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Unraveling unusual X-chromosome patterns during fragile-X syndrome genetic testing

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ABSTRACT

Background: Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID). Together with fragile X-associated tremor and ataxia (FXTAS) and fragile X-associated premature ovarian failure (POF)/primary ovarian insufficiency (POI), FXS depends on dysfunctional expression of the *FMR1* gene on Xq27.3. In most cases, FXS is caused by a > 200 CGG repeats in *FMR1* 5′-untranslated region (UTR) and by promoter hypermethylation that results in gene silencing. Males and females with unmethylated premutated alleles (repeats between 55 and 200) are at risk for FXTAS and POF/POI.

Methods: FXS molecular testing relied on PCR and methylation-specific Southern blot analysis of the *FMR1* 5′UTR. Atypical Southern blot patterns were studied by X-chromosome microsatellite analysis, copy number dosage at *DMD locus*, amelogenin gender-marker analysis and array-comparative genomic hybridization (array-CGH).

Results: Six men affected by ID and three women affected by ID and POF/POI underwent FXS molecular testing. They had normal *FMR1* CGG repeats, but atypical X chromosome patterns. Further investigations revealed that the six males had Klinefelter syndrome (XXY), one female was a Turner mosaic (X0/XX) and two women had novel rearrangements involving X chromosome.

Conclusions: Diagnostic investigation of atypical patterns at *FMR1 locus* can address patients and/or their relatives to further verify the condition by performing karyotyping and/or array-CGH.

1. Introduction

Fragile X syndrome (FXS, OMIM #300624) is an X-linked disorder with a prevalence of about 1 in 3600 to 4000 in males and 1 in 4000 to 6000 in females. FXS is the main cause of inherited intellectual disability (ID), associated with attention deficit, hyperactivity, autistic-like behavior, macroorchidism, and facial dysmorphisms. In 99% of cases, FXS is caused by a full mutation (FM), i.e., an unstable expansion of the CGG repeats (> 200 triplets) in the 5′-untranslated region (5′-UTR) of the fragile X mental retardation 1 gene (*FMR1*, OMIM *309550) and DNA promoter hypermethylation, resulting in gene silencing and absence of the *FMR1* protein product, FMRP. The severity of clinical symptoms in FM heterozygous females may depend on skewed X chromosome inactivation or lyonization, i.e. when > 80% of cells show preferred inactivation of the normal or of the mutant X chromosome; in fact, the degree of methylation of the normal *FMR1* allele correlates with the FMRP levels and the FXS phenotype [1]. In the general population, the CGG repeat region within the 5′-UTR of the *FMR1* gene is highly polymorphic. The normal size of triplet repeats ranges from 5 to 44, and these alleles are stably transmitted to the offspring. The alleles with 45–54 CGG repeats are defined intermediate alleles or grey zone alleles and are rather stable when passed to the progeny. About 17% of the intermediate alleles may expand to the premutation (PM) range (between 55 and 200 unmethylated repeats) when transmitted by the mother. PM alleles are highly unstable when transmitted to offspring, and can give rise to FM, especially in female meioses. Normal and PM alleles are unmethylated in males and subject to lyonization in females. Individuals with a PM allele do not have any FXS symptoms, but are at

Abbreviations: FXS, Fragile X syndrome; FM, full mutation; FXTAS, fragile X-associated tremor and ataxia; ID, intellectual disability; KS, Klinefelter syndrome; PM, premutation; POF/POI, premature ovarian failure/primary ovarian insufficiency; TS, Turner syndrome; UTR, 5′-untranslated region

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risk for fragile X associated tremor and ataxia (FXTAS) (OMIM #300623) [2]. Moreover, approximately 16–25% of females with a PM allele are affected by premature ovarian failure (POF)/primary ovarian insufficiency (POI) (OMIM #311360) [3].

Molecular testing for FXS and *FMR1*-related disorders is based on the identification of the whole range of CGG expansion and the methylation status of the *FMR1* 5′-UTR, traditionally based on PCR and Southern blot analyses. PCR discriminates between normal and PM alleles up to 100–110 CGG repeats in females and to 160 CGG repeats in males [4]. High-performance PCR procedures, as triplet-primed PCR (TP-PCR), allow the identification of PM and FM alleles, but not detect the methylation status. Southern blot carried out on genomic DNA digested with a methylation-sensitive enzyme easily detects PM alleles > 100 repeats, analyzes the methylation status of FM alleles, identifies expansion and/or methylation mosaicisms and reveals skewed X chromosome inactivation in women with PM or FM [4]. More recently, a novel PCR-based method has been developed that detects PM and FM alleles, and determines their methylation status, also when they are under-represented, as it occurs in mosaic individuals [5].

Despite FXS is a rare disease and ID is frequently associated with chromosomal imbalances that often involve the X chromosome [6], most clinicians primarily request molecular diagnosis of FXS for patients affected by any type of ID or developmental delay. Notably, X chromosome deletions and rearrangements give rise to atypical patterns during molecular analysis of the *FMR1 locus*. For this reason, we are not surprised to occasionally find atypical results following FXS molecular diagnosis.

