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Patients with primary cataract as a genetic pool of DMPK protomutation

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Abstract Myotonic dystrophy 1 (DM1) is known to diminish reproductive fitness in its severe form. Since no de novo mutations are known for this disease, it has the tendency to becomeextinct from a population. To explain the preservation of DM1 in a population, a hypothesis that a pool of subjects for the mutated gene exists in the apparently healthy (non-DM1) population was tested. In order to determine the (CTG) repeat number, PCR was performed in 274 patients found to have primary cataract of adult onset who showed no DM1 symptoms, and were not related to DM1 patients. In four cataract patients (1.46%; 95% CI 0.5–3.7), a protomutation in the myotonin protein kinase gene was found which might lead to a complete mutation after transmission through the next generations. The number of (CTG) repeats in the remaining 270 cataract patients did not differ significantly from the control subjects in terms of the distribution of larger $[(CTG)n \geq 19]$ versus smaller $[(CTG)n < 19]$ alleles. We consider the primary cataract patients to be the pool of DMPK protomutation from which DM1 mutation is maintained in the population.

Keywords Myotonic dystrophy type 1 ·
Mutation preservation · Cataract patients ·
Premutation · Protomutation

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Introduction

Myotonic dystrophy 1 (DM1) is the most frequent inherited muscular dystrophy in adults, with a global prevalence of 5/100,000. It is associated with a variety of symptoms, including cataract (Emery 1991; Harper 2001). The molecular mechanism underlying the DM1 phenotype is the expansion of $(CTG)n$ repeat in the myotonin protein kinase gene (DMPK) (Brook et al. 1992). The repeat number is highly polymorphic in the general population, and mitotically and meiotically unstable in DM1 patients (Monckton et al. 1995). In the healthy population, the number of (CTG) repeats is 5–37 (Brunner et al. 1992), while DM1 patients have between 50 and several thousands of copies, the increase being roughly in correlation with the severity of the disease (Harley et al. 1992, 1993). In minimal DM1, which manifests as only minor muscular symptoms or often cataract alone, the number of (CTG) repeats is between 50 and 150 (Brook et al. 1992). The class of alleles with 50–80 repeats is designated a protomutation, because they are associated with the disease but only in its very mild form, and their transmission is mostly stable (Barcelo et al. 1993).

DM1 is an autosomal dominant genetic disorder. The apparent contradiction between the high prevalence of DM1, the occurrence of anticipation, reduced reproductive fitness, the tendency toward gene extinction from the population by neonatal deaths in congenital cases, and the low rate of de novo mutations has been explained by a pool of unstable large-sized normal (CTG) alleles that undergo expansion to alleles responsible for the DM1 phenotype (Imbert et al. 1993; Neville et al. 1994). A multistep process where the (CTG) number changes from being in the normal range to being in the premutation (n = 38–49) (Yamagata et al. 1994; Martorell et al. 2001) and protomutation (n = 50–80) ranges and then to full-size mutation has been proved in studies reporting the expansion of premutation in protomutation and mutation, and the expansion of protomutation in mutation (Barcelo et al. 1993; Yamagata et al. 1994, 1998; Abbruzzese et al. 2002). Studies have been performed investigating the variation in distribution of (CTG) repeat number in healthy populations (Brook et al. 1992; Davies et al. 1992; Goldman et al. 1995; Martorell et al. 2001); a potential pool of unrecognized carriers of (pre/proto)mutation from which DM1 patients might originate has also been investigated (Cobo et al. 1996; Aves et al. 1997).

In our study we tested the hypothesis about the existence of a clinically recognizable pool of subjects for the mutated gene in the apparently healthy (non-DM1) population—subjects with primary cataract were examined as a potential pool of DM1 pre/protomutation carriers.

Patients and methods

Patients with cataract were ascertained through medical records of outpatients and inpatients at the Department of Ophthalmology, University Medical Centre, Ljubljana, Slovenia, and at the Department of Ophthalmology, General Hospital, Pula, Istria County, Croatia. Our previous epidemiological studies of DM1 in these two populations revealed a DM1 prevalence of 5.5/100,000 in Slovenia (Teran et al. 2005), and a high prevalence of 18.1/100,000 in the Istria region of Croatia (Medica et al. 1997). The patients filled in a questionnaire about DM1 symptoms and were examined by a clinical geneticist. After excluding congenital, posttraumatic and diabetic cataracts, 274 patients diagnosed as having primary cataracts, but who had no other DM1 symptoms and had no DM1 relatives, were included in our study: 139 females and 135 males; age range: 21–95 years; mean age at onset: 61 years; 200 of

whom were Slovene and 74 were Croatians from Istria. A special effort was made to exclude any potential cataract patients with DM1 families. The investigated group was not restricted to an older population with senile cataracts. The control group consisted of 210 subjects without any symptoms of DM1, unrelated to anyone with DM1 and anyone from the group of cataract patients; it included 77 females and 133 males; mean age: 58 years.

