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RESEARCH ARTICLE

Williams–Beuren syndrome in Mexican patients confirmed by FISH and assessed by aCGH

AZUBEL RAMíREZ-VELAZCO[1,2], THANIA ALEJANDRA AGUAYO-OROZCO[1,2], LUIS FIGUERA[1,2], HORACIO RIVERA[1,2], LUIS JAVE-SUÁREZ[3], ADRIANA AGUILAR-LEMARROY[3], LUIS A. TORRES-REYES[4], CARLOS CÓRDOVA-FLETES[5], PATRICIO BARROS-NÚÑEZ[1], SATURNINO DELGADILLO-PÉREZ[6], INGRID PATRICIA DÁVALOS-RODRíGUEZ[1,2], JOSÉ ELÍAS GARCíA-ORTIZ[1] and MARÍA G. DOMÍNGUEZ[1]*

[1] *División de Genética, CIBO, Instituto Mexicano del Seguro Social, Sierra Mojada 800, CP 44340, Guadalajara, México*
[2] *Doctorado en Genética Humana, CUCS, Universidad de Guadalajara, Sierra Mojada 950, CP 44340, Guadalajara, México*
[3] *División de Inmunología, CIBO, Instituto Mexicano del Seguro Social, Sierra Mojada 800, CP 44340, Guadalajara, México*
[4] *Departamento de Biología Molecular y Genómica, CUCS, Universidad de Guadalajara, Sierra Mojada 950, CP 44340, Guadalajara, México*
[5] *Departamento de Bioquímica y Medicina Molecular, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey 64460, Nuevo León, México*
[6] *Departamento de Cardiología, Hospital de Pediatría, Centro Médico Nacional de Occidente, Instituto Mexicano del Seguro Social, Salvador Quevedo y Zubieta 724, CP 44360 Guadalajara, México*
*For correspondence. E-mail: madq67@yahoo.com.mx.

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Abstract. Williams–Beuren syndrome (WBS) has a prevalence of 1/7500–20000 live births and results principally from a *de novo* deletion in 7q11.23 with a length of 1.5 Mb or 1.8 Mb. This study aimed to determine the frequency of 7q11.23 deletion, size of the segment lost, and involved genes in 47 patients with a clinical diagnosis of WBS and analysed by fluorescence *in situ* hybridization (FISH); among them, 31 had the expected deletion. Micro-array comparative genomic hybridization (aCGH) confirmed the loss in all 18 positive-patients tested: 14 patients had a 1.5 Mb deletion with the same breakpoints at 7q11.23 (hg19: 72726578–74139390) and comprising 24 coding genes from *TRIM50* to *GTF2I*. Four patients showed an atypical deletion: two had a 1.6 Mb loss encompassing 27 coding genes, from *NSUN5* to *GTF2IRD2*; another had a 1.7 Mb deletion involving 27 coding genes, from *POM121* to GTF2I; the remaining patient presented a deletion of 1.2 Mb that included 21 coding genes from *POM121* to *LIMK1*. aCGH confirmed the lack of deletion in 5/16 negative-patients by FISH. All 47 patients had the characteristic facial phenotype of WBS and 45 of 47 had the typical behavioural and developmental abnormalities. Our observations further confirm that patients with a classical deletion present a typical WBS phenotype, whereas those with a high (criteria of the American Association of Pediatrics, APP) clinical score but lacking the expected deletion may harbour an *ELN* point mutation. Overall, the concomitant CNVs appeared to be incidental findings.

Keywords. deletion 7q11.23; Williams–Beuren syndrome; supra-valvular aortic stenosis; micro-array comparative genomic hybridization; fluorescence *in situ* hybridization.

