmed in French families (5,6). Recently, the clinical data have been supported by single sperm PCR analysis of 1036 meioses using semen samples obtained from six MJD patients of Japanese descent (4).

Whether or not segregation distortion occurs in MJD due to the production of more sperm carrying the disease than the normal allele is significant and has implications for the biology of trinucleotide repeat instability. In addition, there are clinical implications for genetic counseling of patients with MJD. In this study, we quantify the mutation spectrum of the mutant MJD1 alleles and investigate segregation distortion by sperm typing in another ethnic group, MJD patients of French descent. We do not detect segregation distortion of MJD disease alleles in

INTRODUCTION

The trinucleotide repeat disorders are a class of neurogenetic diseases characterized by expansions of normally polymorphic triplet repeats (1). Machado–Joseph disease (MJD), or spinocerebellar ataxia 3, an autosomal dominant neurodegenerative disorder, belongs to this class of disease and is characterized by trinucleotide repeat expansions of the triplet CAG in the MJD1 gene (2). Clinical manifestations include progressive neurological symptoms and signs of both central and peripheral nervous system involvement, including ataxia, pyramidal signs and neuropathy.

Clinical genetic studies of MJD show that the CAG repeat is unstable during parent–offspring transmission and among the factors which have been suggested to influence the degree of instability are paternal origin and an intragenic polymorphism (987 G/C) found in the 3'-end of the wild-type allele of the gene (3,4). It was reported that those MJD patients who carried the 987G/C genotype showed a significantly greater CAG repeat transmission instability when compared with patients who carried the 987C/C genotype. It has been proposed that an interallelic interaction due to a 'trans' effect occurs in which the genotype of the wild-type allele influences the transmission stability of the expanded allele in MJD heterozygotes (4). Furthermore, in comparing the transmission of disease and normal MJD1 alleles it has been noted that the disease allele is preferentially transmitted to the offspring from their affected father. Initially, this observation was based upon the clinical data of 80 transmissions in seven Japanese MJD pedigrees but not confirmed in French families (5,6). Recently, the clinical data have been supported by single sperm PCR analysis of 1036 meioses using semen samples obtained from six MJD patients of Japanese descent (4).

Whether or not segregation distortion occurs in MJD due to the production of more sperm carrying the disease than the normal allele is significant and has implications for the biology of trinucleotide repeat instability. In addition, there are clinical implications for genetic counseling of patients with MJD.

In this study, we quantify the mutation spectrum of the mutant MJD1 alleles and investigate segregation distortion by sperm typing in another ethnic group, MJD patients of French descent. We do not detect segregation distortion of MJD disease alleles in

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RESULTS

Mutation spectrum and meiotic segregation analysis of mutant MJD1 alleles

Single sperm analysis of the triplet repeat portion of the MJD1 gene was performed on a total of 1050 sperm cells and PCR products were obtained from 815, demonstrating an overall PCR efficiency of amplification of 77.6%. The ratio of disease to normal alleles was 379:436 (46.5:53.5%) for the pooled data obtained from all five individuals (Table 1). Assuming no segregation distortion, the efficiency of amplification of the disease alleles was 72.2 and of the wild-type alleles 83.0%. If segregation distortion in favor of the disease alleles did occur, then the PCR efficiency of the expanded alleles would be lower. Analysis of the 379 disease alleles revealed a mutation frequency of 88.3%, with 54.1% expansions and 33.2% contractions and an average change in repeat number of +0.74 (Table 1).

Analysis of a linked polymorphism to study segregation distortion

The marker D14S1050 is closely linked to the MJD1 gene with a genetic distance <2 cM (7.8). It was also informative in all five individuals, with a difference in size between the two alleles of 4-6 bp. By co-amplifying the MJD1 CAG repeat and D14S1050 we were able to determine which of the D14S1050 alleles were linked to the expanded mutant MJD1 allele (D14S1050_L) for all the donors (Table 2). Thus, except for rare recombination events, analysis of D14S1050 will indicate the segregation pattern of the wild-type and disease MJD alleles.

