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Similar prevalence of expanded CGG repeat lengths in the fragile X mental retardation I gene among infertile women and among women with proven fertility: a prospective study

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Purpose: We sought to determine the usefulness of fragile X mental retardation 1 (*FMR1*) carrier testing among young infertile women with or without signs of ovarian insufficiency as compared with fertile women.

Methods: Three cohorts of women were recruited to determine the cytosine–guanine–guanine (CGG) repeats trinucleotide repeat length in the 5′-untranslated region of the *FMR1* gene in lymphocyte DNA. A total of 199 fertile women, who were reported to have conceived within 3 months, were recruited together with 372 infertile women with ongoing menstrual cycles and 48 infertile women with primary ovarian insufficiency. The various ranges of *FMR1* CGG repeat lengths among infertile women were compared with those of fertile controls. In infertile women with ongoing menstrual cycles, the serum concentrations of follicle-stimulating hormone, anti-Muellerian hormone, and inhibin B were measured during the early follicular phase.

Results: None of the three categories of *FMR1* CGG repeat length expansions (premutation, intermediate range, and high normal range) were more prevalent among infertile women than among fertile women. The CGG repeat length was not correlated with any of the ovarian reserve parameters.

Conclusion: In comparison with a generalized preconception screening strategy, infertility as a criterion, even together with reduced ovarian reserve, is not suitable for identifying a higher proportion of women with expanded *FMR1* CGG repeat length.

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Key Words: fragile X syndrome; infertility; ovarian reserve; population screening; primary ovarian insufficiency

INTRODUCTION

The fragile X syndrome is a debilitating brain disease characterized by mental retardation, seizures, and autistic behavior, most frequently affecting males. It is the leading cause of inherited mental retardation and exerts significant impact on both the affected and their familial and social environment. The fragile X syndrome is transmitted as an X-linked disorder, and the disease status depends on the number of cytosine–guanine–guanine repeats (CGG) in the 5′-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene. The expansion of these repeats determines the methylation status of the gene and thereby its functionality. In the presence of more than 200 CGG repeats, aberrant methylation of the gene results in its silencing. The affected individual will have insufficient fragile X mental retardation protein (FMRP). Lower repeat numbers, varying between 55 and 200, are denominated "premutation", whereas those between 45 and 54 are termed as "intermediate" and those between 35 and 44 as "in the high normal range." The relevance of expanded CGG repeat lengths in otherwise symptom-free carrier women not only resides in transmitting the full mutation to the male offspring, but also in the proportional expansion of intermediate and premutational CGG repeat numbers transmitted to the X chromosome of the female progeny[1] and in fragile X-associated primary ovarian insufficiency (POI).[2]

Considering the severity of the syndrome, the frequency of the carrier status and the potential long-term benefits to the daughters knowing about their mother's screening result, the installment of population-based screening programs has been advocated repeatedly.[3–5] In addition, carrier status testing would also enable women into timely reproductive planning.[6] Even in the absence of a family history of the fragile X syndrome, the cost–effectiveness of prenatal genetic testing programs identifying the carrier status of women has been demonstrated.[7] The psychosocial consequences of preconceptional CGG repeat length screening have been studied in great detail and were found to be in favor of a generalized screening.[8–10] In addition, the attitudes of key medical care stakeholders toward the challenges of general population-based screening programs were found to be favorable,[6,11] and preconceptional testing was most endorsed.[12] Despite these well-studied advantages of a population-based screening and the wide acceptance among key stakeholders of medical care, screening programs have so far only been conducted in research settings.

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Because of the high incidence rates of POI among relatives suffering from fragile X syndrome[13,14] and as POI may be preceded by reduced ovarian reserve,[15] an increasing number of women presenting with infertility with or without POI are now being offered a *FMR1* gene diagnostic test. Several medical organizations dealing with reproduction and obstetrics including the American College of Obstetricians and Gynecologists and the European Society of Human Reproduction and Embryology have decided to recommend *FMR1* carrier testing in infertile women with signs of early onset of ovarian insufficiency[16] or to counsel women diagnosed with POI about their risk of carrying the premutation.[17] However, neither the clinically relevant CGG repeat length nor the target population of infertile women have yet been defined.

