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Original Article

The expression of CGG repeats in FMR1 of child-bearing women with abnormal gestation and delivery history and its correlation with pregnancy outcome

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Abstract: Objective: This study aimed to analyze the expression of CGG repeats in the fragile X mental retardation 1 (FMR1) gene of child-bearing women with abnormal gestation and delivery history and its correlation with pregnancy outcome. Methods: A total of 300 child-bearing women with abnormal gestation and delivery history in our hospital were included into the observation group (OG); meanwhile, 300 pregnant women without abnormal gestation and delivery history were included into the control group (CG). Real-time fluorescence PCR was used for assaying of CGG repeats. The two groups were followed until delivery, and pregnancy outcomes were recorded. The correlation between CGG repeats in the FMR1 gene and the pregnancy outcome was analyzed by SPSS Pearson correlation analysis software. Results: Statistical significance was observed between the OG and the CG for CGG repeats in FMR1 (P<0.05), but the OG reported a lower normal rate and a higher rate of permutation and grey area (P<0.05). There was no significant difference in the incidences of premature rupture of fetal membranes, postpartum hemorrhage, hydramnion, giant baby syndrome, and neonatal malformation (P>0.05), but the incidences of premature deliver and gestational hypertension in the OG were lower than those of the CG (P<0.05), and the incidence of fetal distress, asphyxia neonatorum and neonatal mortality rate in the OG were higher than those of the CG (P<0.05). Analysis showed that in child-bearing women with abnormal gestation and delivery history, the CGG repeats in FMR1 had no significant correlation with the incidences of premature rupture of fetal membranes, postpartum hemorrhage, hydramnion, giant baby syndrome, neonatal malformation (P>0.05), but was positively correlated with premature delivery, gestational hypertension, fetal distress, asphyxia neonatorum and neonatal mortality rate (P<0.05). Conclusion: The FMR1 gene has a high incidence of mutation in child-bearing women with abnormal gestation and delivery history, which may account for the rise of CGG repeats and is associated with pregnancy outcome.

Keywords: FMR1, gene CGG repeats, abnormal gestation and delivery history, child-bearing woman, pregnancy outcome, correlation

Introduction

Abnormal gestation and delivery history is defined as a pregnant woman with a history of 2 or more spontaneous abortions, missed abortions, severely malformed fetus, fetal death, stillbirth or neonatal death [1]. In recent years, the incidences of spontaneous abortion, fetus diapause in the early stage of pregnancy and missed abortion is showing a rising tendency in China [2]. In the meantime, with the application of prenatal diagnosis, and chromosome and gene detection abilities; the detection rate of abnormal gestation and delivery history, and chromosome abnormalities in a fetus has also increased [3]. Ovarian reserve, an important mark of female fertility [4], refers to the capacity of follicular growth, development and formation of fertilized oocytes in the ovarian cortex. For women with diminished ovarian reserve (DOR), the number of follicles and the quality of oocytes is decreased. As the diseases progresses, the patient's fertility is further compromised, and the incidence of infertility and premature ovarian failure (PFO) heightens [5]. According to previous studies [6], DOR accounts for most of the repeated abortions with unknown causes. Therefore, en-

Table 1. Instruments and equipment

Name	Place of origin/manufacturer
ABI3500DXl	ABI, USA
Steponeplus quantitative PCR instrument	ABI, USA
20 ul transferpettor	Eppendorf, USA
100 ul transferpettor	Eppendorf, USA
MiniSpin centrifuge	Eppendorf, USA
Desktop high-speed centrifuge	Eppendorf, USA
Nanodrop One spectrophotometer	Eppendorf, USA
High pressure steam sterilizer	Zhixin, Shanghai, China
Super-clean bench	Antai, Suzhou, China
AmplideX kit	Magen, Asuragen, USA
Magen full-blood DNA extract kit	Magen, Asuragen, USA

hanced prediction of child-bearing women with abnormal gestation and delivery history plays a major role in the improvement of pregnancy outcome.

