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Influence of Sex of the Transmitting Parent as Well as of Parental Allele Size on the CTG Expansion in Myotonic Dystrophy (DM)

H. G. Brunner,* H. T. Brüggenwirth,* W. Nillesen,* G. Jansen,† B. C. J. Hamel,* R. L. E. Hoppe,* C. E. M. de Die,‡ C. J. Höweler,§ B. A. van Oost,* B. Wieringa,† H. H. Ropers,* and H. J. M. Smeets*

Departments of *Human Genetics and †Cell Biology and Histology, University Hospital and Medical Faculty, Nijmegen; and Departments of ‡Clinical Genetics and §Neurology, University Hospital, Maastricht, The Netherlands

Summary

In patients with myotonic dystrophy (DM), the severity of clinical signs is correlated with the length of a $(CTG)_n$ trinucleotide repeat sequence. This sequence tends to expand in subsequent generations. In order to examine the kinetics of this process and, in particular, the influence of the mutant-allele size and the sex of the transmitting parent, we have studied $(CTG)_n$ repeat lengths in the offspring of 38 healthy carriers with small mutations (less than 100 CTG trinucleotides, mean length $[CTG]_{67}$). In these studies, we found a weakly positive correlation between the size of the mutation in the carrier parents and that in their offspring. Furthermore, we observed that, in the offspring of male transmitters, repeat lengths exceeding 100 CTG trinucleotides were much more frequent than in the offspring of carrier females (48 [92%] of 52 vs. 7 [44%] of 16, $P = .0002$). Similarly, in genealogical studies performed in 38 Dutch DM kindreds, an excess of nonmanifesting male transmitters was noted, which was most conspicuous in the generation immediately preceding that with phenotypic expression of DM. Thus, two separate lines of evidence suggest that the sex of the transmitting parent is an important factor that determines DM allele size in the offspring. On the basis of our data, we estimate that when both parents are asymptomatic, the odds are approximately 2:1 that the father carries the DM mutation. Because expansion of the CTG repeat is more rapid with male transmission, negative selection during spermatogenesis may be required to explain the exclusive maternal inheritance of severe congenital onset DM.

Introduction

The mutation underlying myotonic dystrophy (DM) has recently been characterized as an expanding CTG trinucleotide repeat sequence in the 3′ untranslated region of a protein kinase gene (Aslanidis et al. 1992; Brook et al. 1992; Buxton et al. 1992; Fu et al. 1992; Harley et al. 1992*a;* Jansen et al. 1992; Mahadevan et al. 1992). Repeats containing over 40 CTG units tend to expand further in successive generations, and both age at onset and the clinical phenotype correlate with the size of the mutation (Harley et al. 1992*b;* Hunter et al. 1992). These findings have provided a biological basis for the progressively earlier onset of DM in consecutive generations, i.e., anticipation (Fleischer 1918; Ravin and Waring 1939; Bell 1947; Klein 1958; Höweler et al. 1989). However, the factors governing the transition, from minimally expanded CTG trinucleotide repeats found in subjects who are asymptomatic or have cataract as their only feature to larger mutations associated with muscle weakness and clinical myotonia, have not yet been elucidated. We performed DNA analysis in DM families to evaluate whether the size of the CTG trinucleotide repeat sequence in the parent might influence the size of the mutation in the offspring. A parental size effect had already been documented by several groups for the CCG trinucleotide expansion associated with the fragile X syndrome (Fu et al. 1991; Heitz et al. 1992; Yu et al. 1992). Because there appears to be an excess of mildly affected or asymptomatic male trans-

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Address for correspondence and reprints: H. G. Brunner, Department of Human Genetics, University Hospital, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

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mitters in DM families (Bell 1947; Klein 1958; Harper 1989; Brunner et al. 1991), we performed genealogical studies in 38 DM families and analyzed previous reports of such studies to examine whether this male excess is specifically associated with the first appearance of the clinical phenotype in the family. We also studied the molecular basis for this phenomenon by analyzing the influence of the sex of the transmitting parent on the size of the DM mutation in their offspring.