FMR1 screening among infertile women with or without POI may be extended toward an overall preconceptional screening. For this purpose, we carried out this study to quantify the prevalence of various *FMR1* CGG repeat lengths among infertile women and a group of women with proven fertility.

MATERIALS AND METHODS

Study design and hypothesis

This study was conceived to determine the incidence rates of the various CGG repeat length groups among three groups of women characterized by different degrees of fertility together with distinct patterns of their menstrual cycle, the latter reflecting various levels of ovarian insufficiency. A part of this cohort has already been used for determining the role of polymorphic gene variants of crucial endocrine mediators of the menstrual cycle in fecundity.[18] In this study, we hypothesized that in the absence of a familial risk for fragile X syndrome, the distribution of *FMR1* CGG repeat lengths would be similar among women with normal fertility as compared with infertile women with still ongoing menstrual cycles or with POI.

Study populations

A total of 620 women were included in this prospective study: (i) 200 women, who were reported to have conceived within 3 months after starting having unprotected intercourse; (ii) 372 women with ongoing menstrual cycles, but unable to achieve pregnancy despite unprotected intercourse over a period of at least 12 months; and (iii) 48 infertile women presenting with secondary amenorrhea and diagnosed with POI.

To obtain blood samples of women with proven fertility, 315 women were approached in the days or weeks after delivery of healthy offspring in two Swiss Hospitals, one located in the eastern part of Switzerland (Spital Uznach) and the other located in the western part of Switzerland (Women's Hospital at the University Hospital of Basel, Basel, Switzerland). Among these, 200 were reported to have become pregnant naturally within the first 3 months (64.0%) and only the samples of those were included into the fertile cohort. One sample provided insufficient DNA for analysis and was excluded leaving us with a control group of 199 fertile women.

In the period between November 2006 and August 2008, a total of 520 infertile women were approached during their initial diagnostic infertility workup in the outpatient department of the Women's Hospital at the University Hospital of Basel to participate in the study. Of these, 386 were recruited (74.2%), but 14 were later excluded because of severe male or complete tubal infertility or loss to follow-up. All couples included underwent an integrated diagnostic workup at the Clinic of Gynecological Endocrinology and Reproductive Medicine of the University of Basel, which included a detailed personal and familial history, early menstrual cycle, and preovulatory vaginal ultrasound examinations. In all patients included in this cohort, serum was taken during early follicular phase for the measurements of the concentrations of follicle-stimulating hormone (FSH), anti-Muellerian hormone (AMH), and inhibin B. The patency of the fallopian tubes was routinely tested with hysterosalpingography or, in the presence of pathology, with laparoscopy. The male partners underwent a physical exam together with an assessment of their endocrine profile and conventional semen analysis. Despite reduced seminal characteristics or impaired ovarian function, all infertile patients included had the potential to conceive naturally.[19,20] Serum FSH levels were quantified with an electrochemoiluminescence immunoassay (Roche Diagnostics, Rotkreuz, Switzerland), with a detection range between 0.1 and 200 IU/l. For FSH, the interassay coefficient of variation varied between 2.0 and 3.5%. AMH concentration was measured with an enzyme immunoassay (Immunotech, Marseille, France), with a detection range between 1 and 150 pmol/l. The interassay coefficient of variation varied between 7.6 and 19.0%. Inhibin B concentration was measured with an enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Webster, TX), with a detection range between 2.6 and 1,000 pg/ml. The interassay coefficient of variation varied between 5.4 and 6.8%.