The Fragile X mental retardation 1 (FMR1) gene lies at Xq23.7, the end of sex chromosome X. Its promoter region is characterized by dynamic triple-duplication in the coding sequence of CGG [7]. Clinically, CGG duplication sequence is divided into four types according to the different amplification probability, i.e., normal (CGG repeats between 5 and 44), intermediate (CGG repeats between 45 and 54), permutation (CGG repeats between 55 and 200), and full mutation (CGG repeats over 200) [8]. Clinical studies have shown that [9] FMR1 is amplified abnormally during the iteration of CGG repetitive sequence in the 5' noncoded region, resulting in a high incidence of fragile X mental retardation. Referring to previous studies, carriers of FMRI permutations have a high incidence of POF, but for patients with mild POF, the CGG repetitive sequence falls within the intermediate region or normal range. Therefore, CGG repeats in FMR1 are associated with a woman's ovarian reserve [10].

Based on the forgoing study results [11], DOR patients are often accompanied with FMR1 gene repeats and extensions, and for patients with FMR1 permutation, atresia follicle may occur, leading to a decreased number of follicles. Hence, in this study, child-bearing women with/without abnormal gestation and delivery history were included to explore the expression of CGG repeats in the FMR1 gene of child-bearing women with abnormal gestation and delivery history and its correlation with the pregnancy outcome.

Materials and methods

Clinical materials

A total of 300 child-bearing women with abnormal gestation and delivery history were included into the observation group (OG). Inclusion criteria: pregnant women with abnormal gestation and delivery history, 2 or more spontaneous abortions or missed abortions, a history of premature delivery, fetus diapauses, fetal death, stillbirth, delivery of baby with severe birth defects (such as chromosome abnormalities, dysgnosia with unknown causes, abnormalities of major organs, induced labor due to birth defects, and neural tube malformation), and neonatal death within 28 days were included. At the same time, 300 pregnant women without abnormal gestation and delivery history or any internal or surgical complications or autoimmune diseases were included into the control group (CG). Exclusion criteria: patients with psychosis, cognitive dysfunction, malignant tumors, organ diseases, immune diseases or diseases in the blood system were excluded from the study. This study was approved by the ethics committee of Mianyang Central Hospital.

Instruments and equipment

Instruments and equipment required for the tests are listed in **Table 1**.

Methods

Sample collection and DNA extraction: (1) Sample collection: On the next day after hospitalization, 5 mL of peripheral venous blood was drawn (in a fasting status) from the patients in both groups, it was centrifuged for 35 min at 3500 rpm, and then placed in a freezer at -80°C for future use. (2) DNA extraction: Serum samples were used to extract DNA by QIAamp full-blood DNA extraction kit (Qiagen, Germany) in strict accordance with the instructions [12, 13].

Table 2. Primer design

Primer type	Primer	Length
β-actin	F: 5' CAGGATGCAGCAGGTGGAAGC 3'	132
	R: 5' TGCTCCAGGCTGTAGTCTGTGG 3'	
FMR1-F	F: 5' AGGTTCCTCTCCTAGCAGATCATTCTC 3'	99
	R: 5' GAGCGGCAACTTCTGAGGTCTTAC 3'	
FMR1-R	F: 5' GCTGAGTCCTCTTGCTGTGCTC 3'	158
	R: 5' GTACCTGGAGGCTTGGCATGAC 3'	
FMR1-CGG	F: 5' CCGCTTGGTGGTGCATGTATCC 3'	184
	R: 5' CCAAGGTGCTGAGTGGCTAAGG 3'	

Assay of CGG repeats: ① RNA isolation and extraction. Serum samples were taken and mixed with 500 uL of Trizol. After blending, the mixture was transferred to a centrifuge in the amount of 1.0 mL for oscillation for 5 min and then let rest. Next, 200 uL of chloroform was added into all serum samples of both groups. The mixture was then oscillated for 15 s, let rest for 5 min, and centrifuged for 15 min at the centrifugal force of 1216. With the supernatant removed, 1 mL of pre-cooled ethanol (concentration: 75.0%) was added and dried under room temperature for 7 min to assay the absorbency with an ultraviolet spectrophotometer A260. ② Detection method. Real-time fluorescence PCR technique was used to measure the CGG repeats in both groups with primers listed in **Table 2** [14].

