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Can FMR1 CGG repeat lengths predict the outcome in ICSI cycles?

Short title: CGG repeat lengths and ovarian response

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ABSTRACT

Objectives: The aim of this study was to assess relationship between CGG repeat lengths and ovarian reserve and response to controlled ovarian stimulation (COH).

Material and methods: This prospective cohort study was carried out on patients (n = 49) who were admitted to the *in vitro* fertilization (IVF) clinic of the Zeynep Kamil Women's and Children's Diseases Training and Research Hospital, University of Health Sciences. Women under 40 years of age with premature ovarian insufficiency underwent genetic analysis to determine CGG repeat lengths. Ovarian reserve was assessed for each participant and participants underwent ovarian hyperstimulation and intracytoplasmic sperm injection (ICSI) cycle. Relationships between ovarian reserve, cycle outcome and CGG repeat lengths were assessed. Variables including fertility assessment including ovarian reserve tests (Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Estradiol (E2), Prolactin (PRL), Thyroid stimulating hormone (TSH), Antimullerian hormone (AMH), antral follicle count (AFC) tests) and some IVF cycle characteristics were assessed in relation to number of

CGG repeat numbers.

Results: None of the ovarian reserve tests and cycle characteristics was found to be correlated with CGG repeat lengths. Comparison of ovarian reserve tests and cycle characteristics revealed no difference between groups of women with CGG repeat length > 55 and CGG repeat length ≤ 55. Antimullerian hormone (AMH) was a significant predictor for cycle cancellation (AUC = 0.779, P = 0.008). AMH level > 0.035 was found to be the optimal cut off value to predict cycles reaching to embryo transfer with 71% sensitivity and 85% specificity. The rate of cycle cancellation was 71% in cases with AMH ≤ 0.035 whereas it was 20% in cases with AMH > 0.035 (p = 0.001). No difference was determined between groups with and without cycle cancellation in terms of CGG repeat lengths (55.3 vs 53.9, p = 0.769). Among cycles reaching to embryo transfer stage, 3 (13.6%) pregnancies were achieved.

Conclusions: Our data showed no relationship between CGG repeat lengths and ovarian reserve and response to controlled ovarian stimulation. This data also showed that no clinical difference between FMR gene mutation related POI and other etiologies.

Key words: CGG repeat length; pragile X; premature ovarian insufficiency; ICSI

INTRODUCTION

Premature ovarian insufficiency (POI) is seen in approximately 1% of the general female population before the age of 40 [1]. Although the main cause of this disease is unknown, common etiologies include genetic causes [2–6] and autoimmune diseases [7–9]. Among all the genetic causes, Fragile X is the most frequently blamed for this disorder. The premutation allele interval (55–200 CGG repetition interval) is important because of the risk of POI and the risk of being transmitted as a full mutation to subsequent generations [10]. Shamilova et al., reported that the < 28 CGG repeat interval is associated with anti-ovarian antibodies. Making this distinction in the etiology may be important in terms of affecting ovarian response to ovarian stimulation in POI patients in the future [11].

Many population studies have evaluated the relationship between FMR1 premutation (55–200) and POI. While a meta-analysis reported an increased risk of POI in pre-carriage carriers, particularly those of European origin [12], some researchers did not show a significant difference in populations.

FMR1 CGG repeat lengths are examined in four categories according to their stability: normal (< 44); intermediate or gray zone (45–54); premutation (55–200); and full mutation (>

200 repetitions) [13,14]. FMR1 premutations are thought to account for ~5% of all POI cases [15]. The clinical significance of these ranges for ovarian function is highly controversial. Studies have investigated the relationship between FMR1 CGG repeat lengths in the normal range and the intermediate range (gray zone) and the presence of POI or low ovarian reserve. Some investigators reported increased frequency of POI with FMR1 CGG repeat alleles in the intermediate range [16, 17], while other investigators failed to demonstrate this association [18, 19]. In addition, some studies have reported a relationship between low-ovarian reserve and FMR1 CGG repeat alleles in the normal range [20, 21], whereas in other publications [22, 23] the low over-overexpression of the normal range FMR1 CGG alleles was reported. The relationship with the reserve has not been shown. There is no consensus on the effect of CGG repeat lengths in the normal and intermediate range on ovarian reserve.

