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Defining the Deletion Size in Williams-Beuren Syndrome by Fluorescent In Situ Hybridization with Bacterial Artificial Chromosomes

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1. Introduction

Williams-Beuren syndrome (WBS, MIM No. 194050) is a contiguous gene deletion syndrome that was described independently by Williams et al. (1961) in patients with supravalvular aortic stenosis, growth retardation and an unusual facial appearance (Williams et al., 1961) and by Beuren et al. (1962) in patients having the same features as well as dental anomalies and friendly personality (Beuren et al., 1962).

The clinical picture of WBS includes a characteristic craniofacial dysmorphology with broad forehead, periorbital fullness, flat nasal bridge, broad nasal tip, long philtrum, full lips and lower cheeks, micrognathia, wide mouth and stellate irides, growth retardation, cardiovascular anomalies (mostly supravalvular aortic stenosis and pulmonary artery stenosis), mild to moderate mental retardation and unique behavioral and neurocognitive profile (relative preservation of linguistic abilities and gross visual-spatial processing dysfunction) (Morris et al., 1988; Pober, 2010). Its incidence is estimated at 1/7,500-1/20,000 (Grimm & Wesselhoeft, 1980; Morris et al., 1988; Stromme et al., 2002).

Williams-Beuren syndrome results from the hemizygous deletion of several genes, including ELN (elastin), encompassing 1.55 to 1.84 Mb on chromosome 7q11.23 (Ewart et al., 1993; Meng et al., 1998; Pober, 2010; Schubert & Laccone, 2006; Wang et al., 1999). WBS usually results from de novo deletion. Twenty-six to 28 genes have been identified within the WBS deletion region (Merla et al., 2010; Pober, 2010; Schubert, 2009).

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The WBS deletion region is flanked by highly homologous clusters of genes and pseudogenes organized into low-copy-repeat (LCR) blocks. Unequal meiotic recombination during meiosis can lead to deletion of the WBS region. The unique genetic architecture of the region explains why the size of WBS deletion is almost the same in most of the patients (Baumer et al., 1998; Dutly & Schinzel, 1996; Pober, 2010; Schubert, 2009; Valero et al., 2000).

Several studies have investigated the size of the WBS deletion using multiplex PCR with several microsatellite markers (Brondum-Nielsen et al., 1997; Perez Jurado et al., 1996; Wang et al., 1999; Wu et al., 1998). Most of the patients were found to carry the 1.5 or 1.8 Mb deletion. In the present study, we performed fluorescent in situ hybridization (FISH) with Bacterial Artificial Chromosome (BAC) clones in an attempt to map the WBS deletion in 14 WBS patients.

2. Patients and methods

2.1 Patients

Fifteen patients were referred to the cytogenetic laboratory of the Brest University Hospital by medical geneticists, pediatricians and pediatric cardiologist for suspicion of WBS between 2002 and March 2011. The reasons for referral included cardiovascular anomalies, dysmorphism or a combination of signs suspecting WBS (Table 1). However, because of limited cell pellet available for one patient, the study could be performed only on 14 patients.

2.2 Conventional cytogenetics

Metaphase chromosomes were prepared from peripheral blood lymphocytes of the 14 patients after having obtained their parents’ informed consent. Standard R banding chromosomal analyses were performed according to the standard procedures and the karyotypes described according to the International System for Cytogenetic Nomenclature (ISCN 2005).

2.3 FISH analyses with commercially available probe

A FISH study using the Vysis Williams Region Probe - LSI ELN SpectrumOrange/LSI D7S486, D7S522 SpectrumGreen was carried out on the metaphase preparations from all 14 patients, as recommended by the manufacturer (Abbott, Rungis, France).

The Williams Region Probe consists of a probe of approximately 180 kb in size for ELN, LIMK1 and the D7S613 locus located in band 7q11.23, labeled in Spectrum Orange, and a control probe for the region containing loci D7S486 and D7S522 located in band 7q31, labeled in Spectrum Green.

2.4 FISH analyses with BAC clones

To delineate the extent of the deletion on chromosome 7, FISH analyses were carried out using BAC clones mapping to the long arm of chromosome 7 (7q11.23).

