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ORIGINAL ARTICLE

Most Martin–Bell syndrome (*FMR1*-related disorder) Venezuelan patients did not show CGG expansion but instead display genetic heterogeneity

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Martin–Bell syndrome is mainly caused by the expansion of CGG trinucleotide repeats (>200 CGG) in the first exon of the *FMR1* gene, leading to hypermethylation of the promoter region and silencing of the FMR1 protein expression. These changes are responsible for a phenotype with varying degrees of mental retardation, a long face with large and protruding ears, macroorchidism and autistic behavior. There may also be, however, patients who exhibit typical features of the syndrome without any expansion in the *FMR1* gene; thus, other mechanisms affecting the expression of the *FMR1* gene were assessed in 25 out of 29 ascertained patients with the typical phenotype without full mutation. Promoter methylation status of *FMR1*, mutations in its sequence and copy number variations (CNVs) in genes associated with intellectual disability were investigated. In 25 independent male patients without expansion, the promoter region was unmethylated; one patient with a full mutation showed methylation mosaicism; and a female patient had 81.2% of CpG sites methylated and 18.8% hemimethylated. One heterozygous duplication in exon 6 of the *PDCD6* gene (programmed cell death 6) and a heterozygous deletion in exon 5 of the *CHL1* gene (cell adhesion molecule L1), respectively, were found in two independent patients.
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INTRODUCTION

The Martin–Bell syndrome[1] (MBS; *FMR1*-related disorder, OMIM no. 300624, also referred to as fragile X syndrome, fragile X mental retardation, marker X syndrome)[2] has been considered the most common monogenic cause of intellectual disability (ID) among European Caucasoid populations. It occurs in about 1 in 4000 men and 1 in 6000 women.[3] The best known genetic etiology is the expansion of a trinucleotide CGG stretch located in the first exon of the *FMR1* gene;[4] deletions in the *FMR1* gene are the second most frequent cause, although they are rare.[5] The mechanisms of the trinucleotide expansion can be grouped into three types: premutation, in which between 52 and 200 CGG repeats are found; full mutation, with >200 CGG repeats; and mosaics, having both full mutation and premutation or partial methylation of the *FMR1* gene. The full mutation occurs with hypermethylation of the *FMR1* promoter region and inhibition of gene expression, leading to an absence of the FMR1 protein.

Sequence variants affecting the expression or function of FMR1 protein represent <1% of cases; only five-point mutations in the coding regions of the gene have been reported plus other three-point mutations in the promoter region.[6–10]

Another possible mechanism that may affect the expression of the *FMR1* gene is the change in the sequence from −850 to −650 bp upstream of the CGG trinucleotide stretch, known as methylation border. In patients with the full mutation this border region is completely methylated, which leads to destabilization of chromatin structure, possibly causing methylation expansion covering the promoter and producing the gene silencing. It has been proposed that changes in the methylation border sequence could be a mechanism affecting gene expression through methylation expansion.[11]

The lack of FMR1 protein produces varying degrees of mental retardation, autistic behaviors, macroorchidism, long face with large, wide and protruding ears, hyperextensible finger joints, single palmar ridge, flat feet, mitral valve prolapse, velvet-like skin, and so on, all typical of the MBS; however, the phenotype may depend on variable expressivity.[12]

Although it has been stated that the main cause of MBS is the expansion of the CGG trinucleotide beyond the normal range, there are patients who exhibit typical clinical features of the syndrome who do not have either full expansions or point mutations in the *FMR1* gene,[8] which have been proposed to be considered as carriers of a Martin–Bell *phenotype* instead.[13]

The study and diagnosis of patients over a period of 32 years in the Human Genetics Laboratory at the Venezuelan Institute for Scientific Research have shown largely that most patients did not carry the expansion at the *FMR1* locus, even though they had the main phenotypic features. This research focused on the study of possible alterations in the sequence and methylation status of the promoter

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region of the *FMR1* gene, as well as on the assessment of copy number variations (CNVs) using multiplex ligation-dependent probe amplification (MLPA), for autosomal subtelomeric and centromeric loci and for X-chromosome genes, associated with ID to search other mechanisms producing the MBS without the *FMR1* full mutation.

