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Method for the Molecular Cytogenetic Visualization of Fragile Site FRAXA

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Abstract—Fragile X syndrome is one of the most common reasons for human hereditary mental retardation. It is associated with the expansion of CGG repeats in the 5'-untranslated region of the *FMR1* gene, which results in the suppression of its expression and the development of the disease. At present, methods based on PCR and Southern blot analysis are used for diagnostics of the fragile X syndrome. The presence of a fragile site FRAXA on the X chromosome is typical for patients with this pathology. We developed a method of visualizing this site in cell cultures obtained from patients using the fluorescent in situ hybridization (FISH) and the combination of two probes. The method allows one to detect five types of signals on the X chromosome, three of which are normal, while two are associated with the emergence of fragile site FRAXA. An analysis of the distribution of all signal types in cell lines from healthy individuals and patients with fragile X syndrome demonstrated that the method allows one to determine differences between lines with a high statistical significance and that it is applicable to detecting cells that are carriers of the syndrome.

Keywords: fragile X syndrome, mental retardation, chromosome fragility, FRAXA, *FMR1*, fluorescent in situ hybridization

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INTRODUCTION

Fragile X syndrome (FXS) was described by Purdon Martin and Julia Bell in 1943 (Martin–Bell syndrome) [1]. This disease is the main reason for human hereditary mental retardation. The population analysis demonstrated that the syndrome frequency around the world is 1 per 5000–7000 among men and 1 per 4000–11000 among women [2, 3]. In more than 99% of cases, the expansion of the CGG repeat in the 5'-promoter region of the fragile X mental retardation 1 (*FMR1*) gene is the reason for the development of FXS. The normal allele of the gene contains 6–54 CGG triplets; premutant allele, 55–200 triplets; the full mutation, more than 200 triplets. FXS develops upon full mutation and a triplet number of more than 200. In this case, CpG-islands in the 5'-untranslated region of the *FMR1* gene promoter are methylated, and the gene transcription is suppressed. The *FMR1* gene encodes the fragile X mental retardation protein (FMRP). This RNA-binding protein plays a key role in the development of neurons (especially in the process of dendrite differentiation) and in the functioning of synapses [4, 5]. Patients with the full *FMR1* gene mutation have a number of specific symptoms, of which autism and mental retardation are the most significant [6]. It should be especially noted that the premutant allele is unstable and has a tendency towards expansion [7].

Molecular genetic detection of FXS in cell lines or for diagnostics is rather laborious. The main method consists of determining the repeat size by means of Southern blot analysis, which simultaneously allows one to find out whether the *FMR1* gene promoter region is methylated. It is also possible to estimate the size of any repeat; however, it requires a large amount of DNA and quite a long time [5, 8].

PCR using the primers that surround the repeat is another method of determining the length of the CGG tract. Since the content of GC pairs in this region is very high, this approach requires a number of special conditions and reagents [9–11]. The size of the obtained product is determined by a capillary analyzer [12]. A special PCR modification (using chimerical CGG-primer simultaneously with the primers surrounding the repeat) was suggested in recent publications [13, 14]. This allows one to detect AGG insertions in the CGG repeat tract (if they exist), which is important for estimating the possibility of expansion in premutation carriers. The suggested primer contains a sequence $(CGG)_4$ at the 3'-end and a unique

Abbreviations: FXS, fragile X syndrome; FdU, fluorodeoxyuridine; FISH, fluorescent in situ hybridization

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sequence at the 5'-end and randomly binds to regions of the CGG tract that, when used in a pair with the primer that flanks the repeat, allow one to obtain a smear on electrophoresis, the size of which indicates the length of the region. The chimerical primer does not bind to them in the presence of AGG insertions, and the PCR product generates multiple peaks on the capillary electrophoresis. The shortcoming in using PCR is that it does not allow one to detect heterozygotes or mosaics in the case when one allele is a full mutation but the second allele is a premutation or normal [15].

The methylation of the promoter occurs during the full mutation of the *FMR1* gene, which results in the suppression of gene expression and the heterochromatinization of this region [16]. The analysis of methylation status is very important criterion both for the diagnostics and for describing cell lines, since it indirectly reflects the presence of expansion. An analysis of methylation status is conducted either by processing the sample with sodium bisulphate with subsequent PCR or by processing genomic DNA with methyl-specific restriction endonuclease with the subsequent amplification of the fragment that contains the restriction site [17–19].