We identified the BAC clones of interest through the Human Genome Browser Database of the Genome Bioinformatics Group at the University of California at Santa Cruz (http://genome.ucsc.edu/). They were then ordered by Internet on the site of the Children's Hospital Oakland Research Institute in Oakland, California (http://bacpac.chori.org/).
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| Patient Nr. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|-------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
| **Sex**     | F | M | F | M | F | M | M | F | M | M | F | M | F |
| **Age at diagnosis** | 3m | 2m | 7m | 1y4m | 3m | 1y7m | 1y9m | 9y11m | 8m | 2y9m | 9m | 2m | 3m | 2m |
| **Reason for referral** | Heart anomaly | Heart anomaly | Pulmonary artery stenosis | WBS suspicion | Aortic stenosis | Pulmonary artery stenosis | Heart anomaly | WBS suspicion | Dysmorphism | Dysmorphism | Heart anomaly | Dysmorphism | Heart anomaly | Heart anomaly |
| **Growth & Development** | | | | | | | | | | | | | | |
| **IUGR** | + | + | + | - | + | NA | - | - | + | + | - | - | - | - |
| **Growth retardation** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **Psychomotor retardation** | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| **Digestive system** | | | | | | | | | | | | | | | |
| **Neonatal feeding difficulties** | + | + | - | + | + | NA | + | - | + | + | + | - | - | - |
| **Inguinal hernia** | + | - | - | + | - | NA | - | - | - | + | - | + | - | - |
| **Gastroesophageal reflux** | + | - | - | + | + | NA | - | + | - | + | NA | + | + | + |
| **Dysmorphology** | | | | | | | | | | | | | | | |
| **Periorbital oedema** | - | + | - | + | + | + | + | - | - | + | + | - | + | - |
Table 1. Clinical characteristics of the 14 patients with Williams-Beuren syndrome.

| Patient Nr. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------------|---|---|---|---|---|---|---|---|---|----|
| Sex         | F | M | F | M | F | M | M | F | M | M |
| Large mouth | - | + | + | - | - | + | + | + | + | + |
| Fine upper lip | - | + | - | - | + | - | + | + | + | + |
| Flat philtrum | - | + | - | - | - | - | + | + | + | + |
| Bulbous nose | - | + | + | - | + | + | + | + | + | + |
| Anteverted nares | - | - | - | - | + | - | + | + | - | - |
| Low-set dysplastic ears | - | + | - | - | - | - | - | + | + | + |
| Prominent forehead | - | + | - | + | - | - | - | - | - | - |
| ORL          | - | + | - | - | NA | NA | + | + | - | + |
| High arched palate or cleft palate | - | - | - | - | NA | NA | + | - | - | + |
| Ophthalmology | - | - | + | - | NA | NA | + | - | - | + |
| Strabismus ± astigmatisn | NA | - | - | - | NA | NA | - | - | + | + |

M: month; y: year
WBS suspicion: combination of signs (facial dysmorphology and cardiovascular anomaly) making the diagnosis
IUGR: intra uterine growth retardation
NA: not available
Patient # 14 - ?: patient still too young (4 months old)
When received, bacterial cultures were prepared from a single colony picked from a selective plate in the presence of chloramphenicol. Plasmids were obtained from bacterial cultures grown in the presence of chloramphenicol (10 mg/L). After having lysed bacteria using SDS 1%/NaOH 0.2 N, DNA was purified from RNA, proteins and other cellular contaminants. Probes were then labeled by nick translation in Spectrum Orange (Nick Translation Kit, Abbott, Rungis, France) or in FITC (Prime-it Fluor Fluorescence Labeling Kit, Stratagene, Amsterdam, Netherlands). All BAC clones were applied to normal lymphocyte metaphases to confirm their chromosomal location.

After hybridization, the slides were counterstained with 4-6-diamino-2-phenyl-indole-dihydrochloride (DAPI). The preparations were examined using a Zeiss Axio Plan Microscope (Zeiss, Le Pecq, France). Images acquisition was performed using a CCD camera and analyzed using the ISIS program (In Situ Imaging System) (MetaSystems, Altlussheim, Germany).