MATERIALS AND METHODS

Subjects

Twenty-nine patients with a clinical diagnosis of MBS (28 males and 1 female) were ascertained. The clinical diagnosis was established by the presence of the following typical clinical features: variable degree of mental retardation, long face with prominent jaw, large and protruding ears, macroorchidism, hyperextensible finger joints, delayed language development and a pattern of X-linked inheritance. Patients showing this phenotype were tested for the number of *FMR1* CGG repeats.

Twenty-four patients were diagnosed clinically in the Human Genetics Laboratory at Venezuelan Institute for Scientific Research; three were from the Concepción Palacios Maternity Hospital of Caracas; and two from the National Child Psychiatry Unit of Caracas. Twenty-one out of 29 individuals were independent (four patients came from the same family (Figure 1) and two pairs of brothers belonged to two different families). The score protocol described by Butler *et al.*,[14] with some modifications introduced at the Human Genetics Laboratory, was used to classify the phenotype.

A 5 ml blood sample was collected, EDTA anticoagulated and DNA was extracted by a saline method.[15] The study was approved by the Bioethical Committee of the Venezuelan Institute for Scientific Research, and the written voluntary informed consent was obtained from all family members, accordingly.

Study of the promoter region of the *FMR1* gene

To study the promoter region (−180 to +28), the following primers were designed with the Primer 3 software (http://ncbi.nlm.nih.gov/tools/primer-blast/): FMR1-PF (forward), 5′-CCCGCGCGTCTGTCTTTC-3′ and FMR1-PR (reverse), 5′-GTGAAACCGAAACGGAGCTG-3′. The methylation border (−650 to −850) was amplified using primers: FMR1-FM-F (forward), 5′-TGAGTTGAGGAAAGGCGAGTAC-3′ and FMR1-FM-R (reverse), 5′CACTCA GTGGCGTGGGAAACT-3′. Both PCR reactions used 62 °C as annealing temperature with between 200 and 500 ng of gDNA.

Bisulfite sequencing

The Epimark Kit of New England Biolabs Inc. (Essex, MA, USA) was used for the treatment of DNA with sodium bisulfite; some modifications such as 20 μl of starting gDNA (100–300 ng μl^{-1}) were introduced. For conversion, the following temperatures were used: 98 °C for 8 min, 65 °C for 45 min, 98 °C for 8 min, 65 °C for 90 min, 98 °C for 8 min, 65 °C for 120 min, and maintaining 18–20 °C over 12 h. For the amplification of the promoter region (−268 to +36) after treatment with bisulfite, the following primers (methylation-specific) were designed with Methyl Primer Express Software v.1.0 (http://products.appliedbiosystems.com/): ProMet-F (forward), 5′-TATTGA GTGTATTTTTGTAGAAAT-3′ and ProMet-R (reverse), 5′-CTCAA AAACRACCCTCCAC-3′. PCR conditions were as follows: 95 °C initiation for 30 s, 38 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s, and then final elongation step of 72 °C for 5 min.

The methylation border region (−650 to −850) was amplified with methylation-specific primers: FrMetf (forward), 5′-GAGGAAAGGTTTATA TTTTGAGA-3′ and FrMetr (reverse), 5′-TAAAAAATCAAATACATCCR AT-3′. The PCR conditions were as before, using annealing temperature of 59 °C. Genomic DNA samples (after bisulfite treatment) had concentrations between 10 and 35 ng μl^{-1}; bisulfite treatment destroys >80% of the DNA. The converted DNA is stable for 1 day at room temperature, 1 week at 4 °C and 2–4 months at –20 °C, using the Bisulfite Kit (New England Biolabs Inc.) according to the manufacturer's instruction.

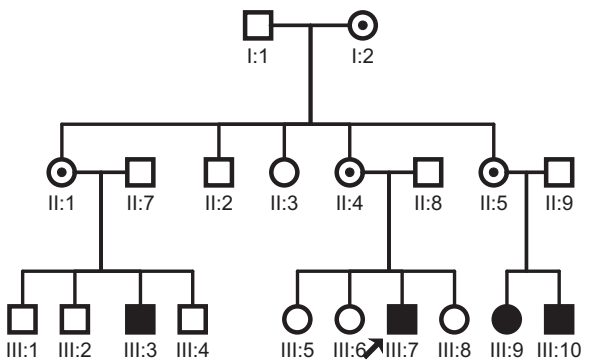

Figure 1 Family 1 with four affected members. The index case III:7 and his first cousin III:3 had both normal CGG number and methylation profile. His cousins III:9 and III:10 had full expansion at the *FMR1* locus and an extended and complete methylation along the promoter region as well.