Introduction

Williams–Beuren syndrome (WBS, OMIM: 194050) is a congenital multisystemic disorder that has a prevalence of 1/7500–1/20000 in newborns and is due to the hemizygous loss of genes in 7q11.23 (Schubert 2009; Ferrero *et al.* 2010). Around 28 genes are lost in typical 7q11.23 deletions (Ferrero *et al.* 2010; Merla *et al.* 2010) whose size is either 1.5 Mb or 1.8 Mb (about 1.2 Mb corresponds to single copy genes) and are flanked by low

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copy repeats (Li *et al.* 2016). The atypical deletions are uncommon and range from 200 Kb to 2.5 Mb (Ramírez and Domínguez Quezada 2017). Usually, the deletion size correlates with the phenotype. Common clinical features are heart anomalies (type A) such as supravalvular aortic stenosis (SVAS) and peripheral pulmonary stenosis (PPS), developmental delay or mild intellectual disability and facial dysmorphisms (Collins *et al.* 2010). The deletion of 7q11.23 is usually detected by molecular techniques such as fluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and micro-array comparative genomic hybridization (aCGH). Significantly, the latter technique gives more information on the size of the deletion and genes involved. In 47 patients with the characteristic facial, developmental, and behavioural WBS phenotype, we looked for the expected 7q11.23 microdeletion by means of FISH and/or aCGH.

Materials and methods

Cytogenetic studies

In the period 2011–2017, we studied 47 patients with a clinical diagnosis of WBS established according to the criteria of the American Association of Pediatrics (AAP) (Committee on Genetics 2001). All patients were karyotyped on preparations obtained from PHA-stimulated peripheral blood cultures and stained for GTG bands (Rooney 2001); at least 16 metaphases were analysed in each patient. A subsequent FISH assay was carried out in each patient with one of three commercial probes (Cytocell, Vysis and Kreatech) containing the *ELN* gene. At least 10 metaphases per case was scored with a fluorescence Olympus AX70 microscope.

Extraction of DNA and quality control

Genomic DNA of 23 arbitrarily selected patients, 18 with and five without the 7q11.23 deletion detected by FISH was extracted from peripheral blood using the Gentra Puregene kit Qiagen (the remaining 24 patients were not tested because of limited resources or they were not available) and signed informed consent was taken from them. The purity and integrity of the gDNA samples were assessed by the NanoDrop 2000c (Thermo Scientific) and electrophoresis gel, respectively. Further, the exact amount of gDNA used in the microarrays was determined by Qubit fluorometer (Invitrogen).

Array based comparative genomic hybridization

DNA samples were analysed with the SurePrint G3 Human CGH Microarray 8 x 60K kit (20 samples) and SurePrint G3 human CGH microarray 2 x 400K kit (three samples) (Agilent technologies, Santa Clara, USA) according to the manufacturer's instructions. The slides were scanned for the two colours simultaneously at 2 μm of resolution with the NimbleGen MS200 microarray scanner (cat. no. 05394341001). Scanned images were processed using the Cytogenomics v3.0 software and analysed with the ADM-2 algorithm (Agilent technology).

Results

We studied 47 patients, aged from seven months to 14 years, with a mean age at clinical diagnosis of six years. All of them had a ≥3 points score according to the AAP; positive patients for the 7q11.23 deletion (31/47) had an average score of 8.29, a range of 3–14 and a mode of 9; in contrast, the nondeleted subjects (16/47) had a mean score of only 5.81, a range of 3–12 and a mode of 5. The difference is statistically significant (student's *t*-test), with *P* value of 0.003.

All 31 patients with 7q11.23 deletion had the characteristic facial phenotype of WBS; the second and third more frequent clinical features were cardiovascular defects type B (including hypertension, heart murmur and other congenital heart disease) and type A present in 26/31 and 19/31 patients, respectively. All patients had at least one 'behavioural and developmental abnormality', but only 17/31 patients reached the total for this category. Less common features were connective tissue, growth and calcium abnormalities with 8/31, 4/31 and 3/31 patients, respectively (table 1). As for the 16 patients without 7q11.23 deletion, all had the characteristic facial phenotype and most of them (14/16) exhibited behaviour and development abnormalities; other characteristics were present in less than 50% of the patients (table 2).

A normal G-banded karyotype was observed in 46 patients; the only exception was a girl (case 23) carrying a 46, XX, inv(X)(p22.3q22)mat complement in addition to the 1.5 Mb 7q11.23 deletion detected by FISH and aCGH (figure 1).