Single sperm cells from each individual donor were amplified at the D14S1050 locus. Amplification products were obtained from 910 sperm, demonstrating a PCR efficiency of 88.7% for the pooled data. The data were analyzed with respect to which allele was linked to the normal and disease MJD alleles; 50.3% disease alleles and 49.6% normal alleles were found (Table 2). These results do not deviate significantly from a ratio of 1:1 expected in the absence of segregation distortion (P = 0.8423). This was also true for each of the MJD sperm donors.

Single genome PCR was performed on somatic DNA obtained from two of the patients and diluted to single copy level to determine whether there were any allelic differences in PCR efficiency for marker D14S1050. The pooled data from amplifying 190 molecules show a ratio of the two D14S1050 alleles of 97:93 and these results are not significantly different from an expected ratio of 95:95 (P = 0.7716).

Likelihood-based segregation distortion analysis

We performed a likelihood-based analysis of segregation distortion in the single sperm data using the SPERMSEG program (http://galton.uchicago.edu/~mcpeek/software/spermseg) of M.S. McPeek (in preparation). The analysis uses a model that explicitly takes into account PCR amplification efficiency, probabilities of 0, 1 or 2 sperm in a sample, contamination and recombination and that also allows for segregation distortion (9; M.S. McPeek, in preparation). The specific model fitted to these data assumed a common probability of segregation of the mutant MJD1 allele across donors. It also assumed allele-specific amplification and contamination rates that are the same across donors. To take into account inter-experimental variability, the probability of one sperm deposited is different for each experiment, while the probability of two sperm deposited is the same across donors. The recombination fraction between MJD1 and D14S1050 was assumed to be 1%. A Monte Carlo goodness-of-fit test (M.S. McPeek, in preparation) did not indicate a misfit of this model to the data (P = 0.3). The amplification efficiencies at the MJD1 locus were estimated to be 78% for the mutant allele and 97% for the wild-type allele. At the linked marker, the amplification efficiencies were estimated to be 98 and 95%, with this difference not being significant (P = 0.29). Probabilities of one sperm deposited in a sample ranged from 85 to 98% and contamination rates ranged from 0 to 2%. Under this model, the segregation probability of the mutant MJD1 allele is estimated to be 0.496, with the 95% confidence interval (CI) extending from 0.461 to

Table 1. Single sperm typing data of the MJD1 gene from five French patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>No. of sperm with mutant allele</th>
<th>Mean change</th>
<th>Expanded (%)</th>
<th>Contracted (%)</th>
<th>Range</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14/73</td>
<td>79</td>
<td>-0.76</td>
<td>48</td>
<td>37</td>
<td>58-78</td>
<td>62.86</td>
</tr>
<tr>
<td>2</td>
<td>23/74</td>
<td>70</td>
<td>+3.44</td>
<td>87</td>
<td>10</td>
<td>57-85</td>
<td>35.36</td>
</tr>
<tr>
<td>3</td>
<td>14/76</td>
<td>88</td>
<td>-0.32</td>
<td>33</td>
<td>43</td>
<td>69-82</td>
<td>39.13</td>
</tr>
<tr>
<td>4</td>
<td>28/72</td>
<td>62</td>
<td>+4.19</td>
<td>93</td>
<td>6</td>
<td>69-83</td>
<td>15.60</td>
</tr>
<tr>
<td>5</td>
<td>14/73</td>
<td>80</td>
<td>+1.67</td>
<td>26</td>
<td>60</td>
<td>64-78</td>
<td>30.85</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>379</td>
<td>+0.74</td>
<td>54</td>
<td>33</td>
<td>57-85</td>
<td></td>
</tr>
</tbody>
</table>

* Number of repeats in the wild-type allele/number of repeats in the mutant allele.