Multiplex ligation-dependent probe amplification

CNVs of autosomal subtelomeric, autosomal centromeric and X-chromosome genes were studied in index cases using three MLPA Kits: (MLPA, MRC-Holland, Amsterdam, the Netherlands) P036-E1 (subtelomeric genes), P181 (centromeric genes) and P106 MRX-B1 (genes on the X chromosome) following the manufacturer's protocols. Capillary electrophoresis was performed on an Applied Biosystem DNA Sequencer 3130XL (Applied Biosystems Genetic Analyzer, Thermo-Fisher Scientific, Waltham, MA, USA), POP7 polymer and 50 cm capillary. Module fragment analysis was used with the following running conditions: injection voltage, 1.6 kV; injection time, 25 s; run voltage, 15 kV; run time: 3000 s; oven temperature 60 °C; and Filter D and ROX 500 as an internal standard. The samples were prepared as follows: 8.5 μl of Hi-Di formamide+0.5 μl ROX 500+1 μl of the MLPA-PCR product. The data generated by the capillary electrophoresis were analyzed with Coffalyzer. Net software by MRC Holland.

RESULTS

Table 1 shows the main clinical manifestations of patients with MBS plus the studied genetic features. All but one were males, diagnosed along the first or the second decades of life. All had ID and a family segregation pattern compatible with X-linked transmission, 93% had large face, 83% had large ears and 50% had macroorchidism.

Patient numbers 1 and 4 were brother and sister (III:9 and III:10) and first cousins of patient numbers 9 and 29 (III:3 and III:7), all belonging to family 1 (Figure 1). Patient numbers 19 and 20 and 24 and 25 were brothers from two unrelated families.

CGG repeats

Twenty-five patients were found to have a normal CGG size (range 18–44 CGG), one patient had 47 CGG repeats (gray zone) and three patients had presumed expansions >200 CGG repeats; as the conventional PCR technique was unable to amplify the product, it is usually seen with expanded alleles.

Promoter and border regions of the *FMR1* gene

The Sanger sequencing of the promoter region from −180 to +28 (250 bp) and from −850 to −650 (400 bp) in all patients ($n=29$) did not show any changes.

Methylation in proximal promoter and promoter border regions of the *FMR1* gene

Thirty-two CG dinucleotides were examined by bisulfite sequencing from −269 to +55 bp at the proximal promoter region and nine CG dinucleotides at the promoter border region (−650 to −850). The methylation profile in the promoter region of affected males without full mutation showed in all cases ($n=26$) that 100% of the CpGs were unmethylated.

Table 1 Phenotypic features of Martin–Bell syndrome in patients and its genetic study

