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Clinical Report

Chromosomal Microarray Analysis (CMA) Detects a Large X Chromosome Deletion Including *FMR1*, *FMR2*, and *IDS* in a Female Patient With Mental Retardation

Frank J. Probst,[1] Elizabeth R. Roeder,[2] Victoria B. Enciso,[2] Zhishuo Ou,[1] M. Lance Cooper,[1] Patricia Eng,[1] Jiangzhen Li,[3] Yanghong Gu,[1] Robert F. Stratton,[2] A. Craig Chinault,[1] Chad A. Shaw,[1] V. Reid Sutton,[1] Sau Wai Cheung,[1]* and David L. Nelson[1]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas
[2]Division of Genetics and Metabolic Disorders, Department of Pediatrics, University of Texas Health Science Center at San Antonio, San Antonio, Texas
[3]Department of Research and Development, Opexa Therapeutics, The Woodlands, Texas

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Chromosomal microarray analysis (CMA) by array-based comparative genomic hybridization (CGH) is a new clinical test for the detection of well-characterized genomic disorders caused by chromosomal deletions and duplications that result in gene copy number variation (CNV). This powerful assay detects an abnormality in approximately 7–9% of patients with various clinical phenotypes, including mental retardation. We report here on the results found in a 6-year-old girl with mildly dysmorphic facies, obesity, and marked developmental delay. CMA was requested and showed a heterozygous loss in copy number with clones derived from the genomic region cytogenetically defined as Xq27.3–Xq28. This loss was not cytogenetically visible but was seen on FISH analysis with clones from the region. Further studies confirmed a loss of one copy each of the *FMR1*, *FMR2*, and *IDS* genes (which are mutated in Fragile X syndrome, FRAXE syndrome, and Hunter syndrome, respectively). Skewed X-inactivation has been previously reported in girls with deletions in this region and can lead to a combined Fragile X/Hunter syndrome phenotype in affected females. X-inactivation and iduronate 2-sulfatase (IDS) enzyme activity were therefore examined. X-inactivation was found to be random in the child's peripheral leukocytes, and IDS enzyme activity was approximately half of the normal value. This case demonstrates the utility of CMA both for detecting a submicroscopic chromosomal deletion and for suggesting further testing that could possibly lead to therapeutic options for patients with developmental delay. © 2007 Wiley-Liss, Inc.

Key words: microarray analysis; X chromosome; chromosome deletion; fragile X syndrome; mucopolysaccharidosis II

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INTRODUCTION

The evaluation of children with developmental delay is often a diagnostic challenge. Chromosomal microarray analysis (CMA) by array-based comparative genomic hybridization (CGH) is a new molecular test that has been validated for the clinical diagnosis of genomic disorders that result from microscopic and submicroscopic deletions or duplications of specific chromosomal regions. The array contains hundreds of BAC, PAC, and cosmid clones covering areas of the genome that are known to be involved in over 70 different genomic disorders, as well as all of the clinically relevant telomeric regions. CMA is a powerful diagnostic tool that detects an abnormality in approximately 7–9% of patients

Frank J. Probst and Elizabeth R. Roeder contributed equally to this study.

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*Correspondence to: Sau Wai Cheung, Ph.D., Director, Kleberg Cytogenetics Laboratory, Baylor College of Medicine, One Baylor Plaza, NAB2015, Houston, TX 77030. E-mail: scheung@bcm.tmc.edu

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tested for dysmorphic features, multiple congenital anomalies, seizure disorder, or developmental delay [Cheung et al., 2005; Lupski and Stankiewicz, 2006; Lu et al., 2007]. We report on the results of a 6-year-old girl with obesity and severe developmental delay who was referred to the Genetics Clinic for evaluation of possible Prader–Willi Syndrome (OMIM #176270). An extensive prior workup had failed to find an underlying diagnosis to explain her symptoms. CMA revealed a loss of copy number with four clones from the genomic region cytogenetically defined as Xq27.3–Xq28. Further analysis done on a research basis has proven that this child has a large submicroscopic deletion involving the fragile site mental retardation 1 (*FMR1*), fragile site mental retardation 2 (*FMR2*), and iduronate 2-sulfatase (*IDS*) genes.

