RESEARCH ARTICLE

Are expanded alleles of the FMR1 gene related to unexplained recurrent miscarriages?

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Abstract

Background: In women with recurrent miscarriages, up to 50 % of those cases remain unexplained. In this study, we evaluated the impact of Cytosine/Guanine/Guanine (CGG) trinucleotide expansions of the fragile-X mental retardation 1 (*FMR1*) gene in women with unexplained recurrent miscarriages.

Methods: This is a prospective case-control pilot study involving 49 women with unexplained recurrent miscarriages and 49 age-matched controls with documented fertility. The case group consisted of women with a history of two or more consecutive miscarriages, in whom no known factor could be identified. The maximum age of recruitment was 40 years. We obtained blood samples that were checked, using polymerase chain reaction with electrophoresis, for the presence of expanded alleles of the *FMR1* gene. We further evaluated using sequencing analysis, those women marked as positive. We set the limit at more than 40 repeats.

Results: The repeat sizes of CGG expansion in the *FMR1* gene differ significantly in the two population groups (p =0.027). We found four women in the miscarriage group and one in the control group positive for carrying premutation alleles (Odds ratio: 4.267, confidence interval: 0.459-39.629). All the positive cases involved intermediate zone carriers. We found no association between the number of abortions each woman had, and her respective CGG repeat number (p =0.255).

Conclusions: Many couples are desperately looking for the cause of their recurrent miscarriage suffering. The CGG expanded allele of the FMR1 gene is possibly to be blamed in some of these cases. More studies are needed to support the results of this prototype study. HIPPOKRATIA 2018, 22(3): 132-136.

Keywords: Unexplained recurrent miscarriages, fragile-X mental retardation 1 gene, *FMR1* expanded alleles, fragile X premutation, abortion

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Introduction

Miscarriage is described as the involuntary loss of pregnancy before the fetus is viable, that is before 24 completed weeks. Recurrent miscarriage is determined as three or more consecutive pregnancy losses, which affects about 1 % of couples[1]. Recently many authors and institutions consider including in the definition above, women with two or more miscarriages[2]. There are many different identifiable causes of recurrent miscarriages, involving genetical, anatomical, infectious, immunological, hematological, and endocrinological factors. Up to 50 % of cases of recurrent miscarriage will remain unexplained, despite having completed the diagnostic work up[3].

In the X chromosome, the fragile-X mental retardation 1 (*FMR1*) gene is located, which encodes a ribonucleic acid (RNA) binding protein, the fragile X mental retardation protein (FMRP), that shuttles specific mRNAs from the nucleus to the cytoplasm for translation[4], and is required for the normal neural development. *FMR1* gene mutations involve an expansion of Cytosine/Guanine/Guanine (CGG) trinucleotide repeat region in the 5' untranslated region[4]. It is extraordinary that different expansions of the FMR1 gene, exhibit distinct disorders; Fragile X syndrome, Fragile-X associated tremor-ataxia syndrome (FXTAS), and premature ovarian failure. It is only the size of the CGG repeat that will ultimately define the clinical phenotype[5].

Healthy people carry up to 54 repeats, while full mutation refers to more than 200 repeats[6]. A premutation is defined as an expansion from 55 to 200 repeats, while the presence of 41-54 repeats is termed as the intermediate or "grey" zone[7,8]. The prevalence of the premutation is about one in 250 women[9], which can reach up to one in 110 women in specific populations[10]. The intermediate or "grey" zone can be found in 1 out of 57 women[11].

It is already known that the premutation status of the *FMR1* gene (55-200 CGG repeats) is associated with premature ovarian failure and elevated serum FSH levels, a

marker of diminishing ovarian reserve[12]. The purpose of this study was to examine whether being a carrier of increased CGG repeats in the *FMR1* gene (intermediate zone, or premutation), entails an increased risk of miscarriage.

Materials and Methods

Patients

We recruited the study population between January 2013 and December 2015 from the recurrent miscarriage outpatient clinic of Papageorgiou University Hospital of Thessaloniki, which is a referral center of Northern Greece. Most of the women suffering from recurrent miscarriages had already completed an extensive set of tests, before approaching the miscarriage clinic. Data were retrieved from their medical records, and additional laboratory examinations were ordered, as needed, including hormonal profiling and thrombophilia tests.