The presence of a so-called fragile site FRAXA in the Xq27.3 region is an important peculiarity of cells that are carriers of the full mutation by the *FMR1* gene. Fragile sites are specific chromosomal loci representing gaps or constrictions on metaphase chromosomes [20]. The fragility of the X chromosome in the FRAXA site is due to the underreplication of the increased repeat, which appears due to the replicative stress caused by the presence of fluorodeoxyuridine (FdU) [21].

Initially, fragile sites were detected based on routine cytogenetic methods and the calculation of constrictions on X chromosomes. At present, this method (assuming the analysis of the chromosome morphology) is considered to be rough and inaccurate and is rarely used. The FRAXA site at the fragile X chromosome syndrome is located very close to the telomere; therefore, it is difficult to find small chromosome fragments arising as a result of the fragility [22]. Furthermore, differences in the chromatid length after the gap cannot be clearly determined. Routine staining allows one to detect the constrictions in the FRAXA region, but does not allow one to find the gaps.

We developed a new molecular cytogenetic method of determining FXS, which allows one to identify any FRAXA type by means of microscopic analysis. The suggested method is based on the fluorescent in situ hybridization (FISH) of the sequence containing the FRAXA region simultaneously with the sequence located distally (with subsequent analysis of localization of the signals). This approach allows one to use cytogenetic method directly for the detection of full mutations that are typical for FXS. It is less time-consuming compared with Southern blot analysis and PCR amplification of GC-rich regions, which allows it to be used as a routine method.

EXPERIMENTAL

Cultivation of human cell lines. All cell lines used in this study (immortalized B lymphocytes) were obtained from the Coriell Institute cell repository (United States). The CGG repeat in the GM04025 cell line with the full mutation is composed of 645 triplets, the promoter is methylated [12, 23]. The CGG repeat in another cell line (GM03200 with the full mutation) is composed of 530 triplets, and the promoter is methylated [24]. Two control cell lines (GM06865 and GM06895) have less than 30 CGG triplets and non-methylated *FMR1* gene promoter [21]. The cells were cultivated in the RPMI 1640 GlutaMAX medium (Gibco, United States) containing 15% fetal bovine serum (Gibco) and antibiotics.

Induction of fragile sites FRAXA and the preparation of preparations. The FRAXA site is folate sensitive rare fragile site, which can be induced by the oppression of dTTP synthesis by means of FdU [25]. To induce the FRAXA, the cells were incubated in medium containing 0.1 μM FdU (Sigma-Aldrich, United States) for 18 h according to previously described protocol [26]. After this, the cells were incubated for 2 h in the presence of colcemide (100 ng/mL) KaryoMAX (Invitrogen, United States) [21]. During the preparation of metaphase spreads, the hypotonic treatment of the cell culture with 0.75 M KCl solution was conducted for 20 min at 37°C, after which it was fixed with Carnoy's solution (methanol : acetic acid, 3 : 1). The cell suspensions were dripped on the slides and for viewing under a microscope.

Preparation of probes and fluorescent in situ hybridization. BAC clones containing the *FMR1* gene (RP11-489K19) and *GPR50* gene (RP11-351H6) (Empire Genomics, United States) were labeled with biotin and digoxigenin in the reaction of NICK translation using the BioNick™ DNA Labeling System kit (Thermo, United States) or DIG-Nick Translation Mix (Sigma-Aldrich) according to the manufacturer's protocols. A volume of 150 ng of labeled probe was taken for FISH, and the suppression of repeated DNA was conducted using 10 ng of human Cot1-DNA repeat fraction for 1 h at 37°C. The hybridization was conducted during the night in the solution containing 50% formamide at 37°C. Probes labeled with biotin were detected using Alexa-555-streptavidin conjugate (Thermo), while the probes labeled with digoxigenin by means of Anti-Digoxigenin-Fluorescein antibodies (Sigma-Aldrich). No less than 100 metaphases were analyzed in each experiment. Fisher's exact test and χ-square criterion (with Yates correction) were used for the statistical processing of the results using Microsoft Excel and GraphPad Prism 7 software (United States).