CLINICAL REPORT

The patient is a 6-year-old girl who presented to the Genetics Clinic with her mother and sister for the evaluation of marked developmental delay. She was born at 36 weeks gestation after an uncomplicated pregnancy to a 27-year-old healthy mother. The delivery and the neonatal period were unremarkable. Birth weight was 3,175 g (25th–50th centile) and birth length was 53.3 cm (90th–95th centile). She did not walk until 24 months of age and did not speak her first words until age 5½ years. She currently has a vocabulary of about 10 words and does not combine words. She was not toilet trained until age 5 years. Past medical history was remarkable only for a single febrile seizure at 3 years of age. She takes no medications. The history was negative for any food-seeking behaviors.

An extensive prior workup included a brain MRI, audiogram, complete blood count, lead level, bone age, thyroid function studies, plasma amino acids, Prader–Willi methylation analysis, and Beckwith–Wiedemann methylation analysis, all of which were normal. Chromosome analysis revealed a normal 46,XX karyotype. *FMR1* trinucleotide repeat analysis yielded a single allele of ~30 trinucleotide repeats.

On physical exam at 6 years of age, the girl had a height of 126.2 cm (75th–90th centile), a weight of 55.9 kg (greater than the 95th centile), and a head circumference of 59 cm (greater than the 98th centile). She was an obese, macrocephalic girl with a happy disposition who was smiling and interactive with examiners, even though she had only one or two recognizable words. She had a prominent forehead, mildly upslanting palpebral fissures, large ears (length slightly greater than the 97th centile) with fleshy earlobes, a small left preauricular ear tag, and a prominent philtrum. The eyebrows were sparse medially and flared (Fig. 1). Acanthosis nigricans was present on the posterior neck. Mild joint hyperextensibility was noted throughout, particularly in the fingers and hands. The fingers were tapered, and there was pes planus with prominent heels. The exam was otherwise unremarkable.

Family history was notable for a 12½-year-old full sister with normal intelligence who was fluent in both English and Spanish. She also had macrocephaly and obesity, with acanthosis nigricans on the posterior neck. Height, weight, and head circumference were all greater than the 97th centile. She had slightly coarse facial features, mild frontal bossing, mild epicanthal folds, large ears with pits and creases on the right ear, a prominent chin, mild joint hyperextensibility, large hands with unusual creases on the palms and digits, and broad feet with pes planus. There was adontia of the permanent teeth (only one secondary tooth—her right central maxillary incisor—was present, and a panorex X-ray of the jaw revealed absence of all of her other secondary teeth). Bone age was that of a 15 year old. Hand radiographs revealed synphalangism of the 2nd and 5th distal interphalangeal (DIP) joints bilaterally. An asymptomatic arachnoid cyst was seen on brain MRI. Laboratory studies were significant for hyperlipidemia and insulin resistance. Past medical history was remarkable for sagittal craniosynostosis,

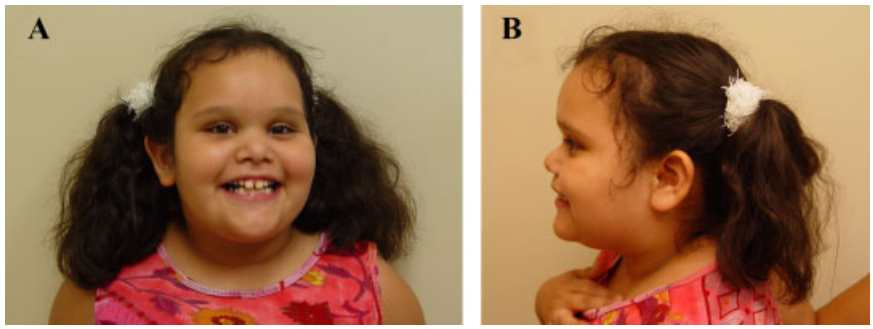

FIG. 1. Front (**A**) and profile (**B**) views of the patient. Note the small ear tag above the left tragus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

status-postsurgical repair. To date, no unifying diagnosis has been made in this individual.