We enrolled in the control group women visiting the outpatient gynecological clinic for a routine diagnostic check-up, and female members of the hospital staff, age-matched within two years, on a 1:1 ratio. Ethical approval was obtained for this study from the relevant Bioethics Committee of Aristotle University of Thessaloniki (protocol 330/4.11.2016) and was conducted in accordance with the Declaration of Helsinki. All patients included in the study signed informed consent. As recurrent miscarriage in this study was considered the presence of at least two, consecutive pregnancy losses.

In order to minimize the risk of bias, a problem inherent in retrospective studies, the inclusion and exclusion criteria were stringent. Cases were excluded if established secondary causes of recurrent miscarriages were identified during the initial visit, or later in the patient work up. Women who were subjected to assisted reproduction were excluded as well. A summary of the eligibility criteria is presented in Table 1. Maximum age at recruitment was set at 40 years, to avoid, as much as possible, ovarian aging contributing to the number of miscarriages.

PCR analysis

We isolated the genomic deoxyribonucleic acid (DNA) from the peripheral blood leukocytes using an affinity purification method (Macherey-Nagel GmbH, Germany). We tested the isolated DNAs for the presence of an expansion in the CGG trinucleotide repeat region using a two-step PCR protocol[13]. This protocol is a rapid, well established, and cost-effective screening method for identification of any expanded allele of the Fragile X gene. In the first step, we amplified genomic DNA using polymerase chain reaction (PCR), with the c and f primers (5'-GCTCAGCTC-CGTTTCGGTTTCACTTCCGGT-3' and 5'-AGCCCC-GCACTTCCACCACCAGCTCCTCCA-3' respectively), which is a primer pair that flanks the CGG repeat region, utilizing betaine as the osmolite[14] and using the Expand Long Template PCR System (Roche Diagnostics Hellas A.E., Marousi, Athens, Greece). The reaction mixtures used, were 500 μmol/L dNTPs, 0.20 μM of each primer, 2.2M betaine and 50 ng of genomic DNA.

We electrophoresed the final PCR products in 2.5 % agarose gel in the presence of ethidium bromide for one hour at 40 Volts. With this method, the expected PCR product was 221 base pairs (bp), excluding the CGG re-

Table 1: Inclusion and exclusion criteria for the 49 women with unexplained recurrent miscarriages and 49 age-matched controls enrolled in the case-control study.

Inclusion criteria
Cases
• Two or more consecutive pregnancy losses up to completed 20 weeks of pregnancy
• Age at recruitment below 40 years
• Controls
• History of documented normal pregnancies
Exclusion criteria
Cases
• Currently in pregnancy or puerperium (6 weeks postpartum)
• History of miscarriage due to infection (TORCH, syphilis, HBV, HCV, HIV)
• Diagnosis of thrombophilic disorder (hereditary or acquired):
○ Hyperhomocysteinemia
○ Deficiency of protein C, S, antithrombin III
○ Mutated factors V Leiden, prothrombin G20210A, Homologous state of MTHFR C677T
○ Anti-phospholipid syndrome or lupus syndrome
○ Activated Protein C Resistance (APC Resistance)
• History of deep vein thrombosis or pulmonary
• Diagnosed anatomical abnormalities of the uterus or fallopian tubes (including submucosal fibroids, uterine septum, Asherman syndrome)
• History of cervical insufficiency
• History of surgical procedures in the pelvis (excluding Caesarean section)
• History of alcohol / drug abuse
• History of cancer
• Abnormal chromosomal karyotype (in the couple)
• Abnormal sperm
Controls
• History of pregnancy loss
• Use of assisted reproduction technology

TORCH: toxoplasmosis, rubella, cytomegalovirus, herpes simplex, HBV: hepatitis B virus, HCV: hepatitis C virus, HIV: human immunodeficiency virus.

peat region. We set the cut-off for identification of the positive cases at 41 repeats. Therefore, the "the gray zone" is defined by the presence of a band between 344-383 bp, while the "premutation state" by a band between 384-821 bp. The gel analysis was performed using Image Lab software (Bio-Rad Laboratories Inc, USA). We verified the results marked as positive with sequencing analysis to confirm the length of the expanded alleles. We subsequently analyzed all PCR reactions that produced a single band, with the second PCR step using the c primer and the CGG-chimeric primer (5'-AGCGTCTACTGTCTCGGCACTTGCCCGCCGCCGCCG-3'), under the same conditions. The 3′ end sequence found in the chimeric primer (CCGCCGCCGCCG) has the potential to bind in the CGG repeat region randomly, and therefore to produce, in the presence of expanded mutated alleles - not amplified in the first step, a "smear" on the gel[13].