*Table 2. Single sperm typing data of segregation distortion of the D14S1050 marker

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>No. of sperm analyzedD14S1050_L (no.)</th>
<th>D14S1050_L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>133/137</td>
<td>187</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>129/133</td>
<td>181</td>
<td>91</td>
</tr>
<tr>
<td>3</td>
<td>133/139</td>
<td>178</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>133/139</td>
<td>202</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>133/137</td>
<td>162</td>
<td>83</td>
</tr>
<tr>
<td>Totals</td>
<td>910</td>
<td>458</td>
<td>50.3</td>
</tr>
</tbody>
</table>

D14S1050_L refers to that D14S1050 allele linked to the expanded MJD1 mutant allele.
DISCUSSION

Mutation frequency

Our results on the mutation frequency can be compared with those obtained from the sperm typing study of Japanese patients with MJD. The overall mutation frequency in both groups is similar, with a 92% mutation frequency observed in the Japanese patients versus 87% in the current study (4). The mean change in the size of the MJD1 alleles during transmission was +0.74 CAG repeats in this study, compared with −1.85 CAG repeats in the previous study. However, the French patients (average disease allele size 73.6 CAG repeats) show more expansions than contractions when compared with the Japanese patients (average disease allele size 75.5 CAG repeats). Although the difference in the frequency of the expansions between these two groups is significant (95% CI 49–59% and 95% CI 28–36%, respectively), there is a large variation in the frequency of expansions in the disease alleles of individual patients within each group.

The sperm typing results can be compared with the clinical data on paternal MJD transmission. In the largest clinical study with available data, 46 paternal transmissions (range of disease allele size 72.5–74.7 CAG repeats) are reported from a variety of ethnic groups and a paternal meiotic instability of +2.8 CAG repeats is obtained (3). In 18 paternal transmissions analyzed in French families the mean increase was only +1.2 CAG repeats (6), which is consistent with the sperm typing data. In a clinical report of Chinese patients with MJD, four paternal transmissions (average disease allele size 74.8 CAG repeats) were reported with a gain of only +0.3 CAG repeats (9). Although there is general agreement between the sperm typing and clinical transmission data, a more reliable comparison can only be made with more data from paternal–child transmissions.

These data on disease allele instability in MJD can be compared with sperm typing results in other CAG repeat disorders where the patients had similar allele lengths. Sperm typing was performed in Huntington’s disease (HD) and dentatorubral-pallidoluysian atrophy (DRPLA) with average disease allele lengths of 62 (11) and 61 (12) repeats, respectively. There were only two donors in each case. The HD and DRPLA disease alleles are >10 CAG repeats smaller than those studied in MJD yet the average gain of CAG repeats (HD 32.51 and DRPLA 9.74) during paternal transmission was much larger. The mean change in repeat size in the disease alleles in MJD is more similar to that observed in spinal and bulbar muscular atrophy disease alleles, with an average length of 48 repeats (13). These sperm typing studies indicate that there are factors independent of the repeat size of the disease alleles that influence the degree of instability.

The trans effect on transmission stability

Those Japanese MJD patients who carried the 987\textsubscript{GC} genotype showed a significantly greater transmission instability of the CAG repeat compared with Japanese patients who carry the 987\textsubscript{GC} genotype (4). The analysis of this instability was carried out by comparing the variance of the change in size of the CAG repeats in single sperm typing from two patients (average disease allele length 75.0 repeats) with the 987\textsubscript{GC} genotype and four patients (average disease allele length 75.6 repeats) who carry the 987\textsubscript{GC} genotype. All of the French patients in our study carry the heterozygous 987\textsubscript{GC} genotype with an average disease allele size of 73.6 CAG repeats (Table 1) and thus we cannot compare their instability with French C/C homozygotes. However, analysis of the variance of the change in the number of the CAG repeats in these French patients shows a wide range (15.60–62.86) which overlaps that of the Japanese patients carrying the homozygous C/C genotype. This inter-population comparison also suggests that there may be factors independent of the 987 intragenic polymorphism which influence the transmission stability of the expanded alleles.

Segregation distortion analysis

The initial analysis of our sperm typing data shows that greater numbers of PCR products containing the wild-type than the expanded disease alleles are obtained. These results are not unexpected, since it has been previously noted that there is greater efficiency of amplification of the wild-type alleles because the target templates are smaller (14). To circumvent these difficulties associated with possible differential amplification of MJD1 alleles of different sizes and to provide an independent analysis of segregation distortion, linked marker analysis was carried out where the difference in size between the two alleles was only between 4 and 6 bp. The results fail to support the presence of segregation distortion in MJD at the sperm level in the French patients.