Twenty-two overlapping BACs covering the WBS deletion region and beyond (about 1.9 Mb) were applied on the 14 patients (Table 2).

| BAC name     | Centromeric start (Mb from telomere) | Telomeric start (Mb from telomere) | BAC length (bp) |
|--------------|--------------------------------------|------------------------------------|-----------------|
| RP11-48D17   | 72,557,570                           | 72,705,204                          | 147,635         |
| RP11-483G21  | 72,688,731                           | 72,875,946                          | 187,216         |
| RP11-614D7   | 72,737,634                           | 72,926,932                          | 189,299         |
| RP11-101D2   | 72,814,548                           | 72,991,974                          | 177,427         |
| RP11-598B14  | 72,850,682                           | 73,042,752                          | 192,071         |
| RP11-622P13  | 73,002,073                           | 73,181,256                          | 179,184         |
| RP11-73G23   | 73,035,513                           | 73,184,825                          | 149,513         |
| RP11-148M21  | 73,153,473                           | 73,327,919                          | 174,447         |
| RP11-1011-F11| 73,166,799                           | 73,342,468                          | 175,670         |
| RP11-1056I4  | 73,437,240                           | 73,639,567                          | 202,328         |
| RP11-7M12    | 73,611,329                           | 73,293,367                          | 182,039         |
| RP11-351B3   | 73,705,662                           | 73,899,619                          | 193,958         |
| RP11-247L6   | 73,872,593                           | 74,038,131                          | 165,539         |
| RP11-196F10  | 73,872,610                           | 74,038,131                          | 165,522         |
| RP11-137E8   | 73,944,720                           | 74,129,587                          | 184,868         |
| RP11-728M8   | 73,959,533                           | 74,148,428                          | 188,896         |
| RP11-926D5   | 73,959,536                           | 74,145,706                          | 186,171         |
| RP11-19F19   | 73,994,434                           | 74,173,462                          | 179,029         |
| RP11-813J7   | 74,091,733                           | 74,261,309                          | 169,577         |
| RP11-1105J19 | 74,128,975                           | 74,321,491                          | 192,517         |
| RP11-1094P22 | 74,129,067                           | 74,321,019                          | 191,953         |
| RP11-379L10  | 74,307,663                           | 74,480,665                          | 173,003         |

The base pairs position (bp) are predicted on Build 39 National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and assembly February 2009 by The UCSC Genome Browser Database (http://genome.ucsc.edu/index.html).

Table 2. BAC library used to delimitate the deletion size in the 14 WBS patients.
3. Results

Fourteen patients were included in the study (Table 1). There were 7 boys and 7 girls. The median age at the time of cytogenetic exam was 7.5 months. Except for a patient who was almost 10 years old, all the other patients were less than 3 years old at the time of cytogenetic analyses, the majority of them being less than 1 year old (9 of 14 patients).

Cardiovascular anomalies and facial dysmorphism were present in all patients, but for one without cardiovascular anomaly and another without dysmorphism (Table 1). Growth and psychomotor retardation was noted in all patients. Stellate irides and/or strabismus was also present in 7 of the 12 patients for whom the data was available. No cognitive nor personality profile was available for the patients, mainly due to their young age.

R-banding conventional cytogenetics showed a normal 46,XY or 46,XX karyotype whereas FISH using the Vysis Williams Region Probe revealed a deletion of the ELN, LIMK1 and D7S613 locus in all 14 patients (Figure 1-A).

![FISH results](image1.png)

Fig. 1. Example of FISH results in a patient with Williams-Beuren syndrome. (A) FISH using the commercially available probe (ELN in SpectrumOrange and D7S486, D7S522 in SpectrumGreen). (B) FISH using two BAC clones (RP11-614D7 in green and RP11-1105J19 in red).

Sequential FISH analyses with BAC clones were applied on metaphases of all 14 patients having a deletion of the Vysis Williams Region Probe (Figure 1-B). They showed the centromeric deletion boundary to be located in a 145 kb interval, between RP11-598B14, deleted in 13 patients (P1 to P13), and RP11-48D17, always present (Figure 2). The telomeric deletion boundary was found to be located in a 229 kb interval, between RP11-351B3, deleted in 13 patients (P1 to P13), and RP11-1105J19, always present. Therefore, the minimum and maximum estimated deletion sizes for these 13 patients (P1 to P13) were 1,048,937 bp and 1,423,771 bp, respectively. Using overlapping BAC clones, both centromeric and telomeric boundaries could be refined. FISH signals with BAC clones RP11-614D7 and RP11-101D2, located at the centromeric deletion boundary, and those RP11-
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247L6 and RP11-137E8, located at the telomeric deletion boundary, showed decreased intensities. The centromeric and telomeric intervals were reduced to 36 kb and 93 kb, respectively, giving an estimated WBS deletion size of 1.17Mb.