Material and Methods

Genealogical Studies

Over a 5-year period (1987–91), all patients with DM seen at the Nijmegen clinical genetics unit were asked to complete a pedigree form that included names, dates, and places of birth and of marriage of their parents, grandparents, and great-grandparents. Pedigrees were constructed that included all affected family members known to the proband. Whenever possible, this information was confirmed by reviewing the medical records or by clinical examination of family members. Affected individuals were considered to belong to a single pedigree if they were linked together through no more than three apparently unaffected individuals. For technical reasons, we limited our studies to pedigrees that originated wholly or partly from the province of North Brabant. There has been no major emigration from, or immigration into, this area over the last 200 years. Its population (approximately 300,000 in 1800 and 2 million in 1991) shows little evidence of inbreeding, similar to the Dutch population as a whole (van Straaten 1986). A total of 38 pedigrees were studied. For each particular pedigree, genealogical studies started at the common ancestral pair and were extended to approximately 1780 (6–8 generations). Sex and age at death were recorded for the obligate carriers who were thus identified. Obligate gene carriers were considered asymptomatic if no muscle weakness or cataract had been noted until at least age 50 years. In the normal population between age 45 and 55 years, approximately 12% of individuals have lens opacities, and approximately 2.5% have lens opacities causing visual loss (Leske and Sperduto 1983). Therefore, subjects who developed cataract as the only symptom after the age of 50 years were also classified as asymptomatic. Generally, medical records or reliable clinical information or both were available for 2–3 generations preceding the appearance of clinical DM in a family. However, for individuals born prior to 1880, documentation was generally lacking.

In addition, data from four published clinical and genealogical studies of DM were included in our analysis (Fleischer 1918; Klein 1958; Höweler 1986; Mathieu et al. 1990). For the study of the sex ratio, we excluded families that showed multiple alternative links. Statistical significance was tested using either the normal approximation to the binomial distribution (two tailed) or the χ^2 test, where appropriate.

DNA Studies

Families were ascertained through the DNA diagnostic service of our department. Care was taken to ensure that all families met previously defined diagnostic criteria (Griggs et al. 1989). Chromosomal DNA was isolated from peripheral blood (Miller et al. 1988). Using a recently described PCR assay (Mahadevan et al. 1992), we determined the length of the CTG repeat in genomic DNA. The CTG repeat was amplified with flanking primers 406 and 409 (Mahadevan et al. 1992). Alleles containing up to approximately 90 CTG trinucleotide repeats were resolved on a 6% polyacrylamide/7 M urea sequencing gel after ^{32}P end-labeling of one of the amplification primers (Brunner et al. 1992). Expanded alleles appeared on the polyacrylamide gel as a mosaic pattern of DNA fragments differing by one or a few trinucleotide repeats. The size of the most prominent band was estimated by comparison with a known sequencing ladder. Accurate sizing was also possible by using fluorescently labeled PCR primers and subsequent analysis on an automated sequencer (ABI 373), with Genescanner software and internal lane standards (Genescan 2500 ^{TR}ROX). Size estimates for larger alleles were obtained from analysis of PCR products on 1% and 2% agarose gels blotted onto GeneScreen Plus filters and hybridized with a ^{32}P end-labeled $(CTG)_{10}$ oligonucleotide. In general, the PCR products produced a smear, and the size of the small end of the smear was determined by comparison with a mixture of lambda × *Hin*dIII and Phi × 174 × *Hae*III digestions as a marker. Finally, unsizable, large smears, partly resulting from heteroduplex formation in the PCR products, were checked by conventional Southern blot analysis of *Hin*dIII-digested genomic DNA, by using probe pGB2.6 (Mahadevan et al. 1992). The same size marker was used. For statistical evaluation, Fischer's exact test, the χ^2 test, Spearman correlation coefficient, Student's *t*-test, and Wilcoxon's rank sum test were used, where appropriate.

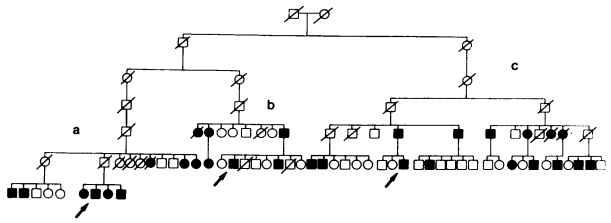

Figure 1 Genealogical connections between three independently ascertained DM kindreds. Only DM patients and their siblings and offspring are shown. Arrows indicate probands. Blackened symbols denote clinically affected individuals. Clinically unaffected individuals were counted as belonging to the last asymptomatic generation if they had a clinically affected offspring. All other unaffected individuals that linked different pedigrees together were counted only once as belonging to the previous generations.