			Martin–Bell syndrome features							*Genetic study*		
Patient number	*Age (years)*	*Sex*	*ID*	*LE*	*LF*	*PR*	*MO*	*HJ*	*H*	*FMR1 (CGG)*	*FMR1 promoter methylation: proximal (%)/border region*	*CNV*
1[a]	3	♂	++	++	++	−	++	++	++	>200	100/M	NE
2	11	♂	++	++	+	++	++	++	−	>200	100/M	NE
3[b]	5	♂	++	++	++	+	++	++	++	>200	87.9/M	NE
4[a]	4	♀	+	+	+	−	−	−	−	30/?	81.2/M	NE
5	9	♂	+	++	++	−	+	+	−	34	0/NE	Dup *PDCD6*
6	6	♂	++	++	+	+	++	−	−	22	0/NE	Del *CHL1*
7	13	♂	++	++	++	−	++	−	−	25	0/NE	NF
8	5	♂	++	++	−	+	+	−	−	27	0/NE	NF
9[a]	22	♂	++	++	++	++	++	−	+	30	0/NE	NF
10	18	♂	++	++	++	−	++	−	−	29	0/NE	NF
11	10	♂	++	+	+	+	−	−	−	47	0/NP	NF
12	5	♂	+	++	+	+	−	−	−	30	0/NP	NF
13	9	♂	++	+	−	−	−	−	−	22	0/NP	NF
14	15	♂	++	++	++	−	−	−	−	29	0/NP	NF
15	9	♂	++	+	+	+	−	−	−	30	0/NE	NF
16	14	♂	++	++	++	++	+	−	−	30	0/NP	NF
17	7	♂	++	−	+	−	++	−	−	30	0/NE	NF
18	8	♂	++	++	+	−	++	−	−	32	0/NP	NF
19[c]	17	♂	++	−	++	−	−	−	+	28	0/NE	NF
20[c]	9	♂	+	−	++	−	−	+	+	30	0/NP	NF
21	9	♂	+	++	++	−	−	−	−	30	0/NP	NF
22	14	♂	++	−	++	++	−	++	++	23	0/NE	NF
23	13	♂	++	-	+	−	−	++	−	29	0/NE	NF
24[d]	14	♂	++	+	++	−	−	++	+	29	0/NP	NF
25[d]	9	♂	+	+	++	−	−	++	−	27	0/NE	NF
26	9	♂	++	+	+	+	+	−	+	22	0/NP	NF
27	15	♂	++	++	++	+	−	−	−	27	0/NP	NF
28	13	♂	++	++	+	−	++	−	−	22	0/NE	NF
29[a]	17	♂	++	++	++	++	++	−	−	27	0/NP	NF

Abbreviations: +, mild; ++, moderate or marked; −, absent; *CHL1*, cell adhesion molecule L1 gene; Dup, duplication; Del, deletion; ID, intellectual disability; LE, large ears; LF, large face; HJ, hyperlaxity of joints; H, hyperactivity; M, methylated border region; MO, macroorchidism; NE, not studied; NP, normal methylation pattern in the border region; NF, not found; *PDCD6*, programmed cell death 6 gene; PR, prognathism.
[a]Non-independent patients (Family 1)
[b]Methylation mosaicism: 87.5%/12.5%.
[c]Brothers (Family 2).
[d]Brothers (Family 3).

On the contrary, in two patients with the full mutation (case nos 1 and 2; Table 1), methylation of 100% of the CpG dinucleotides in the proximal promoter region was observed.

A third case of a patient with full mutation (no. 3; Table 1) showed a methylation mosaicism with a methylation profile of 87.5% methylated sites and 12.5% in mosaicism. These CpG dinucleotides in mosaicism occurred in the recognition site of the transcription factor 3′Sp1 (Figure 2c).

A female patient (III:9 (Figure 1) and case no. 4 (Table 1)) had 81.2% of CG methylated at the promoter (−180 to +28) on both chromosomes and 18.8% hemimethylated. Six hemimethylated CpG dinucleotides were in the binding sites of the transcription factors 5′SP1 and E-box (Figure 2d). She had a brother with full mutation; unfortunately, the methylation profile in the *FMR1* promoter region of the mother of these siblings could not be studied because the amount of DNA in her sample was not suitable for the bisulfite treatment.

The methylation in the border region (−650 to −850) was studied in 16 patients; two males with full mutation had complete methylation (Figure 3d), as in the female patient (Figure 3d); 13 affected males without full mutation had a normal pattern (Figures 3a–c).

MLPA P106-B2 MRX and MLPA P181 centromere-1

There was a normal copy number of the 16 X-chromosome genes evaluated by MLPA P106-B2 MRX probes in all the patients. The mean value of the dose ratio varied between 0.98 and 1.02 with an s.d. of 0.12. Similar results were obtained for the 46 centromeric loci (MLPA P181 centromere-1), with a mean value of the range between 0.99 and 1.0 (s.d. 0.08).