Objectives

Normally, only premutations have a definite relationship with POI, but some studies have shown that POI can develop in normal or gray zone cases. Discussion continues in the literature on the exact relationship between the detection of CGG repeat intervals in the normal and intermediate range with POI, unlike premutation. As we know, 5–10% spontaneous pregnancy can be seen in patients with premature ovarian failure. In patients with normal AMH and AFC, it will be possible to direct the patients in terms of clinical follow-up by looking at the number of CGG repetitions instead of waiting for spontaneous pregnancy. In the light of these data, the aim of our study was to evaluate the relationship between FMR 1 CGG repeat lengths and ovarian reserve and response to ovarian stimulation.

MATERIAL AND METHODS

Patient population

In order to evaluate the relationship between the number of CGG and ovarian reserve and ovarian response to stimulation, this prospective cohort study was carried out on patients (n = 49) who were admitted to the IVF clinic of the Zeynep Kamil Women's and Children's Diseases Training and Research Hospital, University of Health Sciences, Istanbul with infertility between June 2017 and January 2018. This study was conducted in accordance with the Declaration of Helsinki. Approval for this study was obtained from the Institutional Review Board (2017/41). A written informed consent was obtained from all participants.

POI was diagnosed according to ESHRE criteria [24]; under 40 years; cases with oligo-amenorrhea for at least four months and with a high follicle stimulating hormone (FSH) level > 25 IU/L twice every four weeks. Although oocyte donation was reported as the first choice in WHO type III anovulatory patients, since oocyte donation program is not legal in our country, patients were directed to ART. All of these patients underwent initial fertility assessment, conventional fertility assessment include ovarian reserve tests (FSH, Luteinizing hormone (LH), Estradiol (E2), Prolactin (PRL), Thyroid stimulating hormone (TSH), Antimullerian hormone (AMH) Antral follicle count (AFC) tests). Since the relationship between Fragile X carriage and POI is well known, Fragile-X permutation test was requested for all patients not only to reveal the cause of POI, but also because the presence of mutation could have significant effects for the patient and his/her family. Patients were referred to the genetic clinic of our hospital prior to controlled ovarian hyperstimulation (COH) cycle for Fragile X premutation screening.

To avoid multiple comparison statistical bias, only information regarding the initial stimulation cycle of each patient was included. The age of patients during stimulation was also recorded. Initial gonadotropin dose was calculated according to patients' age, AMH and number of antral follicles. Initial gonadotropin doses were between 300 and 450 IU/day (75–150 H LH was added to each case as gonadotropin in total dose). A flexible antagonist protocol was applied for inhibition of the premature LH surge during the COH cycle. For this purpose, when the dominant follicle reached a diameter of 13 mm, 0.25 mg Cetrorelix (Cetrotide; Merck Serono, Switzerland) was started subcutaneously once a day in the morning. Ovulation was triggered with 250 µgr of recombinant hCG (Ovitrelle; Merck-Serono, Switzerland) subcutan was applied when at least one follicle diameter reached ≥ 18 mm. Oocyte aspiration was performed 36 hours after hCG injection under transvaginal ultrasound guidance. The number of mature oocytes obtained in response to stimulation was evaluated as a measure of ovarian response.

Fragile X evaluation was performed using a commercially available kit "Fragile X GScan Kit" (Gene Link-Hawthorne, NY, USA), a standard test procedure mentioned by Sherman et al in their 2005 study [25]. Fragile X genotyping was performed with a DNA sequencer (ABI-310 DNA Sequencer; Applied Biosystems, USA) for direct fluorescent PCR amplification of the CGG trinucleotide repeat region and fragment analysis.