Finally, a third group of women was recruited by contacting 162 women previously diagnosed with POI (secondary amenorrhea and FSH level >30 IU/l before the age of 40 years) and registered in our database (FertiMed, Reinach, Switzerland) by regular mail and asked to participate in this study. All samples were collected between June 2007 and May 2008. Excluded were women with POI caused by medical interventions, such as chemotherapy and/or radiation, and none of them had a family history of the fragile X syndrome. Forty-eight of the 162 women responded (29.6%) and provided a blood sample. In all POI cases, the cause was unexplained and none of them showed an abnormal karyotype.

The extension of this study to include *FMR1* gene was presented to and accepted by our local ethics committee.

Covariates

Both ovarian function and female fertility are strongly age dependent. For that reason, we avoided comparing the age of the participating women at the moment of blood sampling, but instead, we compared the age at conception in the fertile group with the age of onset of the willingness to conceive in the

infertile group of women with ongoing menstrual cycles and with the age of onset of secondary amenorrhea in the group of women with POI.

The ethnic background of the participants in this study was essentially Caucasian (99.8%).

FMR1-CGG repeat size determination

Initial *FMR1*-CGG repeat size was determined using locus-specific primers (FMR1-Fwd, FAM-labeled: AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA and FMR1-Rev: GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT) together with the GC-Rich PCR system according to the manufacturer's instructions (Roche Applied Sciences, Rotkreuz, Switzerland). Fragment length was determined on an ABI Prism 310 Genetic Analyzer and CGG repeat size calculated according to the following formula: number of CGG repeats = ((peak size – 221)/3) + 4. As determined by external quality control experiments, the assay reliably amplifies up to 130 CGG repeats and displays a precision of approximately ±2 CGG repeats.

For validation purposes, 135 pseudo-mono-allelic samples and samples with more than 50 CGG repeats were subjected to a qualitative assessment of CGG repeat length using a previously described method.[21] The assay consisted of amplification of the CGG repeat region by polymerase chain reaction using three primers and a linker. The polymerase chain reaction master mix consisted in 150 ng genomic DNA, 1 × polymerase chain reaction buffer with 20 mmol/l $MgCl_2$ (Roche Fast-Star Taq kit), 6% DMSO (Sigma-Aldrich, Buchs, Switzerland), 1.7× Qsolution (Qiagen, Hombrechtikon, Switzerland), 0.2 mmol/l of each deaza-dGTP (New England BioLabs, Ipswich, MA), dATP, dCTP, dTTP (Invitrogen), 1 U of FST polymerase (Roche Fast-Star Taq Kit, Rotkreuz, Switzerland), 0.6 µmol/l of each of the four primers:

FMR1F: FAM-TGTAAAACGACGGCCAGTGCTCAGCTC CGTTTCGGTTTCACTTCCGGT

FMR1R: CAGGAAACAGCTATGACCCTCGAGGCCCAG CCGCCGCCGCC

FMR1CCGR: CAGGAAACAGCTATGACCCCGCCGCC GCC

M13 reverse linker primer: CAGGAAACAGCTATGACC

Cycling conditions were as follows: 98 °C for 10 min to activate the polymerase, 10 cycles of denaturation at 97 °C for 35 s, annealing at 64 °C for 2 min, extension at 68 °C for 8 min, followed by 25 cycles of denaturation at 97 °C for 35 s, annealing at 64 °C for 2 min, extension at 68 °C for 8 min and 20 s, with an additional 20 s extension in each additional cycle. Four microliters of nondiluted polymerase chain reaction product together with 0.5 µl size standard (GeneScan 500 Rox Size Standard; Applied Biosystems, Zug, Switzerland) and 9.5 µl formamide (Hi-Di Formamide; Applied Biosystems) were added to a 96-well plate (4titude, FrameStar 96 semi-skirted). The plate was heated for 3 min at 95 °C, cooled down and samples were injected on an ABI 3130xl Genetic analyzer (Applied Biosystems) equipped with a 36-cm capillary loaded with Pop-7 polymer (Applied Biosystems). The voltage used was 12 V for 40 s and the run time was 2,000 s. The software used for analysis was GeneMapper (Applied Biosystems).

