Letter to the Editor

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Interphase fluorescent in situ hybridization detection of the 7q11.23 chromosomal inversion in a clinical laboratory: automated versus manual scoring

Keywords: detection carrier; FISH; inversion; quantitative automated analysis; Williams-Beuren syndrome.

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To the Editor,

Williams-Beuren syndrome (WBS) is a rare multisystem developmental disorder characterized by elastin arteriopathy, developmental delay with a specific neurocognitive behavior profile and a recognizable pattern of dysmorphic facial features. WBS is most often due to heterozygous segmental deletion of contiguous genes at chromosome 7q11.23, spanning 1.55 Mb–1.84 Mb [1–3]. This segment encodes 28 genes [4] and is flanked by complex chromosome-specific low-copy repeats (LCRs). LCR sequences cause recurrent genomic rearrangements consisting of deletions, duplications [5, 6] or inversions [1, 7] through unequal meiotic recombinations between flanking repeats [2]. A paracentric inversion of this region has been found in 24%–33% of progenitors transmitting the disease [1, 3, 8, 9] compared to 5.8% in the control population. This may predispose future generations to the WBS deletion. Two types of techniques have been developed for the detection of the inversion: interphase fluorescent in situ hybridization (FISH) analysis [1] and molecular genetic approaches, such as *NotI*–PFGE fragment restriction or site-specific nucleotide (SSN) assays [1, 3]. These molecular methods have several limitations. The reference continues to be the FISH approach but it needs an unbiased counting that is easier to implement in a research laboratory than in a clinical one.

FISH is based on manual counting of nuclear signals. However, of the different experimental protocols, FISH analyses conducted in clinical laboratories are not anonymous. Therefore, this lack of anonymity could introduce a potential bias in the manual counting of spots. Thus, we compared two ways of counting the spots: one manual scoring and one with homemade automated interphase nuclei searching software.

Ten couples (20 parents) whose child presented classic WBS (Williams parent or WP) and 10 couples unrelated to the WP (20 controls) were randomly selected among parents of children without WBS (control population or CP). Cells were isolated from the peripheral blood lymphocytes. All samples were obtained from subjects after an institutional review board approved informed consent (DGS 2004/0341).

Cytogenetic preparations and probes for interphase FISH analysis were performed following standard methods. Three specific FISH probes were used and are shown in Figure 1A. The RP5-1186P10 probe labeled with Alexa488 and the CTA-208H19 probe labeled with Rhodamine are located within the common deletion region. The RP11-815K3 probe labeled with Alexa488 is located just outside at the centromeric side. Their combination detected inversion or normal order (Figure 1B).