Here we describe three women affected by intellectual/psychomotor delay and primary or secondary amenorrhea, and six men affected by mental retardation or psychomotor delay that underwent FXS molecular diagnosis and resulted to have normal CGG triplets in the *FMR1* gene but showed atypical X chromosome patterns. Further diagnostic investigations and, in two females, array-comparative genomic hybridization (array-CGH) revealed the possible genetic cause of their conditions.

2. Materials and methods

2.1. Ethics approval

Informed consent was obtained for each patient according to the procedure established by the local Bioethics Institutional Committee.

2.2. Patients

Patients were referred to our center for one or more of the following signs: mental retardation, speech delay, psychomotor delay and/or amenorrhea.

2.3. Methods

DNA was extracted from 10 mL of whole blood samples from each subject using the Nucleon BACC2 kit (Amersham, Little Chalfont, Bucks, UK). The CGG repeat number of *FMR1* alleles up to 160 CGG repeats was determined by capillary gel electrophoresis of fluorescent-labeled DNA amplicons using primers F (labeled with NED fluorochrome) and C as described elsewhere [4]. The TP-PCR and the Southern blot analysis of genomic DNA digested with *Hin*dIII/*Eag*I restriction enzymes were performed as described previously [4].

The X-inactivation analysis was carried out with the PCR-based method for methylation-dependent amplification of the polymorphic triplet repeats at the androgen receptor (*HUMARA*) gene, as previously described [7]. The HUMARA-1 primer was labeled with the fluorochrome FAM, and the amplification products of *Hpa*II-digested and not digested DNA were separated by capillary gel electrophoresis on the ABI Prism 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The Genotyper 4.0 (Applied Biosystem) software was used for data analysis.

Amplification of the amelogenin gender-determining marker to identify the X and Y amelogenin gene (*AMELX* and *AMELY*) sequences was carried out using primers F, labeled with the fluorochrome FAM, and R (Applied Biosystem) in 25 µl of buffer 2 (Applied Biosystem Roche), containing 200 ng of genomic DNA, 0.2 mM dNTP, 0.2 µM primers (primer F: 5′-ACCTCATCCTGGCACCCTGG-3′; primer R: 5′-AGGCTTGAGGCCAACCATCAG-3′), 1.5 mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystem Roche), using the following PCR thermal profile: 95 °C for 11 min; 96 °C for 1 min; 28 cycles of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, 72 °C for 1 min; 72 °C for 7 min. Amplification products were separated by capillary gel electrophoresis on the ABI Prism 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA).

Copy number of the dystrophin gene (*DMD*) was evaluated by multiplex ligation probe amplification (MLPA) and haplotype analysis was carried out using 15 intragenic STRs, as described [7].

Molecular karyotyping by array-CGH was performed with the Agilent Human Genome Microarray Kit 4x180K (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Genomic positions were defined using the human genome reference NCBI37/hg19. The microarray platform is composed of 180,000 60-mer oligonucleotide probes with an overall median probe spacing of 13 Kb (11 Kb in Refseq genes). Hybridized slides were scanned with a microarray scanner (Agilent Technologies, Santa Clara, CA, USA), and analyzed with Feature Extraction 10.1 and Workbench 6.5.0.1 (Agilent Technologies). Copy number variations (CNVs) were considered if at least three contiguous oligonucleotides had an abnormal log ratio.

3. Results

3.1. Case 1. Mosaic Turner syndrome (X0/XX)

Patient #3062 is a 13-year-old girl affected by mild psychomotor delay. Standard PCR revealed two normal alleles with 20 and 27 CGG repeats, respectively; however, intensity of the electrophoretic signal corresponding to the allele with 27 CGG was abnormally under-represented compared to that with 20 CGG (Fig. 1A and Table 1). TP-PCR did not detect amplification products extending beyond 55 CGG repeats, which is the cut-off to reveal expanded *FMR1* alleles (not shown). Accordingly, Southern blot did not reveal any expansion, but showed that the methylated 5.2 Kb band was much fainter than the 2.8 Kb non-methylated band (Fig. 1B and Table 1). In particular, the signal of the unmethylated fragment was more intense (> 90%) than the methylated band. We hypothesized that this atypical pattern could be attributed to the presence of X0/XX mosaicism in the patient's leukocytes. To verify this hypothesis, we analyzed the *DMD* gene, on chromosome Xp21, by the routine methods used in our laboratory for the molecular diagnosis of Duchenne/Becker muscular dystrophies [7]. The MLPA analysis detected only one copy of the *DMD* gene and of the Xp22, Xq12, Xq13, Xq28 probed regions (Table 1). Moreover, analysis of 15 *DMD*-specific intragenic STRs detected only one allele [8]. Taken together these data supported our hypothesis of X0/XX mosaicism with a high prevalence of the X0 karyotype (> 90%).