PCR parameters were set up as follows according to the test requirements: 10 min at 30°C, 30 min at 42°C, 5 min at 99°C, and 5 min at 5°C; 35 cycles in continuity, extension for 10 min at 72°C. The final product was loaded into 1.5% agar gel for electrophoresis with β-actin as the internal control.

Pregnancy outcome and association analysis: The two groups were followed until delivery, and pregnancy outcomes were recorded, including outcomes of the mothers, such as premature delivery, premature rupture of fetal membranes, postpartum hemorrhage, hydramnion and gestational hypertension, as well as outcomes of the babies, such as fetal distress, giant baby syndrome, neonatal malformation, asphyxia neonatorum and neonatal mortality rate.

Statistical analysis

Statistical analysis was performed with SPSS 18.0. SPSS Pearson association analysis software was used to analyze the correlation between CGG repeats in FMR1 and the pregnancy outcome of child-bearing women with abnormal gestation and delivery history. In the case of nominal data expressed as [n (%)], comparison studies were carried out through chi-squared test. In case of numerical data expressed as Mean ± Standard Deviation, comparison studies were carried out through t test; for all statistical comparisons, significance was defined as $P<0.05$.

Results

Comparison of general data between the OG and CG

The two groups had no significant difference in terms of age, body mass, and abnormal gestation and delivery history; indicating the groups were comparable ($P>0.05$, **Table 3**).

Comparison of CGG repeats between the OG and the CG

Six CGG repeats were found in the patients in the OG, of which, the minimal number of CGG repeats was 20, and the maximal was 200. The most common number of CGG repeats was 30, found in 90 patients, accounting for 31.0%; other values of CGG repeats were 28 in 80 patients, accounting for 26.67%, and 57.67% of the total allelic genes; there were 36 found in 57 patients, accounting for 19%, and 70 of different types, accounting for 23.33% (**Figure 1**). In the patients of the CG, 4 different types of CGG repeats were observed, ranging in number from 10 to 120. The most common was with 16, found in 135 patients, accounting for 45.0%; the other CGG repeats included 15 in 112 patients, accounting for 37.33%, and 57.67% of the total allelic genes; there were 25 in 53 patients, accounting for 17.67%. The two groups exhibited statistical significance in terms of CGG repeats in FMR1 ($P<0.05$, **Figure 2**).

Comparison of distribution ratio of permutations and gray area between for the OG and CG

The normal rate, permutation rate and great area rate were 72.00%, 10.00% and 18.00% in the OG, and 89.67%, 1.33% and 9.00% in the CG, respectively ($P<0.05$, **Table 4**).

Table 3. Comparison of clinical data between the two groups

Clinical materials		OG (n=300)	CG (n=300)	F	*P*
Age (y)		37.49±4.34	36.84±4.31	1.296	0.678
Body mass (kg)		57.36±4.33	58.04±4.30	1.159	0.674
Abnormal gestation and delivery history	Fetal death	11 (3.67)	-	-	-
	Stillbirth	10 (3.33)	-		
	Neonatal death	9 (4.00)	-		
	Missed abortion	45 (15.00)	-	-	-
	Severely malformed fetus	11 (3.67)	-		
	Spontaneous abortion	214 (71.33)	-		

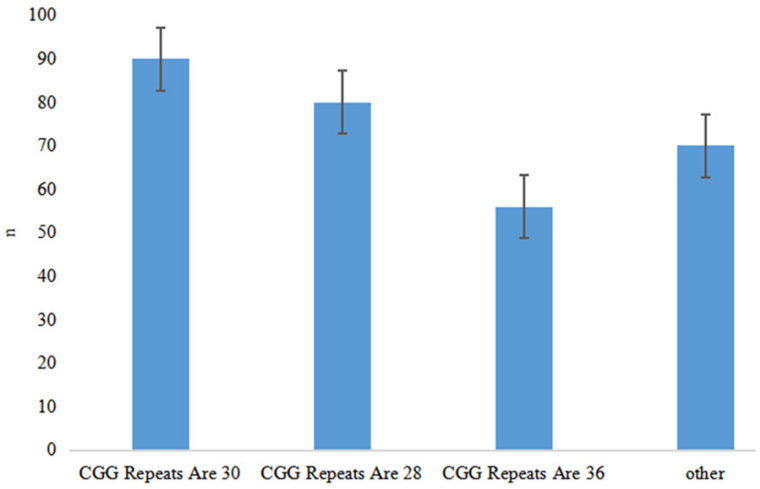

Figure 1. CGG repeats in FMR1 and its composition ratio in the OG.