Sequencing analysis

From the specimens considered as positive, all PCR fragments were gel isolated, purified, and further analyzed by dideoxy-termination sequencing method to accurately measure the number of the repeats. Furthermore, in all six cases, the number of repeats were also analyzed by PCR and capillary gel electrophoresis (Labnet laboratories S.A., Greece).

Statistical analysis

Data analysis was performed using IBM SPSS Statistics for Windows, Version 23 (IBM Corp. Armonk, NY, USA). Continuous variables are described as medians (percentiles 25th, 75th) or means with standard deviation, and dichotomous variables as numbers with percentages in brackets. The Mann-Whitney test was utilized to compare continuous variables between groups and the Kruskal-Wallis test to compare among more than two groups. We created a model of linear regression for predicting values, after accurately measuring by sequencing a number of them. The level of significance was set at 0.05.

Results

After the initial screening process and the diagnostic workup for the common causes of recurrent miscarriages, a total of 49 cases were selected fulfilling the criteria of the study, and another 49 controls were also recruited, age-matched (within two years plus or minus). The total population was 98 subjects. The main characteristics of the study population are summarized in Table 2. Since the values of CGG repeats were not normally distributed, non-parametric tests were applied (Kolmogorov-Smirnov $p < 0.001$)

The women in the patient group had in average 2.65 ± 0.75 miscarriages, ranging from two up to five. The percentage of Women who suffered two miscarriages was 53.1 %, three miscarriages 34.7 %, four miscarriages 10.2 %, and five miscarriages 2.0 % (Table 3). The number of miscarriages each woman had, is not associated with the number of the CGG repeats (Kruskal-Wallis test, $p = 0.255$)

Gel electrophoresis of the first PCR step analysis revealed a distinct, two band pattern in 34 out of 49 of patients and 22 out of 49 of control samples (Figure 1). We further analyzed the samples that showed a "single band", with the second PCR step in order to distinguish homozygosity, from the presence of a non-amplified mutated allele in the first step. No mutated allele was detected. For each woman in the study, we considered the band representing the highest number of CGG repeats.

We identified five women from the patient's group to carry the expanded allele: 46, 50, 55, 58, and 65 repeats respectively (two intermediate zone and three premutation carriers), whereas only one woman in the control group, was identified to carry an expanded allele with 57 repeats (premutation).

The six cases marked as positive by electrophoresis were further examined using dideoxy-termination sequencing analysis, to accurately determine the exact number of repeats present in each allele. After the sequencing analysis, one case had a normal number of repeats, and all the remaining five had from 42-47 CGG repeats, and are presented in detail in Table 4. These results were further verified independently, using a commercially available diagnostic methodology employing capillary gel electrophoresis (Labnet Laboratories S.A., Greece).

This approach revealed that the results obtained from electrophoresis are consistent with sequencing analysis, however, led to an overestimation of the CGG repeat number. The divergence of measured results with sequencing vs electrophoresis was used to construct a regression model in SPSS. As shown in Figure 2, linear regression was a good

Table 3: Number of miscarriages for the 49 women in the patient group.

N of miscarriages	Cases	% group
2	26	53.1
3	17	34.7
4	5	10.2
5	1	2
	49	**100**

N: number.

Table 2: Characteristics and results of the study population of the prospective case-control pilot study.