Results

Genealogical Studies

We found that 22 (56%) of 38 DM pedigrees were linked to at least 1 other DM pedigree. In eight instances, two families were linked together. In two instances, three families were linked by the genealogical studies. Genealogical links involved 4–10 apparently unaffected individuals (average 6.6 individuals). Genealogical linkage of three independently ascertained pedigrees is shown in figure 1. Analysis of our data indicated that genealogical links involve males more frequently than females (62 males vs. 38 females; $P = .02$). Analysis of published DM pedigrees (Fleischer 1918; Klein 1958; Höweler 1986; Mathieu et al. 1990) shows an excess of male transmission in each study. For all studies combined, genealogical links involved 387 males and 267 females ($P < .001$). As shown in table 1, this difference is caused by a disproportionate excess of males in the last asymptomatic generation. Comparison of the paternal/maternal sex ratio between individuals in the last asymptomatic generation and those in all preceding generations shows a highly significant difference (177/90 vs. 210/177, respectively; $P < .005$). This was still true when a large French-Canadian pedigree study (Mathieu et al. 1990) was excluded ($P < .01$). This study classified individuals only as affected or unaffected, without further details, and identified a common ancestral pair for 746 DM patients from a highly inbred area. Inbreeding increases the chance of finding spurious genealogical links, although this is unlikely to be relevant in this case of extensive and detailed genealogical studies of a very large number of patients.

DNA Studies

We studied the offspring of 38 subjects (28 males and 10 females) with a CTG repeat size of less than 100 (range 41–95, mean 67 CTG trinucleotide repeats) and their offspring. Carriers of this type of mutation usually have cataract as their only feature or no symptoms at all (Brunner et al. 1992; Reardon et al. 1992; H. T. Brüggenwirth, unpublished results). These 38 subjects (only 3 of whom were included in the genealogical studies) had transmitted the DM mutation to a total of 68 offspring. In all but two offspring, the CTG repeat had increased in size on transmission, confirming the general tendency for anticipation in this disorder (Fleischer 1918; Höweler et al. 1989; Ashizawa et al. 1992*a*, 1992*b*). As shown in table 2, in 55 (81%) of 68 offspring the DM mutation had undergone expansion to at least 100 CTG trinucleotides. As expected, the size of the mutation in offspring correlated with the size of the parental mutation when all data were analyzed together ($r = .278$, $P = .02$ by Spearman's rank correlation).

Table 1

Comparison of Paternal versus Maternal Transmission of the DM Gene in Five Studies

	Paternal/Maternal Transmission of the DM Gene						
	Fleischer 1918	Klein 1958	Höweler 1986	Mathieu et al. 1990	Present Study	Total	*P* Value[a]
Last asymptomatic generation	7/1	25/6	15/5	99/65	31/13	177/90	<.001
Previous generations	6/8	31/16	7/4	135/124	31/25	210/177	.103
Total	13/9	56/22	22/9	234/189	62/38	387/267	<.001

[a] *P* values were calculated for the ratio of paternal to maternal transmission, compared with the expected 50% segregation, by using the normal approximation to the binomial distribution (two tailed). The last asymptomatic generation shows an excess of male transmission compared with previous generations ($\chi^2 = 9.45$; $P < .005$).

Table 2

Proportion of Offspring with a DM Mutation Exceeding 100, 200, or 300 CTG Trinucleotide Repeats

CTG Repeat of Parent and Offspring	Sex of Transmitting Parent		*P* Value[a]
	Male	Female	
40–59:			
≥100	6/9	1/2	n.s.
≥200	5/9	0/2	n.s.
≥300	1/9	0/2	n.s.
60–79:			
≥100	41/42	3/11	<.0001
≥200	36/42	2/11	.0001
≥300	26/42	2/11	.019
80–99:			
≥100	1/1	3/3	n.s.
≥200	1/1	1/3	n.s.
≥300	1/1	1/3	n.s.
Total:			
≥100	48/52	7/16	.0002
≥200	42/52	3/16	<.0001
≥300	28/52	3/16	.026

[a] *P* values for male vs. female transmission were calculated by Fisher's exact test (two tailed). n.s. = not significant.

However, results were not significant when paternal and maternal inheritance were considered separately (father-child, $r = .129$, $P = .36$; mother-child, $r = .626$, $P = .094$).