RESULTS

Localization Patterns of FMR1 and GPR50 Signals

The FRAXA site, folate-sensitive fragile site, can be induced by means of FdU. This is a replication inhibitor, its effect decreases the metaphase index and complicates the chromosome analysis. The optimal concentration of this substance for the FRAXA induction is 0.1 μM at the time of exposure 16–18 h [24, 30]. Under these conditions, it is possible to stimulate the expression of this site, to preserve the chromosome structure, and to achieve the metaphase index sufficient for the studies. After the cell's incubation in the presence of FdU under the conditions described above, the metaphase spreads were prepared and analyzed by means of the bicolor FISH method with probes specific to *FMR1* and *GPR50* (see Experimental section). The cells that were incubated during the same time without FdU were used as a control. In total, we studied more than 1000 metaphase plates in four cell lines. All patterns of signal localization (normal and typical for the fragile X chromosome) can be divided into five types (Fig. 1, see colored insert).

The localization patterns of types I–III refer to normal X chromosomes that have no fragile sites FRAXA. The type-I pattern represents signals from two used probes on two X chromosome chromatids, and no gaps or constrictions are observed during the DAPI coloration. The localization pattern of type II is associated with the presence of signals on both chromatids from one of the probes and with the absence of signals from the second probe on one of the chromatids, as well as with the absence of gaps or constrictions during the DAPI coloration. The type-III pattern represents signals on two chromatids from only one probe, no signals from the second probe and no visible changes during the DAPI coloration were observed. The absence of one or two signals in type-II and -III patterns can be associated with hybridization difficulties in certain DNA regions and, thus, the use of two probes helps researchers to study these chromosomes and save time in their work.

The localization type-IV and -V patterns refer to X chromosomes that express the fragile site FRAXA. In the type-IV pattern, the signal from two probes is absent on one of the chromatids. In this case, the chromosome can look normal during DAPI coloration or one of the chromatids can be shorter. The gaps and constrictions during DAPI coloration are visible in the type-V pattern of localization. All of these anomalies can be seen on one or two chromatids and have signals from one or two used probes.

In addition, we paid attention to some differences in signals from two sister chromatids of one X chromosome (Fig. 2). One probe (*FMR1* or *GPR50*) can give double signals on one or two chromatids (Fig. 2a) or signals of different size on different chromatids (Fig. 2b), but such variants of signals are observed in all studied cell lines and do not depend on the effect of FdU. We found no statistically significant differences in the distribution of such variants of signals in different cell lines or under different conditions of the cell incubation, and they were not taken into account in further work. We assume that this is a peculiarity of normal hybridization.

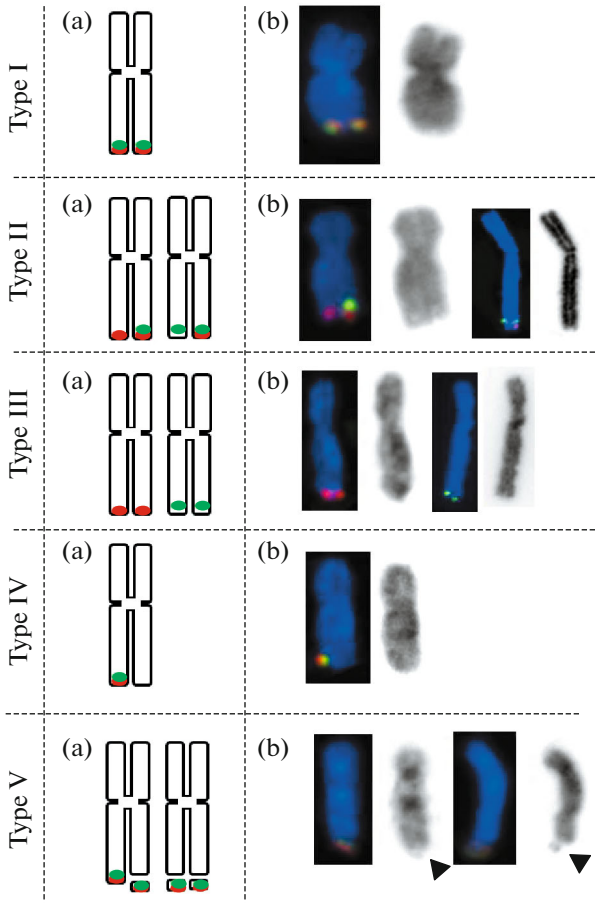

Fig. 1. Types of localization patterns of *FMR1* and *GPR50* probes on normal X chromosomes and X chromosomes of the cells of patients with FXS. (a) Schemes and (b) FISH photos are presented as examples for each type of location. FISH chromosomes are colored with DAPI on photos, the *FMR1* gene is labeled in green, the *GPR50* gene in red. DAPI coloration (black-and-white image) is presented in each FISH example. Arrows indicate visible constrictions or gaps on the chromosomes colored with DAPI.