	Cases	Controls	p value
Number of cases	49	49	
Age (years)	31 (27-35)	32 (27-36)	0.906
Miscarriages	2.65 ± 0.75	-	
Women with three or more miscarriages	23 (47 %)		
CGG repeats *	34 (31-38)	32 (30-34)	0.027
CGG repeats **	28 (26-31)	27 (25-28)	0.027
Intermediate zone (45-54 CGG repeats)	4	1	0.168
Odds ratio: 4.267			

Continuous variables are presented as median (25th-75th percentile in brackets) or mean ± standard deviation and dichotomous variables as numbers (% in brackets). CGG: Cytosine/Guanine/Guanine, *: estimated by electrophoresis analysis, **: calculated by linear regression analysis.

fit, and it was subsequently used to predict the number of CGG repeats in the entire study. The complete set of repeat numbers is plotted in Figure 3. There was a significant difference in the variation of CGG repeats, between the two study groups (Mann-Whitney test, p =0.027).

Out of the five women carrying an allele with an increased number of CGG repeats (intermediate zone), four belong in the patient group (4/49; 8.2 %) and one in the control group (1/49; 2 %). Thus, the reported prevalence of the intermediate zone carriers was calculated to approximately 1/57 (1.7 %) as mentioned before. Therefore, the two groups did not differ in terms of the number of women marked positive for a premutation (Chi-square test, p =0.168, Odds ratio: 4.267, Confidence Interval: 0.459-39.629). Nevertheless, women in the patient group had significantly more CGG repeats than the control group (Mann-Whitney test, p =0.027); Table 2.

Table 4: Measured number of CGG repeats using electrophoresis vs sequencing analysis, on cases marked as positive. It is evident that electrophoresis led to an overestimation of CGG repeats. One of the positive cases was measured as normal after sequencing (underlined).

Number of CGG repeats	Cases					Controls
Electrophoresis	46	50	55	58	65	57
Sequencing analysis	38	42	44	44	47	46

CGG: Cytosine/Guanine/Guanine.

Discussion

We examined, in the current study, the presence of expanded CGG alleles in women with a history of unexplained recurrent miscarriages. Compared to women with documented normal fertility, these women had significantly more CGG repeats at their *FMR1* gene. Nevertheless, there were only six positive cases in total, which hinders the clinical significance of this finding.

Women with *FMR1* gene premutations are likely to develop premature ovarian failure (POF) in up to 20 % of cases[15]. Women with intermediate length alleles, on the contrary, do not seem to carry this risk[16]. Since none of the women in the current study had a premutation of the *FMR1* gene, one could not attribute the number of miscarriages directly to POF.

A study that examined patients with intermediate zone or low-end premutation expansions (40–85 CGG repeats) showed that motor dysfunction and cognitive decline were correlated with CGG repeat size, levels of antisense FMR1, and cytochrome C1 (CYC1) mRNA[17]. Furthermore, several phenotypes like Parkinsonism and ataxia may be associated with the "gray zone". Intermediate length CGG repeats might be associated with trisomy[18].

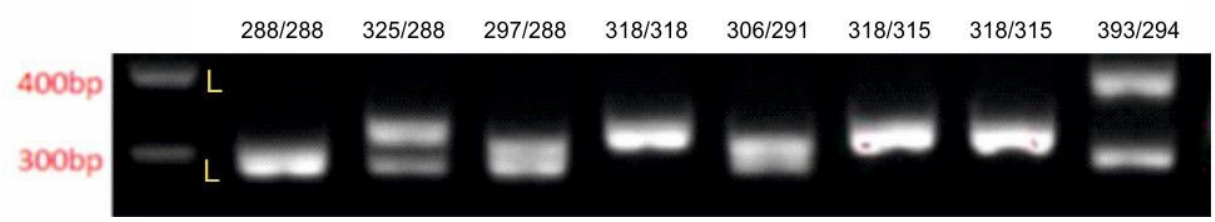

Figure 1: Representative gel electrophoresis analysis of the polymerase chain reaction (PCR) products. The number of base pairs (bp) noted on top. The last specimen on the right edge was positive, with bps close to 400 (measured as having 46 repeats after sequencing). L: 100bp DNA ladder.