Apart from this weak effect of parental allele size, the sex of the parent was more important in determining the size of the mutated allele in the offspring. In the complete sample, a large mutation in the offspring was found in 48 (92%) of 52 paternally transmitted mutations but in only 7 (44%; $P = .0002$ by Fisher's exact test; table 2) of 16 maternally transmitted mutations. To rule out the possibility that genetic factors other than the sex of the transmitting parent were responsible for similar allele sizes in siblings, we repeated these calculations, this time restricting our analysis to the transmitting parents and the oldest offspring carrying the mutation. In this analysis, 23/28 offspring of males and 2/10 offspring of females had inherited a mutation of at least 100 CTG trinucleotides ($P = .0017$ by Fisher's exact test). In light of the effect of parental allele size on the size of the mutation in their offspring, we next examined whether the apparent effect of parental sex was caused by a difference in allele size between fathers and mothers in our study. However, the number of CTG trinucleotides was not different between male and female transmitters (66.9 ± 10.2 vs. 66.9 ± 8.8 [not significant], respectively). Moreover, when we restricted our analysis to parental alleles of 60–79 CTG trinucleotides, there was again a significant difference between paternal and maternal transmission (41/42 vs. 3/11 offspring with a large mutation; $P < .0001$ by Fisher's exact test; table 2). A significant difference between paternally and maternally inherited mutations was also found when we used a threshold of 200 or 300 CTG trinucleotide repeats to define large expansions (table 2). When the absolute size of the repeat sequence was compared, paternally inherited DM mutations were found to be larger than maternally inherited DM mutations (310 ± 29.9 vs. 105 ± 40.7 CTG trinucleotide repeats, respectively; $P = .0008$ by Student's *t*-test, two sided). While the sex of the parent appeared to influence the CTG expansion, no difference in CTG repeat size was noted when male and female sex of the offspring were compared ($\chi^2 = 0.14$; not significant).

Discussion

We have performed genealogical studies, as well as DNA studies, in an attempt to elucidate some of the factors that are associated with the transition of small CTG expansions at the DM locus (with limited phenotypic effect other than ocular cataract) to larger CTG expansions that cause more severe disease.

First, we confirmed previous reports (Harley et al. 1993; Lavedan et al. 1993; Redman et al. 1993) that the size of the parental allele correlates with the size of the DM mutation in the offspring. Second, our study shows that the onset of clinical expression in DM families is preferentially associated with paternal transmission of the DM mutation. In the genealogical studies, an excess of males was found among subjects who linked independently ascertained DM kindreds. Male transmission was especially increased in the last generation preceding onset of clinical symptoms (177 of 267 transmitters male; $P < .005$ for last asymptomatic generation vs. all previous generations combined; table 1). This apparent effect of the sex of the transmitting parent was also observed in the DNA studies. A marked difference in CTG trinucleotide repeat size in offspring was found when we compared the proportion of mutations exceeding an arbitrary threshold of 100 CTG trinucleotide repeats in offspring of male and female mutation carriers (table 2). The differential influence of

paternal and maternal transmission persisted when we restricted our analysis to the first-born affected offspring or to offspring of carriers of mutations between 60 and 79 CTG trinucleotide repeats. The same conclusions were reached when the threshold separating small and large mutations was set at 200 or 300 CTG trinucleotide repeats. We conclude that, although an increase in CTG trinucleotide repeat size is the rule for both sexes, the degree of this expansion is clearly greater for paternally transmitted mutations of this size range.

Our data do not allow us to estimate, in absolute figures, the risk that a small mutation will evolve into a large mutation. However, both the results of the genealogical studies and those of the DNA studies suggest that expansion from a DM mutation of less than 100 CTG trinucleotide repeats, causing only cataract and no muscular disease (Brunner et al. 1992; Reardon et al. 1992; H. T. Brüggenwirth, unpublished data), to a mutation of at least 100 CTG trinucleotide repeats, usually causing muscular symptoms, is approximately two times as frequent with paternal as with maternal transmission.

Although we have taken great care to exclude measurement errors, the somatic heterogeneity of the DM mutation precludes complete accuracy of allele-size measurements. Nevertheless, a significant difference for maternally and paternally derived mutations was evident in each of our analyses. For this reason, the difference in CTG repeat size in paternally versus maternally transmitted minimal DM mutations most likely constitutes a true biological phenomenon.

Our finding that the increase in CTG repeat size is larger with paternal than with maternal transmission of minimal DM mutations explains previous observations of an excess of males in the earliest generations of a clinically ascertained DM pedigree (Bell 1947; Klein 1958; Harper 1989; Brunner et al. 1991; Harley et al. 1993). In our total DM family sample of more than 200 families, when both parents of a DM patient are clinically normal, the odds are approximately 2:1 that the father is the transmitting parent (authors' unpublished data).