FSH, E2 and AMH concentrations were evaluated by commercial experiment (Diagnostic System Laboratories Inc, Texas, USA) using enzyme-linked immunosorbent

assay. All comparisons were performed per participant instead of cycle. The variation coefficients for these three tests were between 2.4% and 8.6%. Individuals with approximately 55–200 CGG repeats were considered premutation carriers. The primary aim of this study was to figure out any association between number of CGG repeat length and cycle outcome. The secondary outcome was to assess possible relationship between number of CGG repeat length and ovarian reserve markers.

Statistical Analysis

The statistical analysis was performed with using the Statistical Package for the Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). The continuous variables were expressed as the mean ± standard deviation. The categorical variables were expressed as the number and percentage. Mann-Whitney U test was used for nonparametric data. Statistical significance was defined as $p < 0.05$.

RESULTS

None of the ovarian reserve tests and cycle characteristics was found to be correlated with CGG repeat length (Tab. 1). Comparison of ovarian reserve tests and cycle characteristics revealed that no difference between groups of women with CGG repeat lengths ≤ 55 and > 55 (Tab. 2). Comparison of groups with and without cycle cancellation did not show any significant difference between groups in terms of age ($p = 0.8$), FSH ($p = 0.06$), CGG repeat number ($p = 0.6$) and total antral follicle counts ($p = 0.2$) but serum AMH ($p = 0.007$) was significantly lower in group with cycle cancellation. AMH was a significant predictor for cycle cancellation (AUC = 0.779, $p = 0.008$). AMH level > 0.035 was found to be the optimal cut off value to predict cycles reaching to embryo transfer with 71% sensitivity and 85% specificity. AMH > 0.035 is associated with cycle cancellation (OR = 0.1, 95% CI [0.02–0.5, $p = 0.001$]). The rate of cycle cancellation was 71% in cases with AMH ≤ 0.035 whereas it was 20% in cases with AMH > 0.035 ($p = 0.001$, Tab. 3). No difference was determined between groups with and without cycle cancellation in terms of CGG repeat lengths (55.3 vs. 53.9, $p = 0.769$). Among cycles reaching to embryo transfer stage 3 (13.6%) pregnancies were achieved.

DISCUSSION

The main reason for investigating triple CGG repeats on the FMR1 gene has been the prevention and/or diagnosis of psychiatric and/or neurological conditions that have historically been associated with extremely high triple re-expansion and full mutation (Fragile X syndrome > 200 CGG repetition) intervals [26, 27]. The current classification of CGG repetition extends to typical (normal), intermediate (gray zone), premutations, and full mutation, so it is based solely on a screening process for psychiatric and neurological risks. Therefore, these risk ranges have nothing to do with other potential risks associated with triple CGG repeats in the FMR1 gene, such as the risk for premature ovarian aging.

The aim of this study was to assess relationship between CGG repeated numbers and ovarian reserve and response to gonadotropin stimulation. Our data showed no relationship between CGG repeat lengths and ovarian reserve and response. This data also showed no clinical difference between FMR gene mutation related POI and other etiologies.

In studies of markers of ovarian function in populations, a relationship was found between the premutation carriers, which was largely based on the family history of fragile X syndrome and both FSH and AMH [28]. No correlation was found between medium number CGG repeats and POI. Therefore, a role of up to 55 repetitions for FMR1 CGG repeat sizes in the ovarian aging process can be questioned. Furthermore, the diagnostic study of women affected by POI shows a limited value for the assessment of normal and moderate FMR1 repeat size or for prognostic purposes in women at risk of developing POI [29]. Some cut off values for CGG repeat length have been proposed in the context of ovarian function, normal values were suggested to be between 26–34, whereas > 34 repetitions were considered to be high and < 26 repeat was considered to be low. These values were suggested to be associated with weaker embryo morphology and an accelerated decrease in functional ovarian reserve [30]. Tang et al., evaluated the relationship between the number of CGG repeats in FMR1 in Chinese patients with POI and DOR. The authors found that the frequency of FMR1 premutation did not differ between POI or DOR and normal menopausal controls; they reported that the most common CGG repeats were 29 and 30, and the repeat length for allele 2 had a secondary peak around 36–39 repeats. In addition, the researchers reported that mean FSH and AMH values did not show any association with different CGG repeats in both the POI and DOR groups [31]. In our study population, there was only one case with CGG repeat length of 2, among all the remaining cases the lowest number of repeat numbers was 38.