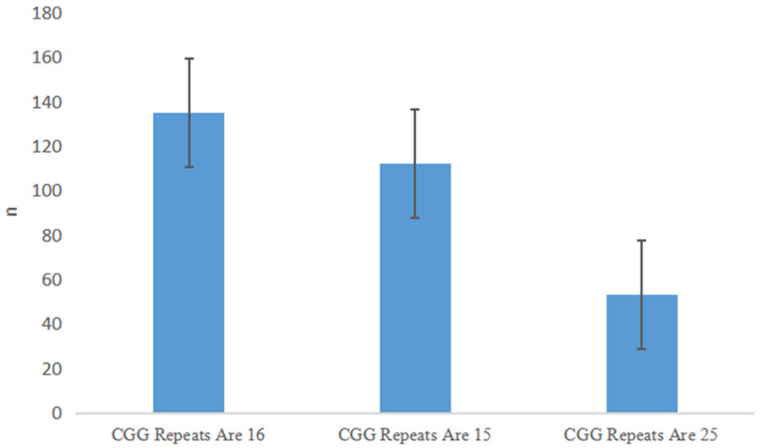

Figure 2. CGG repeats in FMR1 and its composition ratio in the CG.

Comparison of pregnancy outcomes between the OG and CG

The incidences of premature rupture of fetal membranes, postpartum hemorrhage, and hydramnion were 1.67%, 2.67% and 3.67% in the OG, and 1.33%, 4.00%, and 4.00% in the CG, respectively ($P>0.05$). The OG reported a higher incidence of premature delivery and gestational hypertension as 17.33% and 13.67% respectively, while in the CG, the two incidences were only 2.33% and 3.00%, respectively ($P<0.05$, **Table 5**).

Comparison of fetal outcomes between the OG and CG

There was no significant difference in the incidence of giant baby syndrome and neonatal malformation between the OG and the CG ($P>0.05$). The incidences of fetal distress, asphyxia neonatorum and neonatal mortality rate were higher in the OG than those in the CG ($P<0.05$, **Figure 3**).

Analysis of correlation between CGG repeats in FMR1 and the pregnancy outcome of child-bearing women with abnormal gestation and delivery history

SPSS Pearson correlation analysis revealed that, in the child-bearing women with abnormal gestation and delivery history, the CGG repeats in FRM1 had no statistically significant correlation with the incidence of premature rupture of fetal membranes, postpartum hemorrhage, hydramnion, giant baby syndrome, and neonatal malformation ($r=0.223$, 0.431,

Table 4. Comparison of distribution ratios of permutation and grey area between the two groups [n (%)]

Group	n	Normal	Grey area	Permutation
OG	300	216 (72.00)	54 (18.00)	30 (10.00)
CG	300	269 (89.67)	27 (9.00)	4 (1.33)
x^2	/	6.392	5.891	9.324
P	/	0.025	0.033	0.015

0.229, 0.323, 0.215 respectively, P=0.081, 0.074, 0.072, 0.074, 0.068), but was positively associated with the incidences of premature delivery, gestational hypertension, fetal distress, asphyxia neonatorum and neonatal mortality rate (r=0.771, 0.784, 0.667, 0.698 respectively, P=0.021, 0.023, 0.028, 0.027, **Table 6**).