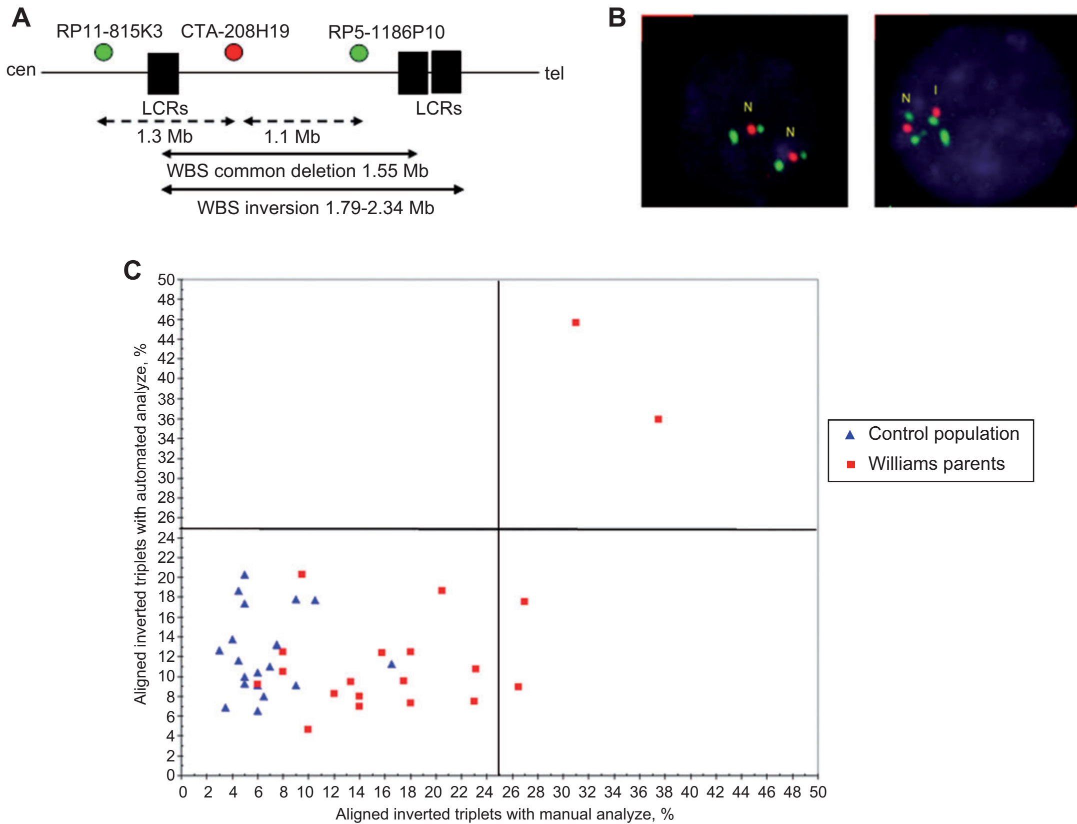

Figure 1 FISH detection of WBS region: automated vs. manual scoring.
(A) Locations of the three specific probes, RP11-815K3 probe (Genbank accession number AC007941), 215 kb, outside the common deletion region, just to the centromeric side, CTA-208H19 probe (Genbank accession number AC007941), 50 kb, and RP5-1186P10 (Genbank accession number AC005074), 100 kb, within the deletion region. They are located within 71, 72.3 and 73.4 Mb, respectively, from short arm telomere. Expected order: RP11-815K3-CTA-208H19-RP5-1186P10, green-red-green. Inverted order: RP11-815K3-RP5-1186P10-CTA-208H19, green-green-red. (B) Left: Concordant nucleus with two normal triplets (N). Right: Discordant nucleus with one inverted triplet (I). (C) Percentage of inverted triplets in the control population and Williams parents groups after manual and automated scoring FISH signals, threshold of 25%.

In the case of manual scoring, 200 triplets (three probe signals) minimum per slide were recorded with an epifluorescent microscope which captured and transferred the image to our image acquisition software (ISIS® Metasystems®, Althuseim, Germany). Triplets were selected when the angle formed between the three signals in the same nucleus were visually >90°. Analysis was entirely manual. For each slide the percentage of inverted or normal triplets was calculated. Technicians were aware of the identity of patients analyzed and their group membership (CP or WP).

For the automated scoring, the images were captured on Metafer® automated image acquisition software (Metasystems®, Altlusheim, Germany). Inversion detection requires the scoring of a large number of interphase nuclei. Therefore, 3000 images per slide were captured for each participant by computer assisted microscopy. Then an algorithm searching for the valid triplets of interest was used. Only a nucleus with six spots was considered, where two triplets with three probe signals were clearly visible and roughly aligned. The program gave the coordinates of the spots, the lengths of the intervals between spots. The program selected exclusively the triplets with an obtuse angle equal or superior to 110° and then identified triplets as inverted or normally ordered. The normal and discordant (one normal triplet and one inverted) nuclei were differentiated. Each step was operator independent. At the end of the automatic selection process between 60 and 210 valid triplets were analyzed. The homemade analysis program under Ana_Wil and ImageJ software is given in the Supplemental data, available from http://www.degruyter.com/view/j/cclm.2013.51.issue-4/issue-files/cclm.2013.51.issue-4.xml.

To assess and compare the manual and automated scoring, the percentage of inverted triplets in all CP and WP were evaluated with each method (Figure 1C). With

the automated method, the percentage of inverted triplets ranged from 6.52% to 20.31% in the CP group and from 4.65% to 45.59% in the WP group. We consider that any subjects with a least 25% of inverted triplets should be considered as an inversion carrier. The chosen threshold of 25% is of biological significance according to Mendelian laws; it is midway between the theoretical 0% expected in non-inverted carriers and the 50% expected in inverted carriers. Therefore, in the WP group, two WP had a significantly higher percentage of inverted triplets (36% and 46%) suggesting a heterozygous inversion carrier status for these two Williams parents (Figure 1C). The gross distributions of both data sets were not statistically different when the two outliers (inversion carriers) in the WP population were discarded (two-tailed t-test, $p=0.08$).

With the manual counting method, the percentage of inverted triplets ranged from 3.00% to 16.58% in the CP group and from 6.00% to 37.50% in the WP group. Four individuals had a percentage of inverted triplets superior to 25% (26.48, 27.00, 31.00 and 37.50%) (Figure 1C). Those with a percentage of 31% and 37.50% were the same Williams parents detected as inverted with the automated method. Conversely, those with the highest percentage close to the threshold were not detected as inverted with the automated method. With the manual scoring, the gross distributions of the percentage of inverted triplets in the CP and WP groups are statistically different even if the four outliers (presumed inversion carriers) in the WP population are discarded (two-tailed t-test, $p<0.01$).

In this study we observed a difference in the detection rate of inverted triplets according to the method used. The percentage of inverted triplets in the WP group is significantly higher than in the CP group with the manual scoring. With the automated method, counting results are homogenous between the two groups. Moreover, with the manual method, two more inversion carriers were detected than with the automated method. Discordant results in the counting of inverted triplets between the manual technique and automated technique could be explained by the fact that the slides were not anonymous, which brought about a bias in the manual counting and influenced the final detection rate of cells with inverted triplets. This suggests that the two inversion carriers detected only by the manual counting method and with a percentage of inverted triplets close to the threshold should be considered as false-positives by the manual method. However, we cannot completely exclude that those two patients could be false negatives by the automated method. These results demonstrated that the manual method for the detection of the inversion is not reliable in determining carrier status. Our interphase fluorescent in situ hybridization automated scoring for the inversion carrier detection improves previous cytogenetic methods because it is operator independent.

In conclusion, quantitative analysis with automated interphase fluorescent in situ hybridization finder software makes possible an unbiased selection of nuclei with inverted or normal triplets and confers better robustness for detection of hemizygous inversion carriers of the 7q11.23 region in WBS. This is of capital importance in order to organize genetic counseling, calculate the risk of recurrence and provide an adapted prenatal diagnosis.

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Conflict of interest statement

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