3.2. Case 2. Chromosomal rearrangement 46,X,der(X)t(X);10(q21.3;q21.3)

Patient #2985 is a 20-year-old women affected by ID and psychomotor delay. She also has hypergonadotropic hypogonadism and primary amenorrhea. PCR analysis revealed one allele with 27 CGG repeats. TP-PCR did not detect amplification products beyond the normal range. Southern blot confirmed absence of expanded alleles but revealed an atypical pattern with only one 2.8 Kb non-methylated normal band (Fig. 2A and Table 1), as occurs in a normal male. Analysis of the

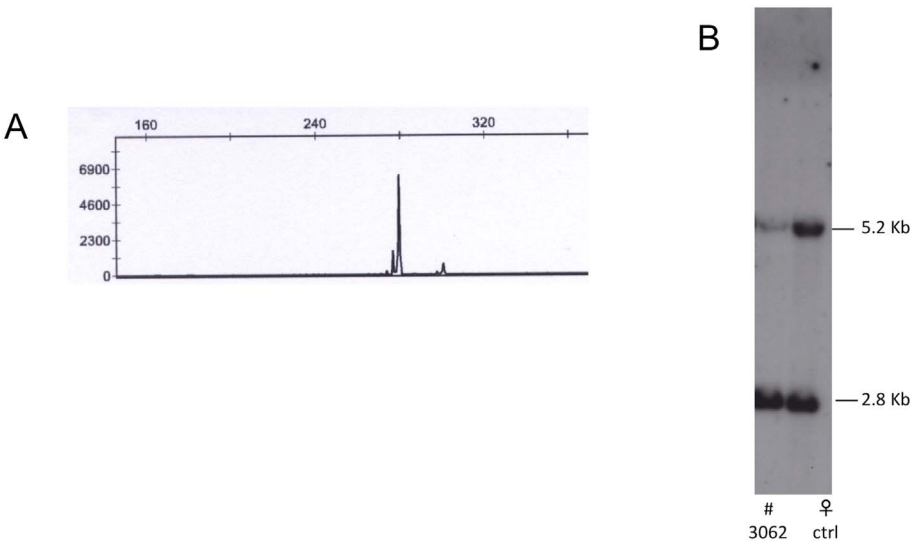

Fig. 1. *FMR1* PCR (A) and Southern blot (B) analyses of female patient #3062. ♀Ctrl: female normal control.

AMEL locus showed only the fragment corresponding to *AMELX* (Table 1), as occurs in females. Molecular analyses of other X-chromosome *loci* detected two copies of the *DMD* gene and heterozygous deletion of genomic sequences within the Xq28 region (Table 1). Array-CGH revealed a 64.7 Mb deletion extending from chrX:90530805 to 155207239 (Fig. 2C) that removed the whole X-chromosome distal region (starting from Xq21.3 to Xqter), which also contains the *FMR1* gene. Concurrently, a 63.5 Mb duplication of the region from 72,032,349 to135434178 (from 10q21.3 to 10qter) of chromosome 10 (Fig. 2B) was identified. These findings indicate that the patient has a chromosomal rearrangement that likely arises in a parent as consequence of a balanced translocation between the distal regions of chromosome 10 and chromosome X [46,X,der(X)t(X);10(q21.3;q21.3)]. In this patient, the undamaged X chromosome is fully active, i.e. unmethylated (Fig. 2A). Analysis of *Hpa*II methylation-sensitive digestion of the *HUMARA* gene confirmed the severely skewed X inactivation in this patient (data not shown).

3.3. Case 3. Chromosomal rearrangement (46,XX); Xdup(Xp22.33-21.3); X,del(X q21.1-ter)

Patient #3223 is a 12-year-old girl affected by ID and secondary hypergonadotropic amenorrhea. PCR analysis detected only one type of allele with 17 CGG, TP-PCR and Southern blot did not identify any expanded allele, but revealed only the presence of the normal non-methylated band, an atypical pattern for a female subject (Fig. 3A and Table 1). Array-CGH revealed a 27.43 Mb heterozygous duplication extending from chrX:594356 to 28024428 (Xp22.33-p21.3), and a 73.47 Mb heterozygous deletion that removed the chrX:81734974–155207239 region, i.e. the X-chromosome distal region that spans from Xq21.1 to Xqter and that also contains the *FMR1* gene (Fig. 3B). In this girl, *Hpa*II methylation-sensitive digestion of the *HUMARA* gene confirmed severely skewed inactivation of the deleted X chromosome (data not shown).

Table 1
Characteristics and experimental results of examined patients.

