CTG amplification in the DM1PK gene is not associated with idiopathic male subfertility

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BACKGROUND: Polymorphism in the CTG triplet number in the myotonic dystrophy type 1 (DM1PK) gene has been proposed as being associated with idiopathic azoospermia. The aim of this study was to investigate whether the CTG trinucleotide amplification in the DM1PK gene is associated with male subfertility. METHODS: We evaluated 107 subfertile patients, male partners of infertile couples, affected by non-obstructive azoospermia (n = 38) and oligoasthenoteratozoospermia (OAT) (n = 69), and 102 men with proven fertility. Main outcome measures were CTG repeat size in the DM1PK gene, testicular volume, sperm concentration, rapid progressive motility, normal morphology, serum FSH levels, testicular histology and Johnsen score. RESULTS: In subfertile males, no minimal mutation or mutation carriers were found. The difference in the number of CTG repeat lengths between the groups was not statistically significant (P = 0.825). There was no correlation between the number of CTG repeats and the clinical parameters of subfertile patients: testicular volume, sperm concentration, rapid progressive motility, normal morphology, FSH level, testicular histology and Johnsen score. CONCLUSIONS: The number of CTG repeats in the normal or mutational range of DM1PK gene is associated with neither idiopathic male subfertility nor with clinical characteristics of male subfertility.

Key words: azoospermia/CTG repeats/DM1PK gene/male subfertility/myotonic dystrophy type 1 (DM1)

Introduction
Male subfertility affects one man out of ten, yet in ~30% of cases the origin of reduced male fertility is unknown. It is a heterogeneous disorder, with several genetic and environmental factors contributing to impaired spermatogenesis (Nieschlag et al., 2000). The former factors include several monogenic inherited disorders, in which male subfertility is part of the phenotypic spectrum. Moreover, it can be speculated that the genetic variation in genes involved in normal development and function of the male reproductive system could contribute to male subfertility. Indeed, several studies have reported that males with an increased number of CAG repeats within the normal range of amplification in the androgen receptor gene are at an increased risk of impaired spermatogenesis (Dowsing et al., 1999; Yoshida et al., 1999; Mifsud et al., 2001).

Myotonic dystrophy type 1 (DM1) is also a trinucleotide repeat disease caused by the expansion of an unstable (CTG)ₙ trinucleotide repeat in the 3' untranslated region of a protein kinase gene DM1PK on chromosome 19q13.3. In the normal population, the number of CTG repeats is polymorphic, ranging from 5 to 37 copies (Brook et al., 1992). A minimal amplification of the repeat, ranging from 42 to 180 copies, designates mild DM1, characterized by no or only mild manifestation of the disease. In classical DM1 the copy number of CTG repeats is usually >80, which causes a more severe phenotype including muscle weakness and wasting, myotonia, cataract, and often cardiac conduction abnormalities (Brewster et al., 1998). Instability of CTG repeats, especially in female meiosis, gives rise to the phenomenon of anticipation, i.e. progressively earlier appearance of the disease in successive generations that is generally accompanied by increasing severity.

Males affected by classical DM1 may have reduced fertility as well as testicular tubular atrophy (Harper, 1989). Male patients with severe DM1 and reduced fertility have been found to have higher levels of serum FSH and LH as compared to infertile patients (Mastrogiacomo et al., 1994, 1996). DM1 patients have also been reported to have a decreased sperm function (Hortas et al., 2000). Recently, it has been proposed that larger CTG repeat alleles in the DM1PK gene might be associated with idiopathic azoospermia (Pan et al., 2002).

In order to test the hypothesis that the CTG repeat amplification in the DM1PK gene, either within the mutational or within the normal range, is associated with male subfertility, we examined the distribution of CTG repeat alleles in a Slovene population of 107 subfertile patients, male partners of infertile couples affected by azoospermia or oligoasthenoteratozoospermia (OAT), and of 102 fertile men. Additionally,
we sought a potential correlation between the CTG repeat amplification and clinical parameters of male infertility (testicular volume, sperm concentration, rapid progressive motility, normal morphology, FSH level, testicular histology and Johnsen score).