All subjects were molecularly analyzed for the exact number of (CTG) repeats in the DMPK gene. The PCR analysis was performed as described (Kunej et al. 2004). Amplification was carried out in a reaction mixture containing 5 ng of genomic DNA, primers 101 and 102 [Brook et al. (1992)] and Taq DNA Polymerase (AmpliTaq GOLD, Perkin Elmer Roche, Foster City, CA, USA). Fragments were amplified using the ''touchdown'' PCR method consisting of four initial cycles at 95 °C (1 min), 68 °C (1 min) dropping 2 °C per cycle, and 72 °C (1.5 min), followed by 35 cycles at 94 °C (1 min), 62 °C (30 s), and 72 °C (1.5 min), with the final extension performed at 72 °C (5 min). Reaction products were electrophoresed on Spreadex EL400 gels (Elchrom Scientific AG, Cham, Switzerland) for 115 min at 55 °C (120 V) and visualized by SYBR Gold nucleic acid staining (Molecular Probes, Leiden, The Netherlands). The products were accurately sized by comparing them to the molecular weight marker M3 (Elchrom Scientific AG, Cham, Switzerland) and to a series of fragments with known numbers of (CTG) repeats (5,11,13,17,...), obtained by sequencing. Alleles containing more than 38 (CTG) repeats were amplified with Platinum Taq Polymerase (Invitrogen, Paisley, UK) under the same conditions as described above. Allele sizes were determined by electrophoresis on Spreadex EL600 gels.

Southern blot-based assay was performed to verify the homozygosity of the samples, which were found to have only one allele (Medica et al. 1997).

The number of (CTG) repeats was correlated between the cataract patients and the control group using the statistical package SPSS (version 12, SPSS, Chicago, IL, USA) using a χ^2 procedure. Correlation coefficients were considered statistically significant at $P < 0.05$.

Results

Two hundred and seventy-four independently ascertained cataract patients were molecularly examined for the presence of an expansion in the DMPK gene and the number of (CTG) repeats; small-scale expansions

were found by the PCR analysis in four of them (1.46%, 95% CI 0.58–3.7) (Table 1).

After the expansions had been detected, molecular examination was performed in all available close relatives of these four patients: for patient 1, an identical small-scale expansion was found in a daughter, while normal results were found in a son; for patient 2, the results from the analyses of two sons and a daughter showed normal results; for patient 3, the analysis of a son showed normal results; for patient 4 there were no available relatives. There was no possibility of examining the parents of these four cataract patients.

In the remaining 270 cataract patients, the number of (CTG) repeats ranged from 5 to 28. The 210 healthy controls were all negative for (CTG)n expansion in the DMPK gene, and the number of (CTG) repeats ranged from 5 to 32 (Fig. 1).

The number of larger alleles containing 19 or more (CTG) repeats detected was 38 in cataract patients versus 25 in healthy control subjects; the number of smaller alleles containing less then 19 alleles detected was 502 in cataract patients versus 395 in controls (Table 2).

There was no significant difference in the number of (CTG) repeat alleles within the normal range between the cataract patients and control subjects: χ^2 ($df = 1$) for (CTG)$n \geq 19$ versus (CTG)$n < 19 = 0.45$; ($P = 0.502$).

Discussion

Our study was performed in order to identify a clinically recognizable group which could serve as a potential pool of DMPK premutation/protomutation. This pool would be the source of the expansion mutation in the DMPK gene, and therefore responsible for the preservation of DM1 in a population. Molecular analysis of the (CTG)n expansion in the DMPK gene in subjects that were diagnosed with cataracts but did not show any sign of having DM1 and were not related to DM1 patients revealed an expansion of the size of the protomutation in four of the 274 patients. An analysis of the origin of the patients showed that three

were from Istria (a total of 74 subjects from this area were included in the study), a Croatian region with a high prevalence of DM1, and one was from Slovenia (200 Slovenes were included in the study). Stable transmission to offspring was also observed in one of the patients with protomutation.

The underlying molecular mechanism for DM1 itself was surprising: a single mutation was found to be responsible for the disease in patients from different ethnic origins, and a total linkage disequilibrium was observed between the DMPK mutation and nearby polymorphisms (Harley et al. 1991; Yamagata et al. 1992; Mahadevan et al. 1993). This implies that there is a single ancestral mutation, which is unexpected because generally diseases with the tendency to become extinct are characterized by a high level of new mutations, which compensates for the loss of abnormal alleles due to decreased reproductive fitness. However, DM1 remains one of the most frequent neuromuscular genetic diseases.