Patient ID	Sex	PCR CGG repeats	TP-PCR	Southern blot	*DMD* gene copy number	Xp22, Xq12, Xq13, Xq28 region probes (copy number)	Amelogenin	Array-CGH
#3062	♀	two normal alleles: 20 CGG; 27 CGG (under-represented)	No expanded alleles	2.8 Kb 5.2 Kb (very faint)	1	Xp22 (1) Xq12 (1) Xq13 (1) Xq28 (1)	ND[a]	ND[a]
#2985	♀	1 normal allele: 27 CGG	no expanded alleles	2.8 Kb	2	Xp22 (2) Xq12 (2) Xq13 (2) Xq28 (1)	*AMELX*	chrX: deletion (90530805- 155207239; Xq21.3 to qter); chr 10: duplication (72032349-135434178; 10q21.3)
#3223	♀	1 normal allele: 17 CGG	no expanded alleles	2.8 Kb	ND[a]	ND[a]	ND[a]	chrX: duplication (594356-28024428; Xp22.33 - p21.3); chrX: deletion (81734974-155207239; Xq21.1 to qter)
#1090	♂	1 normal allele: 28 CGG	ND	2.8 Kb 5.2 Kb	2	ND[a]	*AMELX/AMELY*	ND[a]
#785	♂	2 normal alleles: 27 and 45 CGG	ND	2.8 Kb 5.2 Kb	ND[a]	ND[a]	*AMELX/AMELY*	ND[a]
#1184	♂	2 normal alleles: 16 and 26 CGG	ND	2.8 Kb 5.2 Kb	ND[a]	ND[a]	*AMELX/AMELY*	ND[a]
#141/98	♂	2 normal alleles: and 28 CGG	ND	2.8 Kb 5.2 Kb	ND[a]	ND[a]	*AMELX/AMELY*	ND[a]
#1856	♂	1 normal allele: CGG	ND	2.8 Kb 5.2 Kb	ND[a]	ND[a]	*AMELX/AMELY*	ND[a]
#1858	♂	1 normal allele: 27 CGG	ND	2.8 Kb 5.2 Kb	ND[a]	ND[a]	*AMELX/AMELY*	ND[a]

[a] ND: not determined.

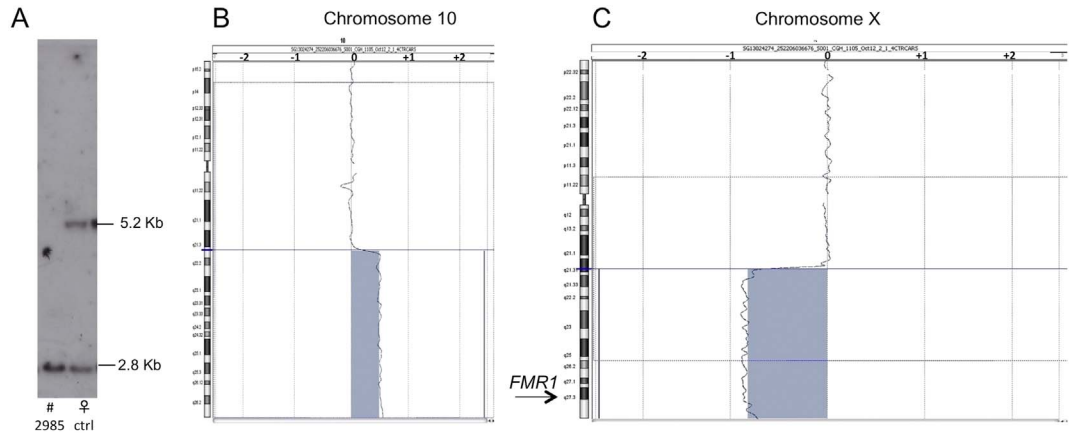

Fig. 2. *FMR1* Southern blot pattern (A) and array-CGH profile of chromosomes 10 (B) and X (C) of female patient #2985. ♀Ctrl: female normal control. Arrow indicates the approximate position of the *FMR1* gene.