There may be several explanations for the discrepancy between those of the sperm typing analysis of the Japanese MJD patients and those of our study. On the one hand, the difference may have a technical explanation. For example there may be a polymorphism present in that portion of the MJD1 gene where the primers anneal. If the PCR primers exactly matched the disease allele but differed from the normal allele, this could affect the amplification efficiency and could account for preferential amplification of the disease allele over the normal allele. This issue could be addressed by performing single genome PCR analysis on somatic DNA diluted to single copy level and comparing the ratio of wild-type to disease alleles amplified. It is also possible that there is a polymorphism where the primers anneal in the D14S1050 marker and by chance all the alleles linked to the disease MJD1 allele were poorly amplified, thereby eliminating any evidence for segregation distortion. We note, however, that our single genome PCR analysis of the D14S1050 marker performed on single molecules of somatic DNA does not show any preferential amplification of either allele. Alternatively, there might be a biological basis for segregation distortion being present in Japanese but not French patients.

The clinical data supporting the presence of segregation distortion in Japanese MJD patients are based upon 80 transmissions, of which 33 were paternal (5). Although suggestive, this analysis did not demonstrate statistical significance for an excess of affected offspring. In an analysis of segregation distortion in Brazilian patients with MJD, the progeny of 40 patients (22 male and 18 female) showed 91 affected versus 64 unaffected (15). This analysis also did not reach statistical significance. In an analysis of our clinical data on MJD in French pedigrees, a total of 127 transmissions were observed with 73 through an affected father (A. Dürr et al., personal communication; 6). In these paternal transmissions, the ratio of carriers to non-carriers was 42:31 and in the maternal transmissions the ratio was 31:23. These data again do
not support the presence of segregation distortion ($\chi^2$ test, $P = 0.092$). Although the data from these clinical studies could be pooled there are difficulties in interpreting the results of such an analysis because of differences in how they were conducted. For example, some of the individuals included in one study were diagnosed as affected without confirmation by genotyping and in another study information on how individuals were diagnosed as affected is not provided. In these clinical studies, where the sample size is small, misdiagnosis of a few individuals could have an impact on the conclusions which have been reached. Moreover, when families are recruited for linkage or other genetic studies, sibships with one or more affected individuals are more likely to be ascertained and sampled than those without.

There have been a series of reports indicating the presence of meiotic drive not only in MJD but in several other of the trinucleotide repeat disorders, including myotonic dystrophy (DM), Fragile X and DRPLA (5,16–19). In a previous single sperm analysis in MJD, we failed to confirm meiotic drive in a series of non-disease alleles thought to be progenitors of disease alleles (9). Moreover, in the initial clinical study reporting meiotic drive in MJD, it was proposed that male-specific meiotic drive also occurred in Japanese patients with DRPLA, but single sperm analysis performed in two patients failed to provide supporting evidence (5,12). Sperm typing studies do not exclude the occurrence of male-driven meiotic drive resulting from events following ejaculation; however, until clinical studies of this phenomenon reach statistical significance, the presence of meiotic drive in these disorders will remain controversial (19).

Our single sperm analysis of French MJD patients confirms and extends the previous study of Japanese patients and shows a high mutation frequency of the disease alleles during paternal transmission. In addition to previous sperm typing studies of HD, DRPLA and SBMA, the results of these studies provide highly accurate data on mutation instability of the disease alleles during paternal transmission. However, in MJD patients of French descent, the results do not support increased MJD1 allele instability associated with the intragenic 987 G/C polymorphism when compared with the Japanese sperm typing data. Furthermore, our results do not support the presence of male-driven meiotic drive at the gamete level.