It is now generally accepted that the ancestral mutation(s) arose on a predisposing haplotype(s), with a mid-size (CTG) repeat allele being the most ancient chromosome (Imbert et al. 1993; Mahadevan et al. 1993; Rubinsztein et al. 1994; Neville et al. 1994; Krahe et al. 1995a, 1995b, 1995c; Goldman et al. 1995; Deka et al. 1996; Tishkoff et al. 1998), and that DM1 could arise due to recurrent expansions occurring on a predisposing allelic form of the normal gene (Imbert et al. 1993), with such a predisposition occurring in the (CTG) repeat itself.

A certain threshold number of repeats is required before replication fidelity is compromised and the gene function/product is disrupted. The molecular mechanisms that determine the instability of triplet repeats during meiosis are unclear, implicating not only meiotic recombination but also DNA replication and repair (Pearson 2003; Malter et al. 1997; De Temmerman et al. 2004), CpG islands methylation, or its absence in the flanking region of the DMPK repeats and other epigenetic germline-specific modifications (Lemmers et al. 2004), the involvement of nucleosomes (Wang et al. 1994), and the defect in the DNA mismatch repair trans-acting mechanism (Kramer et al. 1996).

Table 1 Patients with cataracts who showed (CTG)n expansions

Patient	Origin	Sex/age	Age at onset	Muscle weakness, atrophy, myotonia	Cataract in family	(CTG)n
1/MF	Cro	F/64	47	No	No	$n = 74$
2/KM	Cro	F/60	52	No	Yes	$n = 52$
3/PI	Cro	M/82	79	No	Yes	$n = 80$
3/SS	Slo	F/51	49	No	No	$n = 81$

Fig. 1 (CTG) repeat numbers within the normal range in cataract patients and in control subjects
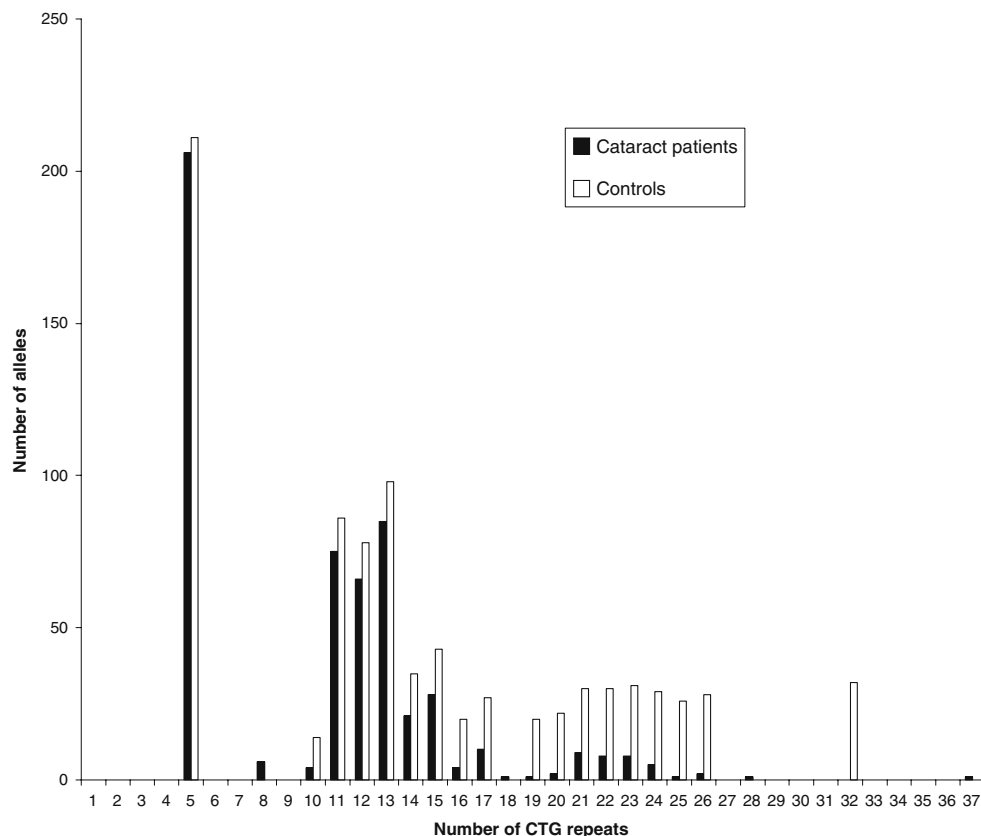

Table 2 Comparison of thenumber of larger alleles to the number of smaller (CTG) alleles in cataract patients and control subjects

Allele size	Number of alleles			
	Cataract patients (Croatia) $n = 74$	Cataract patients (Slovenia) $n = 196$	Cataract patients Σ alleles	Healthy controls $n = 210$
(CTG)$n < 19$	135	367	502	395
(CTG)$n \geq 19$	13	25	38	25
Total alleles	148	392	540	420

Patients with protomutation are excluded

Also, especially in DM1, a role for the perturbation of replication in instability is strongly supported (Yang et al. 2003).