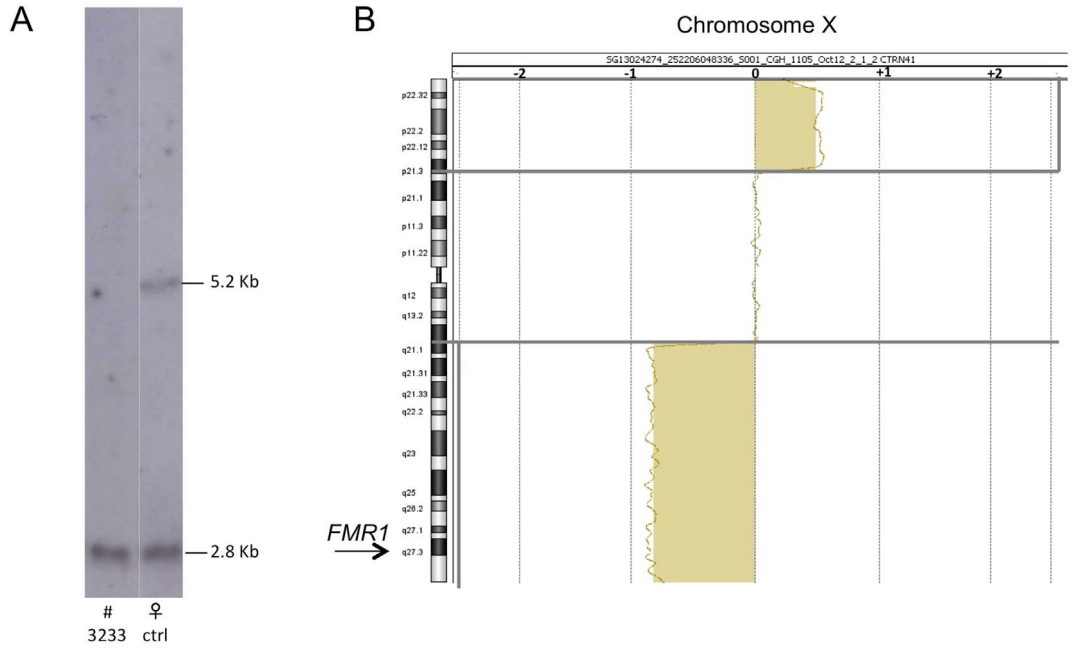

Fig. 3. *FMR1* Southern blot pattern (A) and array-CGH profile of chromosome X (B) of female patient #3223. ♀Ctrl: female normal control. Arrow indicates the approximate position of the *FMR1* gene.

3.4. Cases 4–9. Klinefelter syndrome (XXY)

Six boys (#1090, #785, #1184, #141/98, #1856, #1858) affected by ID or psychomotor delay, had a Southern blot typical of a normal female with two bands, a 5.2 Kb methylated and a 2.8 Kb non-methylated fragment; the PCR analysis detected 1 or 2 normal alleles (not shown). Analysis of *AMEL* revealed the presence of both *AMELX* and *AMELY* (Table 1) and the analysis of 15 *DMD*-specific intragenic STRs [8] detected two alleles for the majority of the analyzed microsatellites.

4. Discussion

Due to the X-linked inheritance and to the specific related mutational mechanism, the molecular diagnosis of FXS with methylation-specific Southern blot analysis revealed, in a small subset of our patients, unexpected patterns that were however compatible with X chromosome abnormalities. Despite sex chromosome abnormalities are a quite frequent cause of ID with an overall incidence of about 1 in 500 births, only 0.25% of > 3500 analyses of the *FMR1 locus* we have performed from 1995 to date [4, and unpublished data] gave such atypical patterns. In any case, such unusual results need further studies to address the patient toward her/his most likely genotype. To solve our cases, it was crucial the availability in our specialized center of various molecular tools, such as those for the analysis of the *DMD locus* on X chromosome, and array-CGH.

In fact, all the six males that had a female-like pattern after the *FMR1 locus* analysis appear as such also after microsatellite analysis of the *DMD locus*. Typing of the length variation in the X-Y homologous amelogenin gene (*AMELX* and *AMELY*) identified *AMELY* and *AMELX* that ultimately indicated the patients were likely affected by Klinefelter syndrome (KS). KS is the most common sex chromosome abnormality; it occurs in 500–1000 newborn males (Genetics Home Reference, http://ghr.nlm.nih.gov/) and in 1:300 spontaneous abortions. Patients affected by KS usually have learning difficulties in childhood, especially related to language and short-term memory, and these signs often lead to poor school grades and subsequent low self-esteem if extra help is not given in early childhood. Consistently, our KS children (#1090, #785, #1184, #141/98, #1856, #1858) were affected by ID or psychomotor delay, thus supporting the demand of FXS molecular diagnosis.

A similar strategy allowed us to reveal a X0/XX mosaicism in a

young female. Notably, PCR-based methodologies we used to analyze the *DMD* gene identified only one normal allele (X0), whereas *FMR1* analysis (standard PCR and Southern blot) were able to reveal the < 10% of cells that carried the normal XX genotype. Therefore, only the complexity of the obtained results allowed us to fully solve the case, by revealing the X0/XX mosaicism with a high prevalence of the X0 karyotype (> 90%). The X0 aneuploidy causes Turner syndrome (TS), which affects approximately 1:2500 live-born girls [9]. The clinical phenotype of TS is characterized by short stature, gonadal dysgenesis with delayed pubertal development, primary amenorrhea and sterility, a variety of dysmorphic features [10], malformations and impairment of various organs [11,12]. The TS phenotype is attributed to haploinsufficiency of genes normally expressed in both the active and inactive X-chromosomes; however, other factors may influence phenotypic expression [13,14]. Although TS patients usually have normal intelligence, moderate to severe ID has been described in some patients [15,16]. In particular, selective impairments in visuospatial and memory areas and significant underachievement in arithmetic, particularly numerical ability, mental calculation, geometry, and reasoning have been described [15]. In this context, it is consistent that our TS patient (#3062), affected by mild psychomotor delay, was referred for FXS molecular diagnosis.