FISH analyses showed the 7q11.23 deletion in 31 of 47 (66%) patients. aCGH confirmed the deletion in 18 patients who were tested positive for FISH and revealed no deletion in the five FISH-negative samples. Among the former, 14 patients had a ~1.5 Mb deletion with the same breakpoints (hg19: 72726578–74139390) and comprising 24 coding genes from *TRIM50* to *GTF2I.* Four patients showed an atypical deletion: two had a 1.6 Mb loss encompassing 27 coding genes, from *NSUN5* to *GTF2IRD2* (cases 4 and 10, table 1); another had a 1.7 Mb deletion involving 27 coding genes from *POM121* to *GTF2I* (case 21, table 1), and the fourth patient presented a deletion of 1.2 Mb that included 21 coding genes from *POM121* to *LIMK1* (case19, table 1; figure 2). We did not find microduplications in 7q11.23.

In our series, we found some benign variants (e.g., gain or loss of 8p11.2, 14q11.2, 15q11.1-q11.2, and 22q11.22) or variants of uncertain significance (VUS) (e.g. 12q24.32

Table 1. Clinical AAP scores in 31 patients with 7q11.23 deletion.

Case	Growth	Behaviour and development	Facial features	Cardiovascular defects (A)	Cardiovascular defects (B)	Connective tissue abnormalities	Calcium studies	Total score
1		1	3		1	2	2	9
2			3	5	1			9
3		1	3		1	2	2	9
4£			3	5	1	2		11
5			3		1			4
6		1	3	5	1			10
7			3	5	1	2		11
8		1	3		1			5
9*		1	3	5	1			10
10£		1	3		1			5
11*	1		3	5	1			10
12*			3		1			4
13*		1	3		1			5
14		1	3	5	1			10
15*		1	3	5	1			10
16*	1	1	3	5	1			11
17		1	3	5				9
18*		1	3		1	2		7
19£		1	3	5	1	2	2	14
20*			3					3
21£			3	5	1			9
22*	1	1	3		1	2		8
23*	1	1	3	5		2		12
24*		1	3					4
25		1	3	5				9
26*			3	5	1			9
27*			3	5	1			9
28*			3	5	1			9
29			3	5	1			9
30			3	5	1			9
31			3		1			4
Trait presence Quantity/%	4/13%	17/55%	31/100%	19/61%	26/84%	8/26%	4/13%	Mean score 8.29

For ‘Growth’, at least 3/5 items checked, score 1 point. For ‘behaviour and development’, at least 3/6 items checked, score one point. For ‘facial features’, at least 8/17 items checked, score three points. For ‘cardiovascular defects (A)’, at least 1/2 items checked, score five points. For ‘cardiovascular defects (B)’, at least 1/3 items checked, score one point. For ‘connective tissue abnormalities’, at least 2/6 items checked, score two points. For ‘calcium studies’, at least 1/2 items checked, score two points. According to the American Academy of Pediatrics (AAP).*Typical deletions by aCGH, £ atypical deletion by aCGH.

Table 2. Clinical AAP scores in 16 patients without 7q11.23 deletion.

Case	Growth	Behaviour and development	Facial features	Cardiovascular defects (A)	Cardiovascular defects (B)	Connective tissue abnormalities	Calcium studies	Total score
1	1	1	3					5
2			3					3
3		1	3					4
4		1	3					4
5		1	3			2	2	8
6		1	3	5		2		11
7		1	3			2		6
8		1	3		1			5
9			3		1			4
10*§		1	3	5	1	2		12
11*		1	3		1			5
12*	1	1	3					5
13*	1	1	3					5
14		1	3					4
15*§	1	1	3		1			6
16		1	3			2		6
Trait presence Quantity/%	4/25%	14/87.5%	16/100%	2/12.5%	5/31.2 %	5/31.2%	1/6.4%	Mean score 5.81

For 'Growth', at least 3/5 items checked, score 1 point. For 'behavior and development', at least 3/6 items checked, score one point. For 'facial features', at least 8/17 items checked, score three points. For 'cardiovascular defects (A)', at least 1/2 items checked, score five points. For 'cardiovascular defects (B)', at least 1/3 items checked, score one point. For 'connective tissue abnormalities', at least 2/6 items checked, score two points. For 'Calcium studies', at least 1/2 items checked, score two points. According to the American Academy of Pediatrics (AAP).*Tested by aCGH, § variants of proven pathogenicity.