MLPA P036-E1. human telomere-3

Twenty-five patients with a normal number of CGG repeats and one in the gray zone (47 CGG) without changes in the promoter methylation pattern were evaluated for CNVs in subtelomeric genes. The DNA sample in three patients was not suitable for the analysis. In 20 patients, a normal dose ratio of patient/controls was observed. Changes were found in two independent male patients: a heterozygous

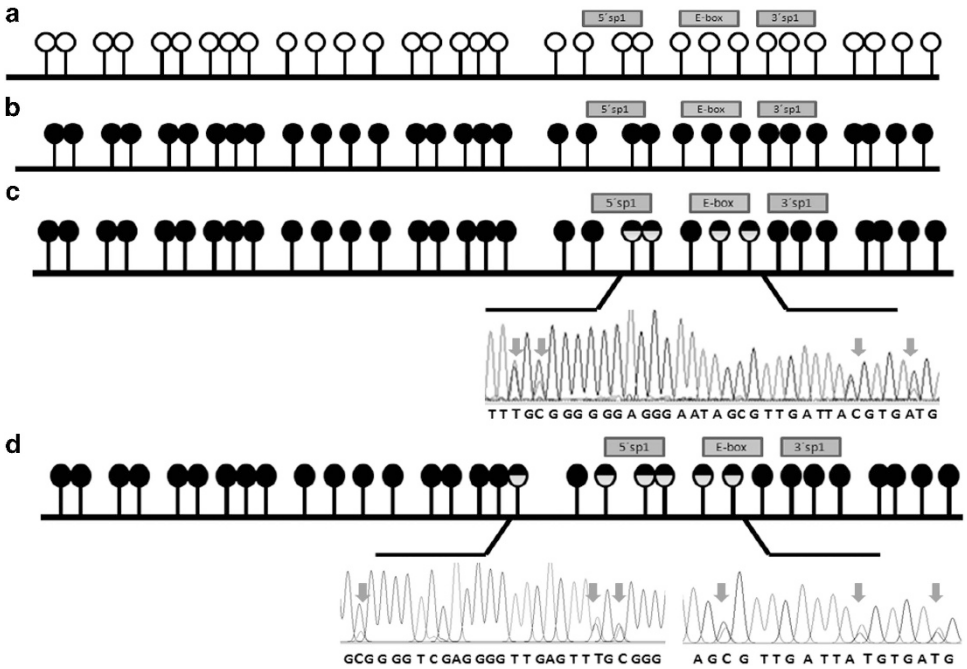

Figure 2 Epialleles in the *FMR1* promoter region. Solid black circles represent methylated dinucleotides; half black circles represent hemimethylated CpG. Electropherograms show the epimethylated alleles. (**a**) The results observed in patients with a number of CGG repeats in the normal range and (**b**) the methylation profile of two patients with full mutation. (**c**) The mosaic methylation observed in a full mutation patient. (**d**) The result of a female patient with a normal CGG allele and another allele with a possible full mutation. Green boxes represent recognition sequences of transcription factors (5′Sp1, E-box and 3′Sp1). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

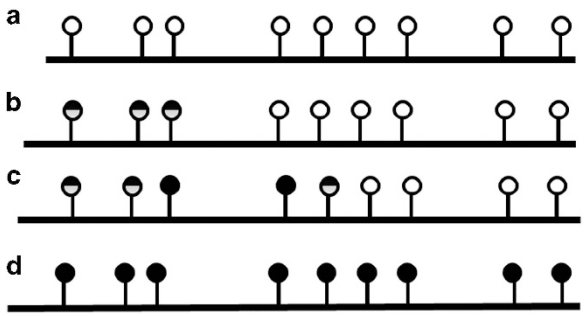

Figure 3 Pattern of epialleles observed in the promoter border region (–850 to –650) of the *FMR1* gene. Epialleles (**a**, **c**) were observed in patients with normal number of CGG repeats, (**b**) in three control subjects and (**d**) in male patients with full mutation, and also in the affected female. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

duplication in the gene *PDCD6* (programmed cell death 6) exon 6 (5p15.33) (dose ratio 1.53 ± 0.12) and a heterozygous deletion (dose ratio: 0.53 ± 0.06) at the locus *CALL* or *CHL1*, exon 5 (3p26.3) (case nos 5 and 6, respectively; Table 1).

These changes were assessed in the parents. In both instances, the mother carried the CNV: a heterozygous deletion at the locus *CALL* or *CHL1*, exon 5 (3p26.3) (dose ratio: 0.6 ± 0.05) and a heterozygous duplication in the gene *PDCD6* exon 6 (5p15.33) (dose ratio 1.39 ± 0.12).

DISCUSSION

MBS (an *FMR1*-related disorder) associated with abnormal functioning of the *FMR1* gene is a major cause for inherited ID. As some similar features may also be observed in patients suffering from other neuropsychiatric disorders, the diagnosis of MBS that is solely based on a patient's physical and behavioral characteristics might not be accurate.