The array-CGH is a powerful tool to solve *FMR1* abnormal patterns because it reveals chromosomal rearrangements with high resolution and accuracy. In fact, a wide number of genomic alterations, including sex chromosome abnormalities, associated to several disorders can be identified with this methodology [17,18]. Accordingly, array-CGH precisely defined the complex rearrangements of the young female cases 2 and 3, both with ID and amenorrhea. However, the genotype-phenotype relationship in these cases is very difficult. In fact, in both cases array-CGH found a huge deletion that removed part of X chromosome long arm (starting from Xq21 to Xqter). Moreover, in case 2, about 64 Mb of the long arm of X is likely replaced with about 64 Mb of the long arm of chromosome 10; in case 3, in addition to the 74 Mb deletion spanning from Xq21 to Xqter, array-CGH revealed a 27 Mb duplication of the Xp22.33-p21.3 region. To our knowledge these precise rearrangement configurations have not been reported before. Moreover, as the *FMR1* and the *HUMARA loci* analyses showed skewed X chromosome inactivation with fully active normal X chromosomes, we deduced that in the two females the rearranged aberrant X chromosomes were completely inactive. The deletions of cases 2 and 3 removed hundreds of distal genes (#241 RefSeq, case 2; #466 RefSeq, case 3) including *FMR1* and various X-linked ID genes such as *IDS*, *MECP2* and *IKBKG/NEMO* [19,20]. Deletions or loss-of-function variants in the *IKBKG/NEMO* gene, for example, have been associated with X inactivation skewing because of the negative selection of cells expressing the mutant X chromosome [21–23]. It has also been reported that, in patients with unbalanced translocations between X and an autosome, the t(X;A) chromosome is generally inactivated [24–26], as in our case 2. Notably, approximately 15–25% of X genes totally or partially escape inactivation [27]. These genes are clustered and map on the distal region Xq13.2 and on the proximal gene-rich region Xq28 [27]. Several cases of POF/POI associated with deletion of Xq regions have been reported [28–31] albeit with different onset ages and different degrees of severity. The haploinsufficiency of X-linked genes, especially in the Xq28 region, has been advanced as a possible cause [31]. The deleted regions of our cases 2 and 3 (patients #2985 and #3223, respectively) contain genes that can escape X inactivation; the expression level of these genes may reach 50% in inactivated X [27]. Therefore, because our cases 2 and 3 have a complete skewed X chromosome inactivation with the normal X chromosomes fully active, the patients' phenotype might depend on the haploinsufficiency of one or more of such genes. Moreover, female patients with primary or secondary amenorrhea and with both partial Xp duplication and partial Xq deletion, as our case 3, have been occasionally reported [32–36]. Finally, the patients' phenotype might be due to a pathogenic variant in at least one of the Xq monosomic genes on the active structurally normal X chromosome. Certainly, high-throughput sequencing of distal X chromosome genes, analysis of the deletion breakpoints and X-inactivation studies of different tissues could help to understand the mechanism underlying the expression of X-linked diseases in these female patients.

Recently, PCR-based diagnostic kits that detect PM and FM alleles and determine their methylation status are replacing the in-house Southern blot analysis [5]. Using these kits, a methylation-sensitive PCR (mPCR) analysis of the *FMR1* 5′-UTR is able to reveal, in female patients, particular features of PM alleles or potential X-chromosome abnormalities that need further testing, especially in cases of infertility [5]. However, on the basis of a cost-benefit evaluation, many laboratories carry out mPCR analysis in females only if they are referred with POF/POI and standard PCR reveals only one normal allele. Ultimately, the results herein presented further stress the importance to evaluate the methylation status of females patients with only one normal allele (cases 1, 2 and 3) and referred with ID (case 1, 2, and 3) and/or primary or secondary amenorrhea (cases 2 and 3).

We emphasize that, for all the presented patients, our diagnostic report concluded for the absence of pathological expansion in the *FMR1* gene and the presence of analytical findings that were suggestive of X chromosome abnormality. We therefore advised doctors, patients and/or their relatives to further verify the condition by performing karyotyping and/or array-CGH.

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Conflict of interest declaration

None declared.

References

[1] R. Martínez, V. Bonilla-Henao, A. Jiménez, M. Lucas, C. Vega, I. Ramos, F. Sobrino, E. Pintado, Skewed X inactivation of the normal allele in fully mutated female carriers determines the levels of FMRP in blood and the fragile X phenotype, Mol. Diagn. 9 (2005) 157–162.

[2] S. Jacquemont, R.J. Hagerman, M.A. Leehey, D.A. Hall, R.A. Levine, J.A. Brunberg, L. Zhang, T. Jardini, L.W. Gane, S.W. Harris, K. Herman, J. Grigsby, C.M. Greco, E. Berry-Kravis, F. Tassone, P.J. Hagerman, Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population, JAMA 291 (2004) 460–469.

[3] A.K. Sullivan, M. Marcus, M.P. Epstein, E.G. Allen, A.E. Anido, J.J. Paquin, M. Yadav-Shah, S.L. Sherman, Association of FMR1 repeat size with ovarian dysfunction, Hum. Reprod. 20 (2005) 402–412.

[4] G. Esposito, R. Ruggiero, G. Savarese, M. Savarese, M.R. Tremolaterra, F. Salvatore, A. Carsana, A 15-year case-mix experience for fragile X syndrome molecular diagnosis and comparison between conventional and alternative techniques leading to a novel diagnostic procedure, Clin. Chim. Acta 417 (2013) 85–89.