Whether the FMR1 CGG repeat length can be used clinically to predict IVF outcome is a controversial issue. In a study performed by Banks et al., with 4690 fresh transfer cycles,

FMR1 CGG repeat lengths was associated with ART response; however, this relationship has been reported to be weak for use during clinical management [32]. The authors argued that CGG repeat lengths do not have a higher predictive ability beyond classical predictors such as age, AMH, FSH, AFC. Banks et al., data reveals a possible role of FMR1 CGG repeat length in the normal zone in ovarian response but failed to demonstrate clinical significance as a predictor of ART results. In another study conducted by Fıçıcıoğlu et al., they suggested that the triple repeat numbers of CGG can predict a reduced ovarian reserve before the onset of ovarian aging, and that in clinical practice CGG repeats can be used to predict premature ovarian aging (FSH > 12–50IU/mL) and ovarian reserve [33].

A recent study by Batiha et al., evaluated the relationship between short CGG repeats (< 26; 26–34; > 34) and poor ovarian response. The researchers reported that CGG median allele sizes differed significantly between cases and controls, and poor ovarian responders carried shorter CGG repeats compared to healthy controls. The authors also noted that women with < 26 alleles showed twice as poor ovarian response as compared to controls. However, the authors also reported that they did not find a significant relationship between CGG repeats and ovarian reserve markers, similar to our study. The authors concluded that although low CGG repeats appeared to be associated with POR as a result of their study, the clinical use of FMR1 to predict ovarian response needs further research [34].

Lledo et al., [19] evaluated the results of oocyte donation cycle. The study cases were examined in three groups with CGG repeat lengths of 35–39 (n = 34), 40–45 CGG (n = 12) and > 45 CGG (n = 17) and the ovarian response was found to be similar between the groups. This study is the first to evaluate the ovarian response in subjects with a normal and intermediate repeat lengths. As a result of this study, the authors recommended that CGG repeats in the intermediate zone does not adversely affect the ovarian response, so fragile X genetic screening should not be taken into account in predicting ovarian response. In a study conducted by Rehnitz et al., they evaluated the COH response in three groups as poor responder, normoresponder and hyperresponder, and divided the patients into six genotypes according to CGG repeat lengths [35]. The authors reported that the ovarian response could be adversely affected by low CGG alleles. They even argued that this poor ovarian response associated with a low CGG allele might be impaired during folliculogenesis independent of stimulation.

In a study conducted by Gustin et al., with 566 patients, it was found that the relationship between CGG repeat length and AMH changes with age in an analysis using a

multivariate regression model [36]. In our study mean age of whole study population was 29 years and our data analysis revealed no association with AMH level and CGG repeat length. We used AMH level to be reference predictor for cycle outcome and AMH significantly predicted cycles reaching to embryo transfer among cycles with high rate of cancellation, overall cancellation rate was 55.1% in all the study groups. In a past study by Pastore et al., the cycle characteristics of seventy-nine women with a diagnosis of low ovarian reserve and no family history of fragile X syndrome were evaluated [37]. As a result of the study, the authors reported that women with a CGG repeat length ≥ 35 had a higher rate of follicular loss starting at later ages.

The impact of smaller repeats at the boundary of premutation and normal is less clear. Eslami et al., compared the FMR1 CGG repeat lengths with the intermediate and premutation group in a study they included the POI, DOR, and healthy control group [38]. In the study, the frequency of premutation was found to be higher in patients with POI and DOR than in control patients; intermediate allele frequency was similar between groups. Based on the results of the study, the authors concluded that FMR1 CGG repeat alleles in the intermediate zone do not pose a high risk for POI and DOR.