The Laboratory of Human Genetics at Venezuelan Institute for Scientific Research has been studying patients with phenotypic characteristics of MBS during the past 32 years. Most of them did not show any expansion of the CGG trinucleotide repeats at the *FMR1* locus (Table 1). Thus, a systematic search for other genetic causes that could affect the expression of the *FMR1* gene as well as CNVs of genes in the X chromosome and other autosomal genes was undertaken to establish the etiology of the syndrome in those patients.

Mutations at the transcription factor sequences Sp1 and AP-2a, transcription start site II, an initiator-like and a TATA-like sequence in the *FMR1* promoter region (−180 to +28 bp) could impair the gene expression.[16,17] In all the studied index cases ($n = 26$), no changes were found in this gene region, discarding such mechanism as the etiology for the syndrome in them.

Mutations in the *FMR1* promoter region are a very infrequent cause of the MBS in almost all populations; to date, <10 point mutations in the coding region have been reported to cause the syndrome. Collins *et al.*[8] found three single-nucleotide changes in the promoter region (+2 A>G, −40 T>C, −74G>C) in independent chromosomes among 963 patients with nonspecific mental retardation without full mutation at the *FMR1* locus. These changes significantly decreased the expression of the *FMR1* gene, and were considered the cause of the ID in only 0.3% of the sample.

The methylation profile of the proximal *FMR1* promoter region in the affected males having a normal number of CGG repeats showed no alteration (Figure 2a) from −269 to +55, indicating that methylation was not the responsible mechanism for the syndrome in them. On the contrary, two out of the three patients with

full mutation (case nos 1 (III:10) and 2; Table 1) had complete methylation of the 32 CG sites. The full mutation led to hypermethylation of the promoter region and silencing of the *FMR1* gene. The third patient (index case no. 3; Table 1) had a methylation profile of 87.5% of 32 CG sites, of which 28 were fully methylated and 4 were a methylation mosaic (Figure 2c). MBS patients with over 200 CGG repeats and a methylation mosaicism may show a milder phenotype than those with a complete methylation profile;[18] in the punctuation protocol for clinical diagnosis of MBS, the score for this patient was 16, whereas in patients with a full mutation and complete methylation it was 19 and 20 (data not shown). The proportion of methylated to unmethylated sites (28:4) and the location of the unmethylated CpG dinucleotides (in the recognition sequence of the transcription factors 5'Sp1 and E-Box) might allow some residual level of FMR1P expression, producing a milder phenotype in this patient; nevertheless, it should be considered that the observed results correspond to a single type of cells (white blood cells), and the mosaicism proportion may be different in other cell lines.

An interesting result was obtained in a female patient (case no. 4 (Table 1) and III:9 (Figure 1)); she was included in the study because she had speech and learning difficulties, besides being the sister of a patient with full mutation and complete methylation (case no. 1 (III:10)). She had at least one allele in the normal size range, with 30 CGG repeats, but the methylation profile of the promoter region showed that 26 out of 32 CG sites on both chromosomes were methylated and 6 of them were in mosaic (Figure 2d). In normal women, the two methylation patterns (methylated and unmethylated) are observed in all the CpG sites,[19] because of the X-chromosome random inactivation process. One possible interpretation of the finding in this female is that inactivation occurred on the X chromosome that has a normal CGG number (30) and the active X chromosome carries an expanded allele, producing 81.2% methylation in the *FMR1* promoter CG dinucleotides. This mechanism may explain the milder observed phenotypic characteristics compared with her brother, as has been reported for most women with the MBS. Furthermore, her methylation profile in the border region showed complete methylation on both X chromosomes (Figure 2d). These findings suggest that the normal mechanism of X inactivation might be affected, as the border region provides the boundary between methylated and unmethylated promoter sequences. Thus, it is important to know the methylation profile of the promoter region of the *FMR1* gene in female patients having disease features, despite having a PCR amplification number of CGG repeats within the normal range.