Finally, for further validation, 10 samples with suspected expanded CGG repeat length were assessed with the AmplideX FMR1 PCR kit (Asuragen, Austin, TX) capable of identifying high-range premutations and full mutations.

In all the experiments, control samples with known CGG repeat sizes (normal female as negative and female with premutation allele as positive control) were included to assure diagnostic accuracy.

Statistical methods

The allele with the longest CGG repeat length was used for all correlations, because in cases with heterozygous alleles, X chromosome inactivation has been shown previously not to impact on the clinical manifestation of POI.[22,23]

Differences in the incidence rates of expanded CGG repeats between fertile and infertile women were assessed using χ^2 analysis. To account for the potential effects of confounding factors, such as age, and the concentrations of FSH, AMH, and inhibin B, multiple regression analysis was performed with the data collected among the infertile women with still ongoing menstrual cycles and confirmed using Spearman rank analysis.

RESULTS

Study population

The entire study cohort consisted of 620 women. The age of the three groups was significantly different ($P < 0.001$), the fertile women being the youngest (mean age: 29.6 years; 95% confidence interval between 29.1 and 30.6 years). The mean age of the infertile women with ongoing menstrual cycles was 31.2 years, with the 95% confidence interval between 30.6 and 31.8 years. The mean age of the women at the onset of POI was 33.5 years, with the 95% confidence varying between 32.2 and 34.8 years.

Prevalence of *FMR1* CGG repeat lengths among the three groups

None of the participants was diagnosed with a full mutation in the *FMR1* gene (>199 CGG repeats). When compared with the fertile controls, more cases with expanded CGG repeat length, including the intermediate range, were detected among infertile women with ongoing menstrual cycles, but the differences were not statistically significant (**Table 1**).

Differences in ovarian reserve parameters among various CGG repeat length categories

Using multiple regression analysis, the correlation between the allele with the longest CGG repeat length and the four major parameters indicating ovarian reserve, measured in the infertile women with ongoing menstrual cycles, was determined. The four parameters included age (either defined by the date of conception or the onset of infertility) and the concentrations of FSH (IU/l), AMH (pmol/l), and inhibin B (pg/ml), all measured during early follicular phase of an untreated menstrual

Table 1 Overview of the prevalence of the various *FMR1* CGG repeat lengths in the three cohorts

CGG repeat numbers	Fertile controls, *n* (%)	Infertile women, *n* (%)	Infertile with POI, *n* (%)	χ^2	*P*
Total number	199	372	48		
<35, low normal	170 (85.4)	303 (81.5)	44 (91.7)		
35–44, high normal	24 (12.1)	55 (14.8)	3 (6.3)	3.26	0.196
45–54, intermediate	4 (2.0)	9 (2.4)	0	1.22	0.543
55–200, premutation	1 (0.5)	5 (1.3)	1 (2.1)	1.24	0.537
≥35	29 (14.6)	69 (18.6)	4 (8.3)	4	0.136
≥45	5 (2.5)	14 (3.8)	1 (2.1)	0.883	0.643

CGG, cytosine–guanine–guanine repeats; POI, primary ovarian insufficiency.

Table 2 Pearson's multiple regression analysis was applied to the ovarian reserve data among the infertile women with ongoing menstrual cycles

Parameter	Correlation coefficient	95% confidence interval	*P*
Age (years)	0.003	−0.116 to 0.122	0.965
FSH (U/l)	0.011	−0.137 to 0.158	0.886
AMH (pmol/l)	−0.001	−0.37 to 0.036	0.976
Inhibin B (pg/ml)	−0.001	−0.17 to 0.015	0.888

The allele with the longest CGG repeat numbers was taken as the dependent variable.

AMH, anti-Muellerian hormone; CGG, cytosine–guanine–guanine repeats; FSH, follicle-stimulating hormone.

cycle. None of these four parameters correlated significantly with the *FMR1* CGG repeat length (**Table 2**).