If alleles of less than 100 CTG trinucleotides enlarge to a greater extent on paternal than on maternal transmission, then only the last asymptomatic generation should show an excess of male transmissions, and the previous generations should show a small excess of female transmissions. As shown in table 1, a small (but not significant) excess of male transmissions was found instead in the previous generations. This discrepancy may reflect biases that are inherent to these genealogical studies. Most important, if indeed paternal transmission of small alleles is associated with greater instability, then the number of transmissions will be larger if many transmissions are through females. Such long genealogical chains would obviously have a smaller chance of being detected in these studies. Also, our own study and those of Höweler (1986) and Mathieu et al. (1990) included all ancestors of a patient. In contrast, Fleischer (1918) and Klein (1958) most likely extended their genealogical studies preferentially along the male line once the same surname had been detected in two families. This may account for the relatively large excess of male transmission in previous generations, found by Klein (1958). However, we emphasize that this male bias should be much stronger for the previous generations than for the last asymptomatic generation. Consequently, this bias cannot explain (and indeed is at variance with) our finding of a significant excess of male transmission in the last asymptomatic generation versus previous generations.

Two studies have concluded that female transmission results in greater enlargements, even for small alleles (Lavedan et al. 1993; Redman et al. 1993). However, in these studies the data were not analyzed separately for parental alleles of less than 100 CTG trinucleotides (0.3 kb). Therefore, it cannot be stated that the greater instability on paternal transmission noted by us for these small alleles was or was not present in their data. Moreover, close inspection of the data presented in these papers suggests that the proportion of parental alleles of less than 0.3 kb (100 CTG trinucleotides) that enlarged by less than 0.5 kb in the offspring may in fact be greater for maternal transmission in both studies. Another study that reported that increases in size of the CTG trinucleotide repeat are not different between male and female carriers of a DM mutation summarized data on the transmission of mutations of all sizes (Tsilfidis et al. 1992).

Harley et al. (1993) have recently suggested that the excess of minimally affected males might be due to a greater tendency to initial instability with male transmission. This suggestion is supported by our findings. However, data supporting that hypothesis were not presented, and the authors concluded that increase in repeat size is similar for both paternal and maternal transmissions when the increase is expressed as a proportion of the parental repeat size (Harley et al. 1993). The data in that paper are presented in a way that does not allow direct comparison between maternal and paternal al-

leles of 60–79 CTG trinucleotides, i.e., the group that showed the largest difference in our study.

We would like to suggest that the conflict between the conclusions in our study and those of others may be more apparent than real and may reflect the fact that the other studies did not specifically compare maternal and paternal inheritance of alleles less than 100 CTG trinucleotides that were matched for size. We postulate that the situation could be different for small or large mutations, because the transmission of larger paternal mutations may be under negative selection during spermatogenesis. It is obvious that there is selection against transmission of very large mutations by males, since males with onset of muscular weakness in early adulthood are usually infertile or celibate or both (Klein 1958; Höweler et al. 1989). In addition, we believe that negative selection could also operate at the level of individual sperm cells, thereby effectively limiting the range of mutated alleles to those with relatively smaller expansions. Indirect support for this hypothesis comes from studies documenting a decrease in CTG trinucleotide number from parent to child. In the large majority of cases of decreasing CTG repeat length, the transmitting parent has been the father (Ashizawa et al. 1992*a*; Shelbourne et al. 1992; Abeliovich et al. 1993; Brunner et al. 1993; Harley et al. 1993; Hunter et al. 1993; Lavedan et al. 1993; O'Hoy et al. 1993; Redman et al. 1993). It is tempting to speculate that a combination of male infertility and selection against individual sperm bearing large expansions at the DM locus could be involved in the exclusively maternal origin of the severe congenital form of DM. In this regard, it is of interest that, in the segregation distorter system in *Drosophila*, allele-specific male gamete dysfunction is dependent on an expanded repeat sequence (Lyttle 1993).

Most of the dynamics of the CTG trinucleotide repeat expansion in DM are presently unknown. It is possible that the increased instability associated with male transmission noted in our study simply reflects the larger number of cell divisions in spermatogenesis than in oogenesis. On the other hand, it is equally possible that expansion of the DM mutation is predominantly a postzygotic event. This would then suggest the possibility that the differential influence of maternal and paternal transmission on trinucleotide repeat length is influenced by genetic imprinting (Reik 1989). In both humans and mouse, the DM-kinase gene is expressed from both chromosomes (Jansen et al. 1993). This finding does not, however, exclude another type of imprinting characterized by differential mutability.

Finally it should be noted that in fragile X syndrome the risk of transition from a small parental mutation (i.e., a premutation) to a large mutation in offspring also depends on the size of the parental trinucleotide repeat but that a large increase in allele size is virtually restricted to maternally inherited mutation (Fu et al. 1991; Heitz et al. 1992; Yu et al. 1992), again suggesting negative selection during spermatogenesis, genetic imprinting, or both. Studies of trinucleotide repeat length in sperm from DM patients and fragile X patients should be of great value for further elucidation of the dynamics of these unstable mutations.

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