MATERIALS AND METHODS

Samples

After obtaining informed consent, blood and semen samples were obtained from five French patients with MJD. DNA was extracted from the blood using standard methods and the CAG repeat number at the MJD1 locus for each individual was determined (Table 1). The genotype of the 987 G/C intragenic polymorphism was determined as previously described (8). All of the patients carried the 987 G/C genotype. The genotype of a closely linked non-intragenic microsatellite repeat marker, D14S1050, was determined by performing two rounds of PCR as described below. The semen was processed for single sperm isolation and individual sperm cells were sorted using a FacStar Plus fluorescence-activated cell sorter and lysed as previously described (20). Single genome analysis of somatic DNA was also performed as previously described (14).

PCR

Two rounds of PCR using a nested strategy were used to co-amplify the MJD triplet repeat region and the linked non-intragenic marker D14S1050. First round PCR was performed in a final volume of 50 µl and the final concentrations for the reagents used were: 50 µM each dATP, dCTP, dTTP and 7-deaza dGTP, 10% (v/v) glycerol, 3% (v/v) DMSO, 1.2 mM Mg(OAc)$_2$, 80 mM KOAc was added for a final K$^+$ concentration of 100 mM. The final pH was 8.7 and 2 U Taq DNA polymerase (Promega) were employed. To amplify the repeat triplet portion of the MJD1 gene, the previously described primers MJD52 and MJD70 were used at a final concentration of 0.5 µM (2). To amplify the linked marker D14S1050, the following primers were designed and used each at a final concentration of 0.5 µM: D14S1050C, 5'-AAGAGAGTACAAATTT-3', D14S1050D, 5'-AGCTATGTGACCT-3'. The thermal cycling profile for this round of PCR was: 94°C for 5 min initial denaturation, 26 cycles of 94°C for 30 s denaturation, 66°C for 5 min for the first eight cycles and 3 min for the next 18 cycles and a 72°C for 5 min final extension.

Figure 1. Representative single sperm PCR products of the CAG repeat portion of the MJD1 gene of a patient with MJD analyzed on a DNA Sequencing Machine (Pharmacia) with internal markers at 250 and 350 bp. Lane 29 contains a sample of amplified genomic DNA from a patient with 76 CAG repeats. The remaining lanes contain products from 16 individual sperm.
modification that MJD25 was fluorescein labeled (4). The thermocycler conditions were: 96°C for 2 min initial denaturation, 38 cycles of 95°C for 1 min, 58°C for 1 min followed by 72°C for 7 min final extension. To amplify D14S1050, primers were designed and used at a concentration of 0.5 μM: D14S1050A, 5’-CCTGGGCAAGGTAAG-3’; D14S1050B, 5’-GGGGGACATTGTGA-3’. The thermocycler conditions were: 96°C for 2 min initial denaturation, 42°C of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min followed by 72°C for 10 min final extension.

To obtain increased efficiency of amplification of the linked marker, some adjustments were made to the protocol. The first round conditions and primers were identical except that 1 U rTth polymerase (Perkin Elmer) was used instead of Taq polymerase. The second round conditions and primers were identical.

Analysis of PCR products
Ten microliters of the PCR reaction were first analyzed on 3% agarose gels for the presence of a product. When the PCR products containing the triplet repeat portion of MJD1 are run on these gels, those products containing the expansions can be distinguished from wild-type alleles. The expanded alleles were then further analyzed on a L.F. ReadyMix Gel (6% acrylamide, 7 M urea, 0.6× TBE) 0.5 mm thick short plate gels (~11.5 cm long) using the A.L.F. Fragment Manager (Pharmacia) with internal standards. Aliquots of 1.2 μl PCR product and 0.6 fmol of each internal molecular weight standard in 42% formamide were denatured at 95°C for 2 min prior to loading and run at 600 V, 50°C. Allele size was defined as the peak with the greatest area. In addition, a sample of PCR products from amplification of genomic DNA from that individual was run simultaneously on the same gel (Fig. 1).

For analysis of the linked marker, alleles were analyzed on 1.0 mm thick, 8 cm long, 1× TBE, non-denaturing, 8% polyacrylamide gels run at 120–160 V (1–3 h) and stained in 0.5 μg/ml ethidium bromide (Fig. 2).

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