On the other hand, patient numbers 9 (III:7; Figure 1) and 29 (III:3; Figure 1) were first cousins of these two siblings (case nos 1 and 4, III:10 and III:9, respectively). Case no. 9 had a very typical Martin–Bell phenotype, being the index case of this family, but did not have any of the *FMR1* alteration (expansion and/or methylation) present in his cousins; the same occurred with case no. 29. Therefore, the etiopathogenic mechanism of the MBS within this family is different, being yet unknown in two remaining family members.

Changes in the sequence of the methylation border region were also evaluated. That region appears to be responsible for the formation of a specific chromatin structure, which delimits the normally hypermethylated area of the unmethylated one, acting as an insulator element between the gene and the condensed chromatin.[11] Based on this finding, it was hypothesized that mutations in this sequence (between −850 to −650 bp in the upstream promoter) could affect the expression of the *FMR1* gene, expanding the methylation into the proximal promoter region in patients without a full mutation. In the present study, no changes were observed in the DNA sequence, thus discarding this scenario.

Methylation of the border region was also investigated in 16 of the patients. In patients carrying a full mutation allele (two males and a female patient), 9 CpG dinucleotides examined were completely methylated (Figure 3d), as has been reported in patients with a full mutation and complete methylation of its proximal promoter region. On the contrary, in individuals without CGG expansion and in controls, a variable pattern of methylation was observed, as found by Naumann *et al.*,[11] suggesting that this gene region tolerates certain degree of methylation. Thus, alteration in the methylation pattern in the border region of patients without CGG expansions was not the etiology of the syndrome.

CNVs of different autosomal and X-linked genes has been reported that accounts for ~3 to 8% of all cases of mental disability. Microinsertions or microdeletions of subtelomeric regions owing to unbalanced translocations have also been implicated as causes of developmental delay, dysmorphic features and other congenital abnormalities.[20]

MLPA P106 for the X chromosome included probes for 16 different genes, associated with mental retardation (syndromic and non-syndromic): *RPS6KA3*, *ARX*, *IL1RAPL1*, *TSPAN7*, *PQBP1*, *HUWE1*, *OPHN1*, *ACSL4*, *PAK3*, *DCX*, *AGTR2*, *ARHGEF6, FMR1*, *AFF2 (FMR2)*, *SLC6A8* and *GDI1*. For the *FMR1* gene, exons 6 and 9 are recognized, and for the *FMR2* gene, probes hybridize to exons 1, 3, 6, 12 and 21, allowing to rule out one of the mechanisms in the etiology of the MBS without trinucleotide expansion, such as deletions in the *FMR1* genes involving multiple exons, leading to the absence of FMR1 protein. CGG expansion of *FMR2* (*AFF2*) was not evaluated; silencing of this gene (either by CCG expansion or by deletion) causes a non-syndromic condition, with mild to borderline mental retardation, cognitive and behavioral deficits and no consistent dysmorphology,[21] unlike *FMR1* mutations, which produce a characteristic syndromic phenotype. Besides *AFF2* mutations are an infrequent etiology in ID.

The dosage of *FMR1* and *FMR2* genes in the group of Martin–Bell patients without trinucleotide expansion was normal, as well as for the other assessed X-linked genes. CNVs at centromeric autosomal regions were also excluded.

The MLPA P036-E1 telomere-3 probes give information on possible deletions or duplications in 46 subtelomeric regions, covering all the autosomal chromosomes and the pseudoautosomal regions of the X and Y chromosomes. Two variations in the number of copies of genes in two independent patients were found: in one case (no. 6; Table 1) a heterozygous deletion was found in the locus *CALL* or *CHL1*, exon 5 (3p26.3). This gene is highly expressed in the central and peripheral nervous systems, and its expression is important for axonal development. It has been demonstrated that a 50% reduction of *CALL* expression in the developing brain results in cognitive deficits; furthermore, its haploinsufficiency contributes to the 3p deletion syndrome phenotype, which includes psychomotor delay, growth retardation, mental retardation, facial dysmorphism and autistic behavior.[22–24] The phenotype may vary among different patients, depending on the size of the deleted region (3p25-3pter), which is frequently a *de novo* event. In a few known familial cases, a great variability between carriers has been observed, as has been reported in a carrier mother with a normal phenotype who had an affected son, or a mildly affected mother having a more severe affected son and a slightly affected daughter.[23] The causes of the variable penetrance of the 3p deletion syndrome are still unknown.