Ranisavljevic et al., investigated that ovarian response to controlled ovarian stimulation in premutation and full mutation carriers and compared the clinical results. They reported that significantly higher FSH doses were needed for ovarian stimulation in premutated patients. However, the researchers found no correlation between the number of oocytes collected and the number of CGG repeats [39].

A current meta-analysis of Pastore revealed no association within subcategories of normal repeat length (< 45 CGG) and IVF pregnancy rates. It was shown that, premutation carriers (CGG 55–200) may have reduced success with IVF treatment than women with a normal CGG repeat length or a full mutation [40]. According to these cited researches, there is no consensus on this issue, majority of the investigations showed no predictive value of CGG repeat lengths and reproduction, while some showed lower number of repeats to be risk factor for poor outcome, on the other hand some showed higher repeat number may be responsible for poor response in IVF. For this reason, we conducted this prospective study, in our study, we included consecutive women diagnosed to have POI, major disadvantage of this study was small sample size and lack of data regarding other etiologies of POI.

In conclusion, our study showed no relationship between CGG repeat lengths and ovarian response to ovarian stimulation. Despite the small number of patients, the results of our study are consistent with the current literature.

Conflicts of interest

All authors declare that they have no conflict of interest.

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Table 1. Correlation between ovarian reserve tests and cycle characteristics with the number of CGG repeats

		Age (years)	FSH	LH	AMH	Total_ AFC	Total_Gonadotropin dose	Stimulation day at oocyte trigger	Peak estradiol	Total oocyte number
CGG repeat #	Correlation coefficient (r)	–0.115	0.079	0.048	0.153	–0.009	–0.072	–0.106	0.282	0.258
	p Value	0.437	0.595	0.751	0.413	0.949	0.625	0.638	0.228	0.203

Table 2. Comparison of ovarian reserve tests and cycle characteristics between groups of women with CGG repeat lengths ≤ 55 and > 55

	Groups	Mean	Std. Deviation	p value
Age [years]	CGG ≤ 55	29.8	6.3	
	CGG > 55	29.9	5.2	0.866
FSH [mIU/mL]	CGG ≤ 55	37.7	35.4	
	CGG > 55	31.6	33.8	0.867
Estradiol [pg/mL]	CGG ≤ 55	42.2	42.6	
	CGG > 55	40.3	52.7	0.810
Progesterone [ng/mL]	CGG ≤ 55	0.4	0.5	
	CGG > 55	0.5	0.2	0.031
TSH [mIU/L]	CGG ≤ 55	1.7	1.09	
	CGG > 55	1.7	0.7	0.652
Prolactin [20 ng/mL]	CGG ≤ 55	15.4	9	

LH [mlU/mL]	CGG > 55	17.2	9.7	0.702
	CGG ≤ 55	18.1	17.5	
AMH [ng/mL]	CGG > 55	17.1	20.06	0.982
	CGG ≤ 55	0.4	0.8	
Total_AFC	CGG > 55	0.8	2.1	0.724
	CGG ≤ 55	6.09	4.01	
Total gonadotropin dose [IU]	CGG > 55	5.4	4.3	0.484
	CGG ≤ 55	3584.03	1493.7	
Menstrual day at ovulation trigger	CGG > 55	3604.8	994.3	0.765
	CGG ≤ 55	10.9	3.2	
Peak estradiol level [pg/mL]	CGG > 55	11.2	2.8	0.748
	CGG≤55	673.3	435.3	
Total number of oocytes	CGG > 55	967.5	359.01	0.274
	CGG ≤ 55	0.7	0.8	
Total number of mature oocytes	CGG > 55	1	0.8	0.497
	CGG ≤ 55	0.4	0.5	
	CGG > 55	0.6	0.5	0.572

Table 3. The rates of cycle cancellation of women with AMH ≤ 0.035 and AMH > 0.035

		AMH ≤ 0.035	AMH > 0.035	Total	p value
uncancelled		10	12	22	
		29.4%	80%	44.9%	
cancelled		24	3	27	
		70.6%	20%	55.1%	P < 0.001
Total		34	15	49	
		100.0%	100.0%	100.0%	