Discussion

The role of the FMR1 gene in women

It is often reported in clinical practice that abnormal gestation and delivery history refers to any previous history of abnormal gestation, including fetal malformation, stillbirth, postpartum hemorrhage, etc., which will bring heavy psychological and mental stress to the pregnant women, accounting for a higher incidence of pregnancy complications and risk of abnormal pregnancy, thus affecting the outcomes of the mother and the baby [15]. Clinical studies have revealed that [16] the ovarian reserve directly affects a woman's fertility and pregnancy outcome; which, if reduced, will lead to a compromised decrease in the number and quality of ovarian follicles. FMR1, also known as the gene of familial mental retardation, is located on chromosome Xq27.3 and consists of 17 exons sized at 38 Kb, corresponding to mRNA4.4Kb in the human body. Its codeable sequence length is 19 kb. According to previous studies [17], FMR1 gene is highly polygenetic and divides into normal repeats, intermediate repeats, permutations and full mutations according to the stability of the repetitive sequences. Therefore, the CGG stability can accurately reflect any possible extension during its iterations. However, the definition of CGG repetition scope is short of an absolute boundary. In general cases, the larger the CGG repeat is, the more unstable it is [18]. In this study, the CGG repeats in the FMR1 gene of the OG and CG have statistical significance when compared (P<0.05); as compared with the OG, the normal rate of CGG repeats in FMR1 was lower than that of the CG (P<0.05), but the permutation rate and the grey area rate were higher (P<0.05), indicating that the child-bearing women with abnormal gestation and delivery history had a higher incidence of FMR1 gene mutation, which mainly concentrated in the permutation rate and grey areas, affecting pregnancy outcome. There are many reasons for this phenomenon. There are a variety of factors for child-bearing women with abnormal gestation and delivery history, such as genetic variables and drug exposure history, which can cause genetic mutations that disrupt fetal growth, and the mutation region of the FMR1 gene is the most obvious [19].

Effect of FMR1 gene mutation on pregnancy outcome

Previous studies [20] have shown that the FMR1 gene is responsible for the regulation of human reproductive cells. Its expression is observed in the ovarium of the fetus and follicular cells of adult females. Some scholars [21] have performed tests in mice and found that FMR1 gene permutations in the ovarium have reduced number of follicles, and abnormal FMR1 protein expression was found in most of the follicular nuclei. Compared with healthy mice, there were permutations of the FMR1 gene in the ovarium, the ovaries had smaller follicles and fewer granulosa cells, indicating that the POF was inhibited to various degrees [22]. For permutation carriers, the anti-mullerian hormone level may be reduce, accompanied by compromised ovarian reserves [23]. In this study, the incidences of premature rupture of fetal membranes, postpartum hemorrhage, hydramnion, giant baby syndrome, and neonatal malformation were not found to have significant differences between the OG and the CG (P>0.05), but the rates of premature delivery and gestational hypertension in the OG were lower, and the fetal distress, asphyxia neonatorum and neonatal mortality rate were higher than those of the CG (P<0.05). This indicates that the FMR1 gene is clearly mutated in child-bearing women with abnormal gestation and delivery history, which can influence the pregnancy outcomes and adversely affect the health of the mother and

Table 5. Comparison of pregnancy outcomes between the two groups [n (%)]

Group	n	Premature rupture of fetal membranes	Postpartum hemorrhage	Premature delivery	Gestational hypertension	Hydramnion
OG	300	5 (1.67)	8 (2.67)	52 (17.33)	41 (13.67)	11 (3.67)
CG	300	4 (1.33)	12 (4.00)	7 (2.33)	9 (3.00)	12 (4.00)
x^2	/	1.295	0.437	6.463	5.398	1.068
P	/	0.637	0.893	0.026	0.035	0.351

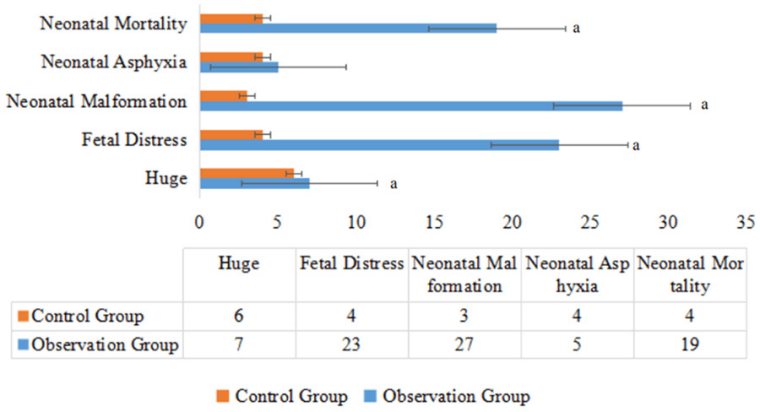

Figure 3. Comparison of outcomes of babies between the two groups [n (%)].