Fig. 2. Size of the deletions in the 14 WBS patients estimated by BAC clones (ELN: elastin gene)

The fourteenth patient (P14) was found to have a centromeric deletion boundary to be located in a 109 kb interval, between RP11-101D2 (deleted) and RP11-48D17 (present). Using overlapping BAC clones RP11-483G21 and RP11-614D7, the centromeric boundary was refined to a 77 kb interval. The telomeric deletion boundary was found to be located at the junction of two overlapping BAC clones (RP11-926D5 and RP11-1105J19). Therefore, the estimated WBS deletion size for this patient was 1.41Mb.
4. Discussion

Molecular genetic studies have shown that hemizygosity of the elastin (ELN) gene accounts for the cardiovascular abnormalities observed in WBS patients. The other signs (facial dysmorphism, growth and mental retardation, etc.) observed in WBS are likely to be due to hemizygosity of other genes flanking the ELN locus. Although the unique combination of signs and anomalies is highly evocative, WBS patients usually present phenotypic variability, which could be explained by different deletion size and, therefore, by different sets of genes showing hemizygosity.

Several studies tried to define the WBS critical deletion region. Using sequence tagged site (STS) markers flanking the ELN gene, Perez-Jurado et al. (1996) and Wu et al. (1998) looked for the minimal deletion region responsible for the WBS phenotype in 123 patients. Markers from D7S489B (also named D7S489U) to D7S1870 were shown to be consistently removed, defining a 2 cM deletion in all informative patients (Perez Jurado et al., 1996; Wu et al., 1998).

Using BAC and PAC (P1-derived artificial chromosome) clones, Meng et al. (1998) constructed a physical map of the common deletion region. They localized the centromeric and telomeric deletion breakpoints to two genomic clones (containing D7S489B and D7S1870) flanking a 1.5 Mb deletion interval (Meng et al., 1998).

Based on the work by Valero et al. (2000) who found that 3 large region-specific segmental duplication or low copy repeat (LCR) elements flanked the common deletion region (Valero et al., 2000), Bayes et al. (2003) defined two common deletion regions in a set of 74 patients with WBS. Most of the patients (95%) had a 1.55 Mb deletion whereas the remaining 5% exhibited a larger deletion of approximately 1.84 Mb (Bayes et al., 2003).

Botta et al. (1999) reported a deletion of about 850 kb in two patients showing the full spectrum of the WBS phenotype (Botta et al., 1999). Furthermore, the analysis of the deletion size in WBS patients with “incomplete” phenotype revealed even more heterogeneity (Antonell et al., 2010). Using quantitative real-time PCR to scan 2.5 Mb of the WBS deletion region at a resolution of 100-300 kb among 65 patients with strong clinical indication of having WBS, Schubert and Laccone (2006) found that 21 patients had a deletion in the WBS region. Nineteen patients had a deletion ranging from 1.4 to 1.8 Mb in size whereas one had a 200 kb deletion and the remaining one a 2.5 Mb deletion (Schubert & Laccone, 2006).

FISH using BAC clones has been extensively used to construct physical maps of the 7q region deleted in WBS and to define the commonly deleted region (Meng et al., 1998; Peoples et al., 2000; Perez Jurado et al., 1996; Wu et al., 1998). However, to our knowledge, no study has been conducted to determine the deletion size in WBS individuals using overlapping BACs. Indeed, the level of resolution is a limit to this technique. A BAC is considered as partially deleted by FISH analysis when the fluorescent signal of this clone is much stronger on one chromosome 7 than on the other. Furthermore, a BAC could be considered as present or absent (not partially deleted) if only a small part of the DNA sequence is removed or kept (usually less than 50 kb). Having resource to overlapping BAC clones can increase the level of confidence but some uncertainty will remain.
5. Conclusion

Thirteen patients had a deletion estimated at 1.17 Mb in size and the remaining patient a deletion size estimated at 1.41 Mb. All the patients had the “classical” WBS phenotype, but for the cognitive profile which was not evaluated. The margins of uncertainty of the centromeric and telomeric boundaries of the deletion were less than 100 kb in all 14 patients, a level obtained by microsatellite analysis or quantitative real-time PCR. Therefore, this approach is efficient to define the deletion size among WBS patients.

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This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel Bacillus subtilis host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

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