MATERIALS AND METHODS

Chromosomal Microarray Analysis (CMA)

CMA was performed on DNA isolated from the patient's peripheral blood leukocytes. Version 5.0 of our array-CGH contains over 850 BAC, PAC, and cosmid clones encompassing over 70 genomic regions and additional coverage of all clinically relevant subtelomeric regions. This version of the array also includes clones from 43 pericentric regions (for detecting marker chromosomes with centromeres) and clones broadly spanning chromosomes 13, 18, 21, X, and Y (for detecting the most common aneuploidies). This array-CGH is now routinely used in our Cytogenetics Laboratory and is built upon the previously published version of the microarray (Baylor College of Medicine, Chromosomal Microarray Analysis, V.5, http://www.bcm.edu/cma/assets/abnormalities.pdf) [Cheung et al., 2005; Lupski and Stankiewicz, 2006]. Clone preparation, chemical modification of the BAC DNA, and efficient attachment of the DNA to an unmodified glass surface to produce the arrays was performed as previously described [Cai et al., 2002]. Hybridization and microarray data analysis were performed as previously described [Yu et al., 2003].

Human Subjects

After the initial CMA result was obtained, informed written consent was obtained from the patient's mother to draw blood on all available family members for additional research studies. Assent was also obtained from the patient's 12-year-old full sister. Blood samples were then obtained from the patient, her mother, and her sister for further analysis. This research was approved by the Institutional Review Board (IRB) for Baylor College of Medicine and Affiliated Hospitals.

Fluorescence In Situ Hybridization (FISH)

FISH was performed on metaphase chromosome preparations from peripheral blood leukocytes to confirm the loss in copy number in the region identified by CMA. Miniprep BAC DNA was labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis, Downers Grove, IL) according to the manufacturer's instructions and used as probes for FISH analysis using standard protocols [Trask, 1991].

Oligonucleotide Microarray Analysis

The Agilent 44K Whole Human Genome Oligo Microarray Kit (Agilent Technologies, Inc., CA) contains 44,000 formatted 60-mer oligonucleotides, representing a compiled view of the human genome at an average resolution of 35 kilobases. The procedures for DNA digestion, labeling, and hybridization were performed according to the manufacturer's instructions, with some modifications. Briefly, we digested genomic DNA from experimental and reference samples with *Alu*I and *Rsa*I. The labeling reaction was performed with Bioprime CGH labeling Module (Invitrogen Corp., Carlsbad, CA) in presence of Cy5-dCTP (for the experimental sample) or Cy3-dCTP (for the reference sample) (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Experimental and reference targets for each hybridization were pooled and incubated with human Cot-1 DNA (Invitrogen), blocking agent, and hybridization buffer. The sample was applied to the array by using an Agilent microarray hybridization chamber, and hybridization was carried out for more than 20 hr at 65°C in a rotating oven. The arrays were then washed in Wash Buffer, and the slides were rinsed with acetonitrile (Sigma-Aldrich Corp., St. Louis, MO) and Stabilization and Drying Solution. The resulting fluorescent signals on the slides were scanned into image files using an Axon microarray scanner (GenePix 4000B from Axon Instruments, Union City, CA).

Microarray image files were quantified using Feature Extraction software (version 9, Agilent Technologies) with default settings. The feature extraction file was then analyzed with CGHAnalytics3.3.28 software with the default filter setting, 1 Mb moving average, and 9.0 threshold setting.

X-Inactivation Studies

The patient's X-inactivation status in peripheral leukocytes was assessed via a PCR-based assay of the methylation pattern at the human androgen receptor locus, essentially as described [Allen et al., 1992]. The PCR products were internally labeled with α^{32}P-dCTP and visualized via SDS polyacrylamide gel electrophoresis.

RESULTS

CMA on the patient revealed a loss of copy number with four adjacent clones (RP11-37P24, RP11-949I9, RP11-489K19, RP11-164A8) on the X chromosome (Fig. 2). This deletion was not visible on routine chromosome analysis, even when the karyotype was re-examined with particular attention to this region (Fig. 3). Subsequent FISH analysis with one of these clones (RP11-949I9) confirmed a deletion at Xq27.3–Xq28 on one of the patient's two X chromosomes in all cells examined (Fig. 4). Additional FISH studies with two more clones that map to this region (RP11-557D6 and RP11-367K15) also showed a deletion in all cells examined (data not shown).