Table 6. Analysis of the correlation between CGG repeats in FMR1 and the pregnancy outcome of child-bearing women with abnormal gestation and delivery history (r, P)

Pregnancy outcome	R	P
Premature rupture of fetal membranes	0.223	0.081
Postpartum hemorrhage	0.431	0.074
Premature delivery	0.693	0.025
Gestational hypertension	0.771	0.021
Hydramnion	0.229	0.072
Giant baby	0.323	0.074
Neonatal malformation	0.215	0.068
Fetal distress	0.784	0.023
Asphyxia neonatorum	0.667	0.028
Neonatal death	0.698	0.027

the baby. FMR1 gene mutations can increase the incidence of abnormal gestation and delivery history, increase the incidence of fetal distress and deformity, and cause neonatal death in severe cases. In recent years, with the continuous development of medical technologies, more and more clinical studies have been carried out on the FMR1 gene and the ovarium reserve. For FMR1 genes with CGG repeat numbers between 26 and 34, the ovarium function is within the normal range. When the CGG repeat numbers exceed this scope, ovarium function will be compromised [24]. Therefore, the number of CGG repeats in the FMR1 gene can be used to evaluate a female's ovary function and predict the pregnancy outcome, which plays a major role in the improvement of patients' prognosis [24].

Relationship between FMR1 gene CGG repeats and pregnancy outcome

To further analyze the relationship between the number of CGG repeats in FMR1 gene mutations and the pregnancy outcome of child-bearing women with abnormal gestation and delivery history, a SPSS Pearson correlation analysis was carried out in this study, and the results indicated that the number of CGG repeats in FMR1 had no statistically significant correlation with the incidence of premature rupture of fetal membranes, postpartum hemorrhage, hydramnion, giant baby syndrome, or neonatal malformation ($P>0.05$). However, it was positively correlated with the premature delivery, gestational hypertension, fetal distress, asphyxia neonatorum and neonatal mortality rate ($P<0.05$), indicating that the number of CGG repeats in the FMR1 gene and pregnancy outcome were closely related to the child-bearing ability of pregnant women with abnormal gestation and delivery history. Therefore, FMR1 gene screening should be strengthened for pregnant women with abnormal gestation and delivery history, and for those with abnormal FMR1 genes, regular pregnancy inspections should be performed to improve the pregnancy outcome of patients. According to the recommendations of American College of Medical Genetics and Genomics

(ACMG) and American College of Obstetricians and Gynecologists (ACOG); female patients with reproductive problems related to elevated FSH level, in particular, those accompanied with POF, FX or hypophrenia with unknown causes, FMR1 gene screening can be enhanced to evaluate their ovarian reserve in order to achieve gestation before the drop of their ovarian reserve and possibly avoid IVF and such other operations in the late stages [25, 26]. For pregnant women, prenatal diagnosis can be regularly enhanced to assess the patients' physical conditions, and folic acid can be administered regularly in the early pregnancy period [27]. The pregnant women may inform doctors about her previous special heath conditions to fully assist in the evaluation of predicting the pregnancy outcome, in order to adopt corresponding measures in the case of possible complications. Patients are guided to count the number of fetal movement by themselves, and complete ultrasound screening and prenatal diagnosis based on the doctor's advice. In case of an abnormal fetus found in pregnancy screening, the risks can be discussed in a timely manner to inform the mother and her family members, or the pregnancy may be terminated in the case of server malformations [28].

This study has limitations. Since China has not yet clearly defined the relevant populations that need to be tested for FMR1 gene screening, more clinical references need to be drawn from the experience of pediatrics, obstetrics and gynecology and related departments. Furthermore, this study included a relatively small number of patients, and requires further study and exploration.

In conclusion, child-bearing women with abnormal gestation and delivery history have a high incidence of FMR1 gene mutations, which can lead to a rise in the number of CGG repeats, and is correlated with pregnancy outcome. Enhanced assaying of CGG repeats in FMR1 can be used to predict pregnancy outcomes and guide clinical treatment.

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Disclosure of conflict of interest

None.

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