The detected patient (case no. 6) carrying the deletion was referred at 6 years and 7 months of age because of the neurological delay and school learning difficulties. He had a mild language disorder, generalized bradykinesia, clumsy movements and delayed acquisition of fine motor skills. His physical characteristics included elongated face, a prominent jaw, slightly protruding ears, macroorchidism and high-arched palate (Table 1). Thus, he must be a case of 3p deletion syndrome involving the *CALL* gene; his mother carried the same change in heterozygosis, without any clinical manifestations, as has been reported.[24]

The second patient with altered copy number of a gene was a 9-year-old boy with mild ID, long and protruding ears and facial dysmorphic features compatible with a Martin–Bell phenotype, hyperextensible joints and macroorchidism (index case no. 5; Table 1). He had no family history. A heterozygous duplication was observed in the gene *PDCD6* exon 6 (5p15.33).

Duplications of the short arm of chromosome 5 produce a variable phenotype, depending on the size and location of the duplication (ORPHA no. 1742, at http://www.orpha.net). Patients with duplications spanning from 5p13.3 to 5p15.3 show moderately affected phenotypes, which include facial dysmorphism, low-set ears, hypotonia, speech delay, ID, motor skill delay and autistic behavior,[25–27] whereas with the duplication of the 5p10-5p13.1 segment, the anomalies are more severe, including cardiac defects and other severe clinical features. Duplications of the *PDCD6* gen locus have not been reported; the herein reported case suggests that genes at the most distally segment of the 5p chromosome are involved in autosomal syndromic ID. Further studies are needed to establish the possible association of the *PDCD6* gene with this phenotype.

As in the case above, the patient's mother carried the same duplication in heterozygosis, without any clinical manifestations. A variable penetrant phenotype- and sex-dependent gene expression variability for the 5p15.33 duplication might be proposed to explain this finding; the specific mechanisms of which are still unknown.

In both cases of the autosomal heterozygous, deletion in the *CHL1* gene or duplication in the *PDCD6* gene, patient phenotypic features overlap with the MBS phenotype. Therefore, our report being the first instance of those apparent associations, besides their very low frequency, the actual etiological significance of those autosomal loci regarding the syndrome phenotype cannot be argued at this time, although a case for genocopy seems plausible.

Exhaustive studies of the known mechanisms of suppression of expression of the *FMR1* gene in the patient group have shown that there are other causes different to CGG expansion yet unknown that cause the phenotype of MBS. Factors in trans, affecting cellular mechanisms mediated by *FMR1*, or other genes with yet unknown functions could cause the syndrome phenotype. The finding of two CNVs in autosomal genes (*CALL* and *PDCD6*) in two separate cases out of 21 patients (9.5%) supports this hypothesis.

Fragile X syndrome was the first identified disorder discovered in association with the fragile site of the X chromosome in two brothers in 1969 by Lubs,[28] and retrospectively with the X-linked pedigree of ID reported by Martin and Bell in 1943.[1] Repeatedly, it has been stated that fragile X syndrome (*sensu stricto*), a cytogenetic marker, is almost always due to a full expansion mutation in *FMR1*, and the main cause for ID in males of populations of European descent and in other ones as well; however, in the MBS (or phenotype) *sensu lato*, as well as in many called fragile X syndrome cases, other genetic causes can produce the typical MBS phenotype. Therefore, the MBS should not be considered as a straight synonym of the former, according to the discussed findings, despite that the princeps family carried, indeed, both a full expansion and the chromosome marker.[29] However, evidently in many cases it is not so. In several studies in which the prevalence of expanded *FMR1* alleles in male individuals with ID was estimated, it has been found between 0.54% (United Kingdom),[30] 1.04% (Netherlands),[31] 2.8% (Spain)[32] and 1.15–6.3% for different ethnic groups;[33] besides in a massive study among ID males in Cuba, it reached only 0.3%.[34] Other etiopathogenesis as point mutations searched in European populations have been negative.[8,35] In patients from the Venezuelan populations, the full mutation is not the predominant etiology for most cases with a typical phenotype of the MBS (Table 1), being present only in $<10\%$ of the cohort. Therefore, the search for the genetic causes that produce the syndrome without trinucleotide expansion is justified, as it is a still unresolved problem in our populations, and apparently so far not detected but eventually present in other Iberoamerican ones.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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