Materials and methods

Study and control group

In the study group, we enrolled consecutively 107 male partners of infertile couples, aged 27–53 years, attending the Andrology Centre, Department of Obstetrics and Gynecology in Ljubljana. All these men were candidates for infertility treatment by ICSI due to abnormal spermiogram consisting of non-obstructive azoospermia, oligozoospermia and/or asthenoteratozoospermia. Patients with congenital bilateral absence of vas deferens or other causes of obstructive azoospermia, cytogenetic abnormalities and microdeletions of the Y chromosome were excluded from the study.

In the control group we enrolled 102 men, aged 28–64 years, with proven fertility (at least one offspring).

Clinical assessment

Each man in the study group underwent an andrological examination. Semen was assessed according to World Health Organization (1992) guidelines with regard to volume, sperm count, rapid progressive motility and normal morphology.

Of the 107 men, 38 had non-obstructive azoospermia and 69 oligozoospermia (sperm concentration < 20 × 10^6 sperm/ml of ejaculate) and/or asthenoteratozoospermia (sperm rapid progressive motility < 25% and normal morphology < 30%). Serum FSH was measured using the Microparticle Enzyme Immunoassay (AxSYM System; Abbott, USA); FSH levels < 8 mIU/ml were considered normal. In azoospermics, testicular biopsy under local anaesthesia was performed prior to ICSI.

Following unilateral scrototomy, a small testicular incision was made and at least two samples of testicular tissue were taken from each testis. One sample was screened for the presence of sperm to be used in ICSI, and one was fixed in Bouin’s solution, routinely embedded in paraffin and cut at a section thickness of 5 µm. The sections were stained with haematoxylin–eosin. A systematic histological evaluation was performed under light microscopy by the same observer. More than 100 seminiferous tubules were scored for each patient. The results were expressed as a relative number of tubules showing Sertoli cells, spermatogonia, spermatocytes, round and elongated spermatids, and sperm. The diagnoses were as follows: normal spermatogenesis, hypospermatogenesis, early and late maturation arrest and Sertoli cell-only syndrome. Testicular histology showed Sertoli cell-only syndrome in 21, maturation arrest in seven and hypospermatogenesis in 10 patients. Additionally, testicular histology was evaluated according to the Johnsen modified classification (Holstein et al., 1994) from score 1 (no germinal cell in sclerotic seminiferous tubules) to score 10 (sperm present).

In the control group, blood samples of 102 men with proven fertility were analysed.

All patients were Slovene or of Slavic origin. Informed consent was obtained from each patient. The study was approved by the national medical ethics’ committee.

DNA analysis of CTG repeat

Genomic DNA was isolated using a Qiagen genomic DNA isolation kit (USA) according to the manufacturer’s protocol. The PCR reaction was carried out in a total volume of 15 µl containing 5 ng of each DNA sample, 50 mmol/l KCl, 15 mmol/l Tris–HCl (pH 8.0), 2% formamide, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP, 0.18 µmol/l primer 101 and 102 (Brook et al., 1992) and 1 IU Taq DNA Polymerase (Amplitaq; Perkin Elmer, USA). One microlitre of PCR reaction products was electrophoresed on Spreadex EL400 gels (Elchrom Scientific AG, Switzerland) for 115 min at 55°C (120 V) and visualized with SYBR Gold nucleic acid gel staining (Molecular Probes, The Netherlands) (Figure 1).

The trinucleotide repeat length was determined by comparison to molecular weight marker M₃ (Elchrom Scientific AG) and to the fragments with a known trinucleotide repeat number obtained by sequencing, using Genetic Analyzer ABI PRISM 310 (Perkin Elmer).

In most patients, PCR revealed two bands indicating heterozygosity, whereas in patients with a single band, southern blot analysis was additionally performed, as previously published (Brook et al., 1992; Medica et al., 1997).