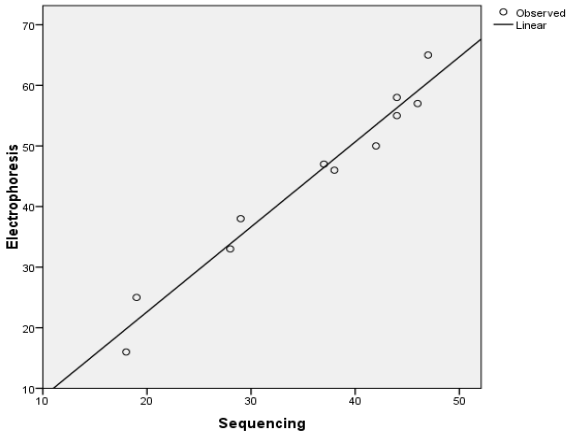

Figure 2: Regression analysis of electrophoresis vs sequencing, as methods of measuring Cytosine/Guanine/Guanine (CGG) repeats. It allowed us to measure the divergence between the two methods, and subsequently estimate the remaining values of the study.

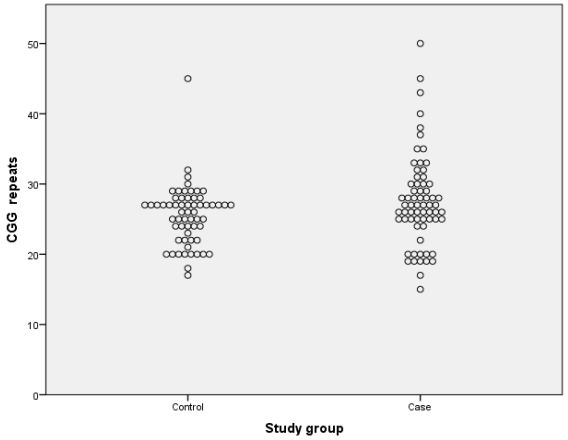

Figure 3: Total number of subjects in the study plotted according to allele representing their highest number of Cytosine/Guanine/Guanine (CGG) repeats. There are clearly more CGG repeats in the case group, a difference which is statistically significant.

FMR1 alleles the size of 45 to 200 are meiotic unstable and can be inherited as such or with increased size in the offspring[19], and also the *FMR1* gene undergoes abnormal methylation[20,21]. Possibly these unstable mutations can result in defects, incompatible with life, and thus lead to miscarriage. This could be proven by performing, in women with recurrent miscarriages, DNA analysis in the products of conception, looking for expanded alleles or otherwise altered *FMR1* gene (e.g., methylation). Therefore, the mechanism of meiotic instability regarding the *FMR1* gene occurs solely during meiosis in oocytes and not in sperm[22]. Hence, women with premutations could have daughters with full mutations, whereas men could only pass premutations to their daughters. Thus, when examining couples for expanded CGG alleles, it seems reasonable to focus the screening to women.

In women with recurrent miscarriages, future pregnancy prognosis is dependent on the number of previous abortions, the duration of the medical condition, and possible coexistence of infertility[23]. The success rates are improved when medical care is provided from specialized staff, offering close monitoring and emotional support[24]. Also, success rates are practically similar after two or three miscarriages, while the history of a live birth is not improving prognosis. This is an argument for commencing the diagnostic workup in women with two miscarriages, since delaying might impair future reproductive potential, through aging.

While women with abortions have significantly more CGG repeats than the control group, this difference was not significant when examined as intermediate group carriers. This study has its design related limitations. As a case-control study, it is prone/vulnerable to bias. The positive cases are relatively few in total, so any conclusions should be interpreted with caution. Also, secondary causes of recurrent miscarriages could not be ruled out and have potential confounding effects.

Recurrent miscarriages are a significant cause of distress for the affected couples and at the same time pose for the involved physicians a diagnostic and therapeutic challenge. This is particularly true for those cases that remain unexplained, after the full diagnostic workup (approximately 50 %). Since even a small elevation in the number of CGG repeats can have clinical implications, it could possibly account for a number of unexplained miscarriages. To the best of our knowledge, there is no other similar study in the medical literature. More studies are needed towards this hypothesis in the future, which could verify it, and uncover the molecular mechanism responsible.

Conflict of interest

The authors declare not having any competing interests.

Acknowledgment

This manuscript was submitted, peer reviewed and accepted in 2017 but its publication was delayed due to similarity issues with another paper that was eventually retracted.

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