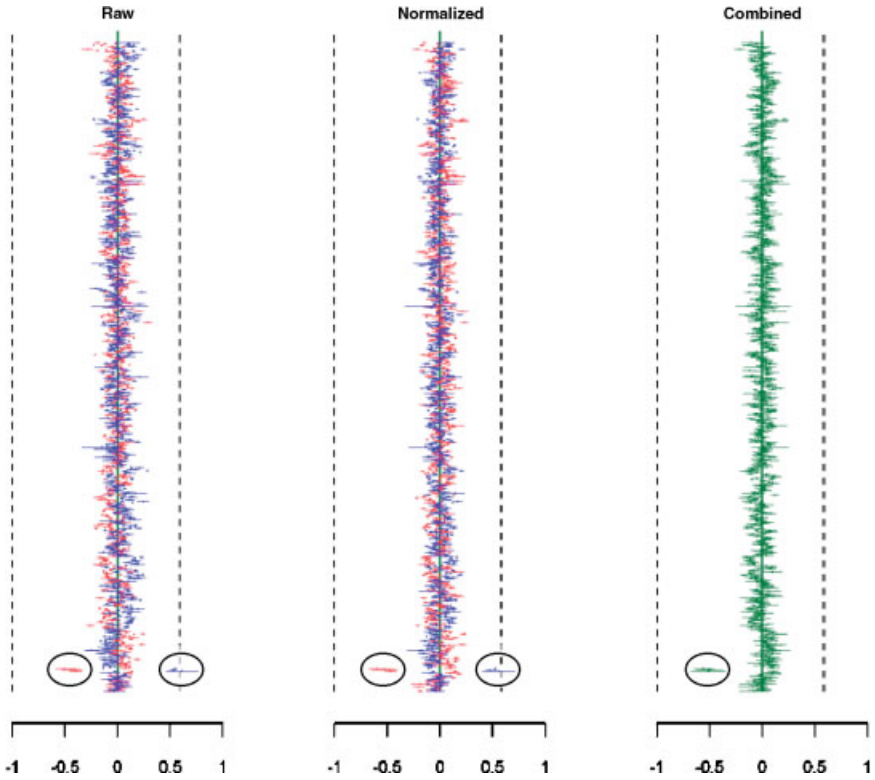

FIG. 2. Chromosomal microarray analysis (CMA) results on the patient. In the column marked "Raw", the mean values of the Test/Reference ratios (with error bars) for each hybridization experiment on the array are shown in red, and the dye reversal data are shown in blue. The effect of normalization of the data is shown by comparing the middle set of data (marked "Normalized") with the raw data. In the "Combined" column on the right, the information from the two hybridizations is combined and depicted as gain or loss in the test sample compared to control sample. In all cases, there is a strong indication of a loss of 4 clones corresponding to Xq27.3–q28 region (circles). See Reference Cheung et al. [2005] for an explanation of the experimental methods and the statistical methods used to convert the raw data to normalized and combined data.

The nearest flanking clones on the microarray (RP11-387H19 and RP11-477H13) both yielded normal signals. These clones have both been sequenced (accession numbers AL358174 and AC136957, respectively) so the estimated size of the deletion based on the CMA and FISH data is less than 4.4 megabases [Lander et al., 2001; Kent et al., 2002; Ross et al., 2005].

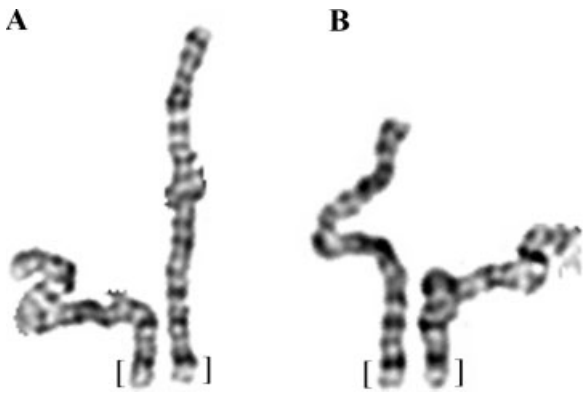

FIG. 3. The deletion is not cytogenetically visible. The patient's X chromosomes from two different cells (**A**,**B**) are shown. The deletion involves material from the Xq27.3 and Xq28 bands, indicated by the brackets, and cannot be seen on the patient's karyotype (~600 band resolution level).