Statistical analysis

Statistical analysis was performed using the statistical package SPSS (version 11, SPSS, Inc., USA). The median, mean and SEM CTG repeat length were calculated. The difference in the number of CTG repeats between the subfertile and control groups were tested by the Mann–Whitney U-test. Trinucleotide CTG repeat length in subfertile patients was correlated to clinical characteristics (testicular volume, sperm concentration, rapid progressive motility, normal morphology, FSH level, testicular histology and Johnsen score) by using Spearman’s correlation coefficient. P < 0.05 was considered statistically significant.

Results

In subfertile patients, no minimal mutation or mutation carriers were found. The CTG number of repeats ranged from 5 to 27 (mean = 10.26 ± 0.37; median = 11) in the group of subfertile patients, and from 5 to 32 (mean = 10.72 ± 0.39; median = 12) in the control group. The distribution of the number of CTG repeat lengths compared between subfertile.
and fertile patients was not statistically significant ($P = 0.825$) (Figure 2).

The comparison of the variable number of CTG repeats with the clinical characteristics of subfertile patients showed no correlation: left testicular volume ($P = 0.799; n = 107$), right testicular volume ($P = 0.817; n = 107$), sperm concentration ($P = 0.394; n = 107$), rapid progressive motility ($P = 0.612; n = 107$), normal morphology ($P = 0.581; n = 107$), testicular histology ($P = 0.157; n = 39$) and Johnsen histological score ($P = 0.728; n = 35$).

**Discussion**

In this study we did not find any difference in the distribution of CTG repeat number in the DM1PK gene between a group of subfertile patients, male partners of infertile couples, and fertile men. Additionally, no correlation between the number of CTG repeats and the clinical characteristics of subfertile men including testicular volume, FSH level, and sperm and histological characteristics was shown.

We speculated that minimal mutations or even full mutations could be responsible for subfertility in some men experiencing subfertility problems. Namely, minimal mutations have been found in the group of patients with cataract, the leading symptom of mild DM1, who did not otherwise have neurological signs and symptoms of DM1 (Kidd et al., 1995; Cobo et al., 1996; Peterlin et al., 1996). On the other hand, a full mutation in the lower range of CTG amplification could be expected in younger DM1 males experiencing subfertility, yet not perceiving muscular symptoms due to reduced expression of the disease. However, neither this study nor two previous reports (Dean et al., 2002; Pan et al., 2002) have identified subfertile patients with a minimal mutation or full mutation in the DM1PK gene. Nevertheless, it might be expected that in rare cases a mutation in the DM1PK gene could be found in subfertile patients with no overt clinical symptoms of myotonic dystrophy.

On the contrary, Pan et al. (2002) has reported an association between the CTG amplification in the normal range and idiopathic azoospermia in a small group of patients and controls. This observation implies that the CTG repeat number in the normal range of amplification is associated with a change in the DM1PK gene expression, which has not been supported experimentally (Sasagawa et al., 1996). Moreover, the polymorphism of CTG repeats in the normal range has not been associated with specific disease symptoms.

In our case–control study, we included 69 patients with oligozoospermia and/or oligoasthenoteratozoospermia in addition to 38 azoospermic patients. We found no evidence of an association between the amplification of CTG repeats in the DM1PK gene and male infertility. Similarly, Dean et al. (2002) have reported a lack of deviation from the normal length distribution in the sample of 118 male partners of couples undergoing ICSI, yet no patient in their group was azoospermic, and male infertility factor was attributed to 52 males.

Also, we did not find evidence of a correlation between the CTG repeat amplification and clinical parameters of male infertility.

We conclude that the amplification of CTG repeats in the normal or mutational range of the DM1PK gene is associated with neither idiopathic male subfertility nor with the clinical parameters of male subfertility.

**Acknowledgements**

We are grateful to Dr. I. Verdenik for advice on statistical analysis, Ms S. Zitko for technical assistance and Ms M. Pirc for revising the English text. The study was supported by Grant J3-2370 provided by the Ministry of Education, Science and Sport, Ljubljana, Slovenia.

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Submitted on October 21, 2003; accepted on June 2, 2004