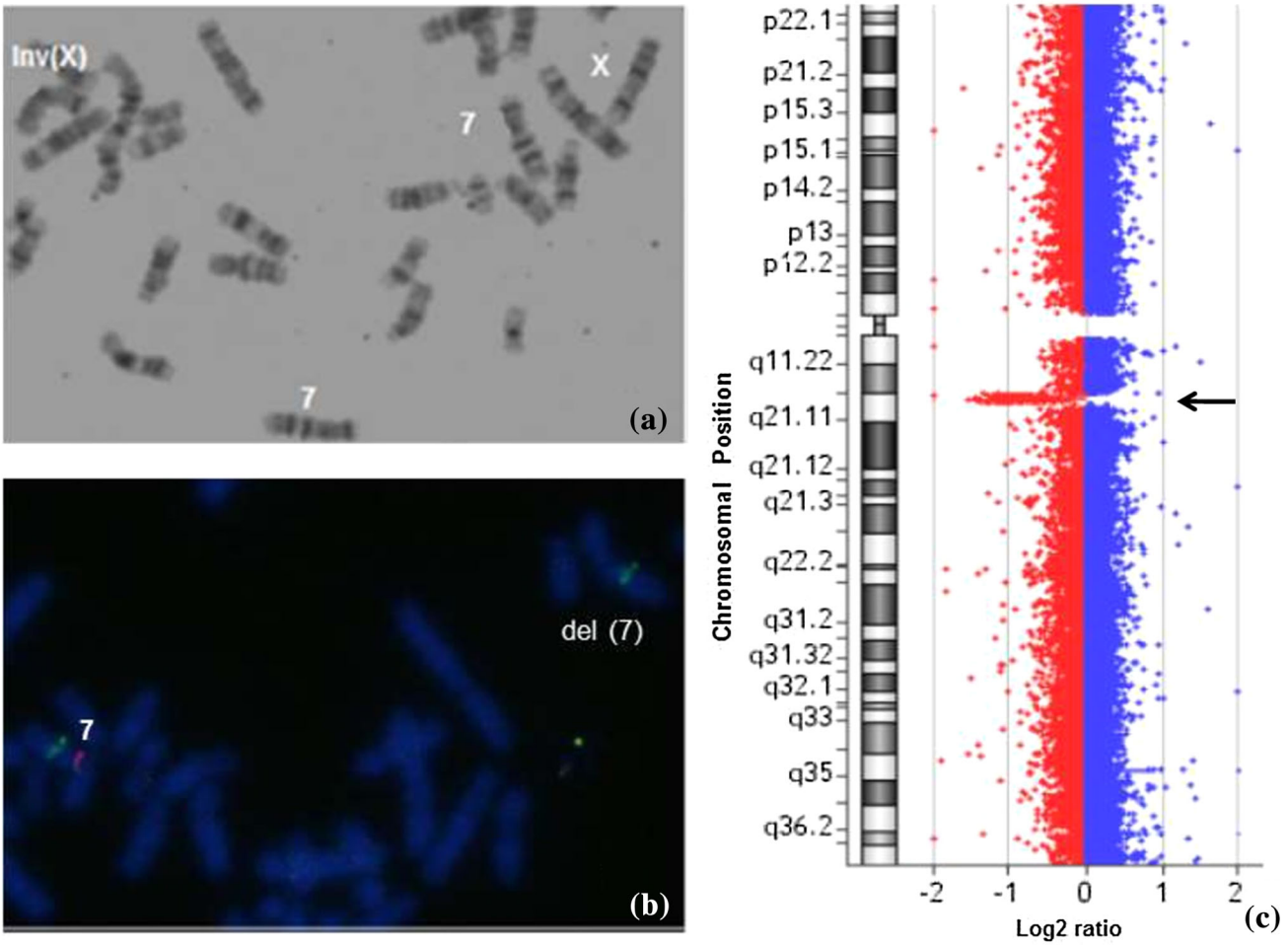

Figure 1. Two partial metaphases of case 23: (a) G-bands at a 400-band resolution showed an inv(X)(p22.3q22) and normal chromosomes 7; (b) deletion 7q11.23 as revealed by FISH with the dual probe targeting the *ELN* and *D7Z1* loci. Note the lack of the 7q11.23 orange signal in one homologue; the green signal marks both centromeres. (c) aCGH analysis showed at 7q11.23 deletion of 1.5 Mb (hg19:72726578-74139390) in the same patient.

duplication of 2.2 Mb, and 21q21.1 duplication of 1.8 Mb in the same patient with a clinical score of five points, case 13, table 2) in patients with or without 7q11.23 deletion. Also, there were four variants of proven pathogenicity, namely a terminal deletion of 3.8 Mb in 6q27 (case 15, table 2), a duplication of 3.8 Mb in 7p21.3p21.2 (case 10, table 2), a duplication of 3 Mb in 9p23 (case 21, table 1) and a duplication of 1.8 Mb in10q21.1 (case 24, table 1) found in a single patient each (the first two without and the other two with 7q11.23 deletion). Whether these variants were inherited or *de novo* remains unclear because the parents were not tested (Ramírez-Velazco 2017).

Discussion

In this study, 66% of the patients with WBS had the 7q11.23 deletion detected by FISH (confirmed by aCGH in all 18 deletion-positive patients tested), a reduced ratio in comparison with >95% reported by other authors (Lowery *et al.