[5] M. Grasso, E.M. Boon, S. Filipovic-Sadic, P.A. van Bunderen, E. Gennaro, R. Cao, G.J. Latham, A.G. Hadd, D.A. Coviello, A novel methylation PCR that offers standardized determination of FMR1 methylation and CGG repeat length without southern blot analysis, J. Mol. Diagn. 16 (2014) 23–31.

[6] M. Bartnik, B. Nowakowska, K. Derwińska, B. Wiśniowiecka-Kowalnik, M. Kędzior, J. Bernaciak, K. Ziemkiewicz, T. Gambin, M. Sykulski, N. Bezniakow, L. Korniszewski, A. Kutkowska-Kaźmierczak, J. Klapecki, K. Szczałuba, C.A. Shaw, T. Mazurczak, A. Gambin, E. Obersztyn, E. Bocian, P. Stankiewicz, Application of array comparative genomic hybridization in 256 patients with developmental delay or intellectual disability, J. Appl. Genet. 55 (2014) 125–144.

[7] A. Carsana, G. Frisso, M. Intrieri, M.R. Tremolaterra, G. Savarese, G. Scapagnini, G. Esposito, L. Santoro, F. Salvatore, A 15-year molecular analysis of DMD/BMD: genetic features in a large cohort, Front. Biosci. (Elite Ed) 2 (2010) 547–558.

[8] A. Carsana, G. Frisso, M.R. Tremolaterra, E. Ricci, D. De Rasmo, F. Salvatore, A larger spectrum of intragenic short tandem repeats improves linkage analysis and localization of intragenic recombination detection in the dystrophin gene: an analysis of 93 families from southern Italy, J. Mol. Diagn. 9 (2007) 64–69.

[9] V.P. Sybert, E. McCauley, Turner's syndrome, N. Engl. J. Med. 351 (2004) 1227–1238.