Analysis of the Distribution of FISH Signal Localization Patterns from FMR1 and GPR50 Probes in Different Cell Lines

Two cell lines obtained from patients with FXS and two control cell lines were studied to determine the frequency of different signal localization patterns from two probes (without the effect of FdU and after the addition of FdU) (see experimental section). All results on the studied cell lines are presented in the Table 1. The highest frequency in the cells not exposed

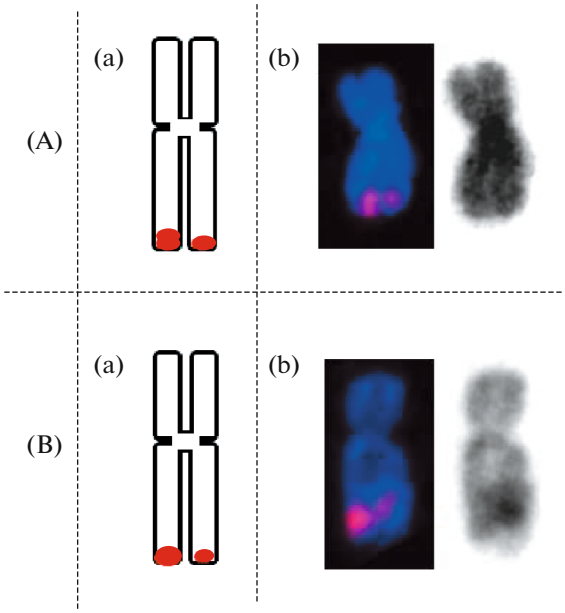

Fig. 2. Peculiarities of FISH that are not a result of FRAXA expression: (A) double signal from *FMR1* gene on one chromatid and normal on the second chromatid; (B) different signal size from *FMR1* gene on two chromatids. (a) Scheme and (b) FISH photo as an example are presented for each FISH result. In examples, FISH chromosomes are colored with DAPI, the *FMR1* gene is labeled in red. DAPI coloration (black-and-white image) is presented in each example.

to the effect of FdU is typical for the pattern with type-I localization (in both the control cell lines and cell lines from patients with FXS). The frequency varies from 0.83 to 0.89 in all studied cell lines. The portion of other types of patterns of normal signal (II and III) location is significantly smaller and varies from 0.03 to 0.09. The localization that corresponds to the pattern IV occurs sporadically and is observed in both the control cells and the cell lines of patients with the syndrome with a frequency less than 0.02. No localization pattern of type V was found in the cell lines not exposed to the effect of FdU. No significant differences between the norm and pathology are observed both during the pairwise comparison and when combining indices by two normal cell lines and two cell lines from patients with FXS of χ-square criterion (with Yates correction) and Fisher's exact test without the effect of FdU.

However, significant differences between the control cell lines and the cell lines obtained from patients appear after the cells were treated with FdU. This substance has no visible effect on both control cell lines (χ-square criterion, Fisher's exact test). It was the most important in the control lines that there are no changes in them in the amount of cells with localization patterns of types IV and V. All observed changes in the amount of metaphases with localization patterns I–III are statistically insignificant. This means that FdU does not have any effect on the amount of FRAXA sites in the control cell lines. The opposite situation is observed in the cell lines from patients with the syndrome. The amount of localization patterns of types IV and V (corresponding to fragile site FRAXA) is increased after the effect of FdU, and these changes have a high statistical significance ($P < 0.001$ for two cell lines from patients with FXS, Fisher's exact test).

These data indicate that the FISH method using two probes after the effect of FdU is a good and promising method of the fragile site FRAXA visualization in the cell lines. Statistically significant increase in the portion of patterns of type IV and V signal localization after the treatment with FdU is a criterion that the cell is the FRAXA carrier.