DISCUSSION

In an unselected cohort of infertile women with or without POI and in the absence of a family history of the fragile X syndrome, the prevalence of expanded CGG repeats, including those in the high normal range, was not higher than that in women with proven normal fertility. In addition, among infertile women with ongoing menstrual cycles the CGG repeat length of the *FMR1* gene was not correlated with any of the four major determinants of ovarian reserve, including age and the concentrations of FSH, AMH, and inhibin B.

Reported differences in the prevalence of expanded *FMR1* CGG repeat lengths decisively depend on the characteristics of the selected cohorts. In two earlier observational studies, which included large cohorts of unselected women, the prevalence of premutations and full mutations during their reproductive age span was shown to vary between 1.43[24] and 0.5%,[25] whereas among women with close relatives suffering from fragile X syndrome and diagnosed with POI, the prevalence rate of the full mutation was reported to be as high as 16.8% and that of the premutation as high as 52%.[14] By contrast, among women with POI but without cases of the fragile X syndrome among close family members, the prevalence of full mutations and premutations was much lower, with the latter varying from 2.2[26] and 2.5[27] to 4.9%.[4] Among infertile women with reduced ovarian reserve but without POI, the prevalence rates of the premutation tended to be even lower, varying between 1.3[28] and 5.6%.[29] In this study, in which the cohorts were selected based on (in) fertility issues entirely unrelated to the fragile X syndrome, the prevalence rates of expanded *FMR1* CGG repeats among fertile women were comparable with known population-based incidence rates, whereas those of infertile women were comparable with the incidence rates found in another large scale survey among infertile women.[28] The number of women with expanded *FMR1* CGG repeat numbers in this cohort among the women with overt POI was comparable to the incidence rates reported previously.[4,26]

The review of all published controlled studies on the prevalence rates of expanded *FMR1* CGG repeat numbers revealed that in the absence of a familial risk, the relative risk of carrying the premutation among infertile women with reduced ovarian reserve and/or POI was marginally increased[13,27] or similar[4,28,29] in all but one study.[30] The prevalence rates of CGG repeat lengths of the intermediate range were similar among infertile women with reduced ovarian reserve and/or POI in all reviewed studies.[4,27–30] Recently, no association with *FMR1* CGG repeat length with the onset of natural menopause could be demonstrated.[31]

Some of the discordant conclusions given by earlier studies may be explained by the characteristics of the control groups. Three studies compared the incidence of expanded *FMR1* CGG repeat lengths among women with POI with that of infertile controls,[13] of healthy family members of fragile X relatives,[4] and of postmenopausal women with timely onset of menopause.[30] Two other studies[28,29] compared the incidence of expanded CGG repeat lengths in women with reduced ovarian reserve with that of infertile women with normal ovarian reserve. This is the only study to have a control group with women of proven fertility.

In conclusion, we present the first systematic study comparing the prevalence of *FMR1* CGG repeat lengths in women with proven normal fertility and an unbiased cohort of infertile women with or without ongoing menstrual cycles. Although both in infertile women with still ongoing menstrual cycles and in infertile women with POI somewhat higher incidence rates of expanded CGG repeat lengths were found than among the fertile controls, the differences were statistically insignificant. Although an even larger cohort size would likely have

demonstrated such a difference, infertility as the sole criterion or even in combination with reduced ovarian reserve or POI does not seem to be more suitable for identifying silent carriers of *FMR1* CGG repeat expansions than a generalized preconception screening.

If a broad genetic screening program for the *FMR1* carrier status is to be installed on a voluntary basis, treatment options preventing transmitting *FMR1* mutations to the offspring need to be available to those infertile women found to be carrying a full mutation or a premutation. These include costly and burdening procedures such as preimplantation genetic diagnostics, the sorting of X chromosome bearing spermatozoa, egg donation, or even adoption. When taking these consequences into account, the cost–effectiveness of a screening program of the *FMR1* carrier status among women aiming for pregnancy must be established.

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DISCLOSURE

The authors declare no conflict of interest.

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