[10] B.E. Hjerrild, K.H. Mortensen, C.H. Gravholt, Turner syndrome and clinical

treatment, Br. Med. Bull. 86 (2008) 77–93.
[11] C. Bondy, D. Rosing, R. Reindollar, Cardiovascular risks of pregnancy in women with Turner syndrome, Fertil. Steril. 91 (2009) e31–32.
[12] M.L. Davenport, J. Roush, C. Liu, A.J. Zagar, E. Eugster, S. Travers, P.Y. Fechner, C.A. Quigley, Growth hormone treatment does not affect incidences of middle ear disease or hearing loss in infants and toddlers with Turner syndrome, Horm. Res. Paediatr. 74 (2010) 23–32.
[13] A. Araújo, E.S. Ramos, Cryptic mosaicism involving a second chromosome X in patients with Turner syndrome, Braz. J. Med. Biol. Res. 41 (2008) 368–372.
[14] R.M. Oliveira, I.T. Verreschi, M.V. Lipay, L.P. Eca, A.D. Guedes, B. Bianco, Y chromosome in Turner syndrome: review of the literature, Sao Paulo Med. J. 27 (2009) 373–378.
[15] J.F. Rovet, The psychoeducational characteristics of children with Turner syndrome, J. Learn. Disabil. 26 (1993) 333–341.
[16] A.V. Bispo, L.O. Dos Santos, P. Burégio-Frota, M.B. Galdino, A.R. Duarte, G.F. Leal, J. Araújo, B. Gomes, E.M. Soares-Ventura, M.T. Muniz, N. Santos, Effect of chromosome constitution variations on the expression of Turner phenotype, Genet. Mol. Res. 12 (2013) 4243–4250.
[17] M. Tarsitano, C. Ceglia, A. Novelli, A. Capalbo, B. Lombardo, L. Pastore, G. Fioretti, L. Vicari, M.A. Pisanti, P. Friso, M.L. Cavaliere, Microduplications in 22q11.2 and 8q22.1 associated with mild mental retardation and generalized overgrowth, Gene 536 (2014) 213–216.
[18] B. Lombardo, C. Ceglia, M. Tarsitano, I. Pierucci, F. Salvatore, L. Pastore, Identification of a deletion in the NDUFS4 gene using array-comparative genomic hybridization in a patient with suspected mitochondrial respiratory disease, Gene 535 (2014) 376–379.
[19] P. Chiurazzi, C.E. Schwartz, J. Gecz, G. Neri, XLMR genes: update 2007, Eur. J. Hum. Genet. 16 (2008) 422–434.
[20] H.A. Lubs, R.E. Stevenson, C.E. Schwartz, Fragile X and X-linked intellectual disability: four decades of discovery, Am. J. Hum. Genet. 90 (2012) 579–590.
[21] F. Fusco, T. Bardaro, G. Fimiani, V. Mercadante, M.G. Miano, G. Falco, A. Israël, G. Courtois, M. D'Urso, M.V. Ursini, Molecular analysis of the genetic defect in a large cohort of IP patients and identification of novel NEMO mutations interfering with NF-kappaB activation, Hum. Mol. Genet. 13 (2004) 1763–1773.
[22] N. Martinez-Pomar, I. Munoz-Saa, D. Heine-Suner, A. Martin, A. Smahi, N. Matamoros, A new mutation in exon 7 of NEMO gene: late skewed X-chromosome inactivation in an incontinentia pigmenti female patient with immunodeficiency, Hum. Genet. 118 (2005) 458–465.
[23] K.H. Ørstavik, M. Kristiansen, G.P. Knudsen, K. Storhaug, A. Vege, K. Eiklid, T.G. Abrahamsen, A. Smahi, J. Steen-Johnsen, Novel splicing mutation in the NEMO (IKK-gamma) gene with severe immunodeficiency and heterogeneity of X-chromosome inactivation, Am. J. Med. Genet. A 140 (2006) 31–39.
[24] C.M. Disteche, K. Swisshelm, S. Forbes, R.A. Pagon, X-inactivation patterns in lymphocytes and skin fibroblasts of three cases of X-autosome translocations with abnormal phenotypes, Hum. Genet. 66 (1984) 71–76.
[25] M. Paciolla Fusco, E. Chen, X. Li, R. Genesio, A. Conti, J. Jones, L. Poeta, M.B. Lioi, M.V. Ursini, M.G. Miano, Genetic and molecular analysis of a new unbalanced X;18 rearrangement: localization of the diminished ovarian reserve disease locus in the distal Xq POF1 region, Hum. Reprod. 26 (2011) 3186–3196.
[26] A.M. Cotton, C.Y. Chen, L.L. Lam, W.W. Wasserman, M.S. Kobor, C.J. Brown, Spread of X-chromosome inactivation into autosomal sequences: role for DNA elements, chromatin features and chromosomal domains, Hum. Mol. Genet. 23 (2014) 1211–1223.
[27] L. Carrel, H.F. Willard, X-inactivation profile reveals extensive variability in X-linked gene expression in females, Nature 434 (2005) 400–404.
[28] A. Beke, H. Piko, I. Haltrich, J. Csomor, A. Matolcsy, G. Fekete, J. Rigo, V. Karcagi, Molecular cytogenetic analysis of Xq critical regions in premature ovarian failure, Mol. Cytogenet. 6 (2013) 62.
[29] G. Ceylaner, S.O. Altinkaya, L. Mollamahmutoglu, S. Ceylaner, Genetic abnormalities in Turkish women with premature ovarian failure, Int. J. Gynaecol. Obstet. 110 (2010) 122–124.
[30] T. Eggermann, D. Meschede, H. Schüler, S. Palm, D. Gläser, B. Horsthemke, K. Eggermann, F. Haverkamp, K. Zerres, Premature ovarian failure associated with a small terminal Xq deletion: narrowing the POF1 region down to Xq27.2/Xq27.3-qter, Clin. Genet. 67 (2005) 434–437.
[31] S.I. Ferreira, E. Matoso, M. Pinto, J. Almeida, T. Liehr, J.B. Melo, I.M. Carreira, X-chromosome terminal deletion in a female with premature ovarian failure: Haploinsufficiency of X-linked genes as a possible explanation, Mol. Cytogenet. 3 (2010) 14.
[32] K.A. Adamson, I. Cross, J.A. Batch, G.A. Rappold, I.A. Glass, S.G. Ball, Trisomy of the short stature homeobox-containing gene (SHOX), resulting from a duplication-deletion of the X chromosome, Clin. Endocrinol. 56 (2002) 671–675.
[33] M.K. Kim, H.H. Seok, Y.S. Kim, M.U. Chin, S.R. Sung, W.S. Lee, S.H. Shim, T.K. Yoon, Molecular genetic and cytogenetic characterization of a partial Xp duplication and Xq deletion in a patient with premature ovarian failure, Gene 534 (2014) 54–59.
[34] K.A. Leppig, C.J. Brown, S.L. Bressler, K. Gustashaw, R.A. Pagon, H.F. Willard, C.M. Disteche, Mapping of the distal boundary of the X-inactivation center in a rearranged X chromosome from a female expressing XIST, Hum. Mol. Genet. 2 (1993) 883–887.
[35] T. Ogata, N. Matsuo, Y. Fukushima, M. Saito, O. Nose, N. Miharu, S. Uehara, B. Ishizuka, FISH analysis for apparently simple terminal deletions of the X chromosome: identification of hidden structural abnormalities, Am. J. Med. Genet. 104 (2001) 307–311.
[36] G. Tachdjian, A. Aboura, M.F. Portnoï, M. Pasquier, N. Bourcigaux, T. Simon, G. Rousseau, L. Finkel, M. Benkhalifa, S. Christin-Maitre, Cryptic Xp duplication including the SHOX gene in a woman with 46,X, del(X)(q21.31) and premature ovarian failure, Hum. Reprod. 23 (2008) 222–226.