* 1995; Nickerson *et al.* 1995; Morris 2010) but greater than the 26% found in another series (Pani *et al.* 2010). These discrepancies are mainly due to selection criteria based on either gestalt phenotype or heart anomalies such as SVAS or PPS. The relatively low proportion (29%) of SVAS found in our series as compared with 70% of patients with 7q11.23 deletion and SVAS (Kalis *et al.* 2017) seemingly reflects the sizeable proportion of patients without deletion in our series. Otherwise, among 100 patients with familial or sporadic SVAS but without 7q11.23 deletion, 35 patients had point mutations in the *ELN* gene (including 23 frameshift mutations, six with splice site mutations, four missense mutations and two deletions in exon 1); yet, a clear genotype–phenotype correlation could not be established (Metcalfe *et al.* 2000). It has been suggested that sequence variations in the nondeleted *ELN* allele may be responsible for the variable expressivity of the cardiovascular phenotype depending on the amount

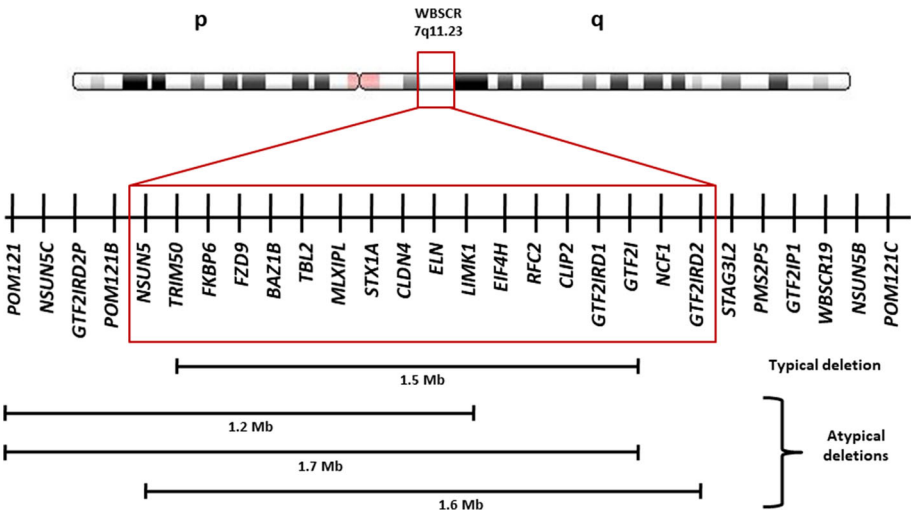

Figure 2. Schema of all four atypical 7q11.23 deletions (two of 1.6 Mb, one of 1.7 Mb and another of 1.2 Mb) revealed by aCGH.

of available tropoelastin (Delio *et al.* 2013). Such a variable expressivity could explain the significant differences between percentages for certain clinical features observed in several studies (Dutra *et al.* 2012).