DISCUSSION

The development of FXS diagnostics is one of the urgent problems of medical genetics. It is possible to obtain the most accurate results using PCR. However, the high GC content of the sequence responsible for

Table 1. Distribution of localization patterns of BAC carrying *FMR1* and *GPR50* genes in control cell lines and cell lines obtained from patients with FXS

GM06895			GM06865			GM03200			GM04025		
pattern type	without effect ($N = 150$)	FdU ($N = 102$)	pattern type	without effect ($N = 100$)	FdU ($N = 103$)	pattern type	without effect ($N = 204$)	FdU ($N = 131$)	pattern type	without effect ($N = 108$)	FdU ($N = 104$)
Norm			Norm			Norm			Norm		
I	0.84	0.85	I	0.88	0.83	I	0.89	0.73	I	0.87	0.77
II	0.09	0.10	II	0.06	0.11	II	0.05	0.03	II	0.07	0.05
III	0.06	0.03	III	0.06	0.07	III	0.03	0.06	III	0.04	0.01
FRAXA			FRAXA			FRAXA			FRAXA		
IV	0.01	0.02	IV	0.00	0.00	IV	0.01	0.12	IV	0.02	0.06
V	0.00	0.00	V	0.00	0.00	V	0.00	0.06	V	0.00	0.12

the syndrome development makes its amplification a nontrivial task. In connection with this, alternative diagnostic methods, such as the determination of the methylation status of the *FMR1* gene promoter and the determination the expression level, have been developed and used.

We developed a method of FRAXA detection using two probes and tested it on two control cell lines obtained from healthy individuals with normal repeat sizes and on two cell lines obtained from patients with FXS and expansion of repeats. Five types of signal-localization patterns were detected, three of which are typical for a normal X chromosome (the FRAXA site is absent), while two of them for the X chromosome in patients (the presence of the FRAXA site). The analysis of FISH results demonstrated that there is a statistically significant identity in the distribution of signal patterns in all cell lines in the absence of the effect of FdU (regardless the genotype). The addition of FdU in the culture medium during the incubation only results in an increase in the amount of cell carrying the X chromosome with the fragile site FRAXA (the localization patterns of types IV and V) in lines from patients with the syndrome. We found no differences in the character of distribution of the signal localization patterns after the effect of FdU or in its absence in the control cell lines. Changes in the cell lines from patients and their differences from the control lines after the effect of FdU are statistically significant.

The use of two probes allows one to avoid false-positive results. For example, in the case when we use only one probe (at a type-II localization pattern), the signal loss on one of the chromatids can be regarded as the presence of FRAXA; the use of two probes allows one to avoid this error. The use of two probes in the case of a type-III localization pattern allows one to study metaphases that should be excluded from analysis when using only one probe, since in the latter case, the picture would be similar to unsuccessful hybridization.

The type-IV and -V localization patterns, which are typical of the fragile site FRAXA, in two cell lines obtained from patients with FXS are present at approximately equal rates. The type-V pattern can be also detected without hybridization, since it affects the chromosome morphology. In this case, the use of two probes specific for the X chromosome allows one to reduce the time required to search for and analyze this chromosome on the metaphase plate.

The localization patterns of the *FMR1* and *GPR50* probe signals depend on the morphology of fragile site FRAXA and FISH specifics on individual chromatids. The use of two probes allows one to analyze a larger amount of metaphases (compared to the use of one probe) and, thus, to avoid the incorrect genotyping of the cell lines. Some problems associated with FISH on individual chromatids (that emerge when using one probe) usually do not affect the second probe. This method allows one to reduce the time of the analysis, which is very important, since it is necessary to analyze at least 100 metaphases for the genotype determination (and obviously two probes allow one to accelerate the process).

Taking into account frequently encountered difficulties during the FXS diagnostics, it is necessary to have several alternative instruments that would allow one to accurately diagnose the full mutation in the *FMR1* gene in patients with mental retardation and autism. The high statistical significance of differences in the character of the distribution of the localization patterns of fragile site FRAXA markers between the control cell lines and cell lines from patients with the indicated syndrome after the effect of FdU makes the two-probe method of the fragile site FRAXA detection a promising diagnostic instrument.

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