While a few models have been proposed, the only differences between them are the haplotype background and the choice of the most ancient (CTG) repeat number (Imbert et al. 1993; Rubinsztein et al. 1994; Deka et al. 1996; Tishkoff et al. 1998) in which the full-blown DM1 mutation arose through a series of mutational events in preceding generations: the first event was a limited number of duplication steps that resulted in the generation of (CTG)$n \geq 19$, with the lack of a predominant allele in the (CTG)$n = 19$–30

suggesting a very limited number of initial duplication events, followed by significant instability of repeats of (CTG)$n \geq 19$. Larger normal-sized alleles with more than 19 (CTG) repeats have significant instability, and they are the predisposing alleles for DM1 mutations (Imbert et al. 1993). This model suggests that the length of the (CTG) repeat in the DMPK gene is the driving force of DM1 mutations. The eventual result would be a chromosome where the repeat number exceeds 50 and becomes highly unstable. This model provides an explanation for the origin and preservation of DM1 throughout evolution.

The passage from the normal range (5–37) of (CTG) repeat number to the full-blown mutation has not been yet reported. There are reports of the advent of a completely expanded mutation from an allele of 37–50 (CTG) repeats (premutation), including an intermediate generation with alleles of 50–80 repeats (protomutation) (Yamagata et al. 1994, 1998). The class of 50–80 repeats is designated as protomutation because it is the mutation itself at an early stage of intergenerational evolution and it should be associated with specific, mild symptoms or no symptoms at all. The upper limit to protomutation size is 80 repeats; this is the upper threshold for stable transmission (Barcelo et al. 1993).

The gap between 37 and 50/80 (CTG) repeats, whether it is named a pre-, a proto- or a full mutation, could correlate to a concrete clinical phenotype. To supplement the theory of a molecular phenomenon that replenishes the pool of DM1 mutations, we searched for a clinical group in a healthy population that could provide a pool of premutation/protomutation alleles. Hypothetical and practical implications of the theory could not have been investigated if a clinically and molecularly available group had not been identified. Patients with primary cataracts were chosen because cataract may be the only symptom of minimal DM1 associated with 50–150 (CTG) repeats. In this group, we found a protomutation in the DMPK gene in 4/274 (1.46%; 95% CI 0.58–3.74) patients [(CTG)n = 74, 52, 80 and 81, respectively]; in one instance we observed the stable transmission of the protomutation from a parent with cataract to an asymptomatic child. Depending on the origins of our cataract patients, the frequencies of protomutation differ, and the theoretical and practical impact of this on the hypothesis of a protomutation pool is important: in a region with a high DM1 prevalence such as Croatian Istria, the protomutation was detected in three of the 74 cataract patients (4.05%; 95% CI 1.4–11.2), while in Slovene cataract patients the protomutation was detected in one of 200 (0.5%; 95% CI 0.1–2.78) patients analyzed.

Similar investigations on a pool of subjects in a healthy, non-DM1 population with repeat numbers in the range of premutation or protomutation have already been performed in two groups of cataract patients—although some methodological bias exists in both, and contradictory results were obtained: the work of Cobo et al. (1996) could not exclude a possible relation between the investigated cataract patients and DM1 families; the work of Aves et al. (1997) was restricted to patients with senile cataracts only. In our study, special efforts were made to exclude a possible relationship between primary cataract patients and DM1 families. Also, the selection criteria used for the the patients did not include any restrictions to senile or mature cataracts.

However, the finding that cataract carriers transmit the DMPK protomutation, and maintain the DM1 mutation in a population, does not exclude the hypothesis that specific multiple polymorphisms nearby predispose to the generation of DM1 mutations (Boucher al 1995; Krahe et al. 1995a, 1995b, 1995c; Harris et al. 1996), or that other mechanisms such as meiotic drive might be involved in the maintenance of the mutation.

We consider the primary cataract patients to be the pool for DMPK pre/protomutation. The high frequency of protomutation detected in the Croatian region of Istria begs the question of whether all patients with primary cataracts should be tested for (CTG)n status in the DMPK gene.

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