Because the phenotypical traits in WBS patients are more evident with age, the probability of finding a 7q11.23 deletion is greater in older subjects (Borg *et al.* 1995). In the present study, the mean age at diagnosis was six years, a similar figure to that reported elsewhere (Pober *et al.* 1993; Ferrero *et al.* 2007).

The remarkable behavioural and developmental traits in WBS (friendly personality, hypersensivity to sound, anxiety, developmental delay, visuospacial problems and delayed speech acquisition followed by excessive talking) have been related to an impaired neuronal migration in the anterior region of the insula due to haploinsufficiency of the genes *LIMK1* and *CLIP2*. This implication of such a region in several personality traits is consistent with the significantly reduced volume seen in patients with the deletion (Jabbi *et al.* 2012).

Among the stigmata related to connective tissue abnormalities in our series, the most frequent feature was hoarse voice followed by long neck, joint laxity and inguinal hernia. These peculiarities are explained by the hemizygous deletion of *ELN,* as it has been illustrated in mice where a significantly decreased concentration of elastin in the lamina propria of vocal folds was documented (Watts *et al.* 2011).

Hypercalcaemia was observed in 10% of the 31 deleted patients, compared with 22% reported in a larger study of 55 patients (Kim *et al.* 2016). Although this feature had been related to the gene *TRPC3* at 4q27, Kim *et al.* (2016) found no evidence supporting such a proposal and still considered hypercalcaemia to be idiopathic. In our patients studied with aGCH, no CNV involving this region was found.

The typical WBS phenotype of case 23 who had a concomitant familial inv(X) (Rivera *et al.* 2016) can reliably be ascribed to her 7q11.23 deletion, a conclusion supported by the lack of CNVs in the X-chromosomes.

Aside from some benign variants and other CNVs of uncertain clinical significance, we found four gains or losses of proven pathogenicity in a single patient each. To illustrate the possible phenotypical effects of these imbalances, we highlight that the 6q terminal deletion is of clinical relevance because some of the lost genes such as *DLL1*, *THBS2*, *PHF10* and *C6orf70* are candidates for the causation of structural brain abnormalities (Peddibhotla *et al.* 2015). Moreover, 6qter deletions ranging from 0.4 Mb to 10.8 Mb are associated with a variable phenotype that includes intellectual disability, hypotonia, epilepsy, cardiac defects, retinal abnormalities, ear anomalies, facial dysmorphisms and malformations of the brain, spinal cord and vertebrae (Zhou *et al.* 2014; Peddibhotla *et al.* 2015). Noticeably, our patient with a 6q27 deletion had a typical WBS (in absence of the 7q11.23 deletion) but no specific clinical features ascribed to this imbalance except for hypochromic spots that may be related to *SMOC2* variants according to UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGene?showAllRef=Y\&db=hg19\&hgg_gene=SMOC2\#gad). No specific clinical features related to the remaining three concomitant pathogenic variants were observed. Although, we did not test the 11 deletion-negative patients by aCGH, our finding that all four atypical deletions diagnosed by aCGH were already detected by FISH and the 35% rate of *ELN* point mutations among patients without deletion (Metcalfe *et al.* 2000) which suggest that at least some of our 16 nondeleted patients actually harbour such a point mutation.

In conclusion, despite the remarkable diagnostic concordance of FISH and aCGH assays in this series as

well as the greater resolution and information given by aCGH, FISH remains as the most feasible and economical approach in countries like Mexico. Our observations further confirm that patients with a classical deletion present a typical WBS phenotype whereas those with a high clinical score but lacking the expected deletion may harbour an *ELN* point mutation. Overall, the concomitant CNVs appeared to be incidental findings.

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