CMA was subsequently performed on the patient's mother and full sister. Neither had any evidence of the deletion (data not shown). Assuming that there was no mistaken paternity and that the father is a healthy 46,XY male, this demonstrates that the patient's deletion was a de novo event. (The paternal X chromosome was screened via the full-sister, as both girls should have inherited the same paternal X chromosome.)

In order to more precisely define the size of the deletion, oligonucleotide microarray analysis was performed. The results (Fig. 5) indicate that the patient has a ~2.7 megabase deletion of the X chromosome. This deletion encompasses three known disease genes: *FMR1*, *FMR2*, and *IDS*. The *MTM1* gene, which is mutated in X-linked myotubular myopathy [Laporte et al., 1996], is located distal to the deletion and does not appear to be involved.

X-inactivation studies on the patient's peripheral leukocytes revealed a random pattern of X-inactivation; no significant skewing was observed (Fig. 6). IDS enzyme activity was subsequently examined in peripheral leukocytes at a clinical laboratory and

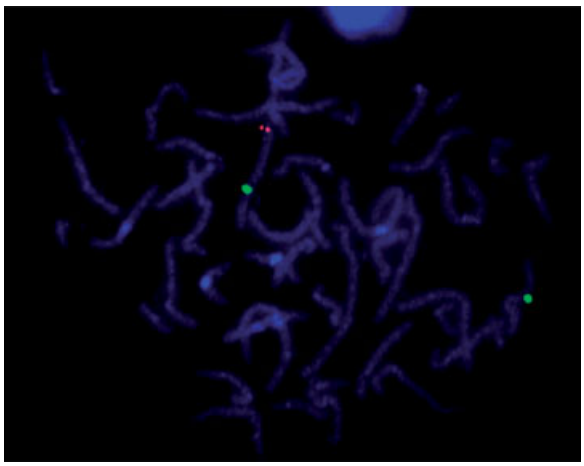

FIG. 4. FISH analysis confirms the presence of an X chromosome deletion. FISH on the patient's peripheral blood leukocytes with BAC RP11-94919 (red) and Vysis' centromere X probe (green) revealed a deletion of one of the two X chromosomes in 10 out of 10 cells examined. A representative cell is shown.

was found to be ~50% of the control specimen, consistent with the finding of random X-inactivation (data not shown).

DISCUSSION

The diagnostic evaluation of the child with developmental delay is a multi-step process that often ends without the establishment of a specific diagnosis for the underlying cause of the child's delay. CMA is a new clinical diagnostic tool that can detect hundreds of different chromosomal deletions and duplications, including submicroscopic lesions that cannot be identified with routine chromosome analysis [Cheung et al., 2005; Lu et al., 2007]. In the present case of a severely delayed 6-year-old girl, CMA detected a ~2.7 megabase microdeletion on the X chromosome involving the *FMR1*, *FMR2*, and *IDS* genes.

The *FMR1* gene is the gene responsible for Fragile X syndrome (OMIM +309550) [Fu et al., 1991; Kremer et al., 1991; Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991]. This disease shows an X-linked pattern of inheritance, and affected males typically have mental retardation, large ears, a long face with a prominent jaw, and postpubertal macro-orchidism [Martin and Bell, 1943; Richards et al., 1981]. Joint hypermobility, particularly in the fingers, is common [Hagerman et al., 1984; Opitz et al., 1984]. Since *FMR1* is located on the X chromosome, typical males have one copy of the gene, while typical females have two copies. Most females who are heterozygous for an *FMR1*, mutations are much less severely affected than their hemizygous male counterparts, and many are phenotypically normal. Intelligence quotients of heterozygous females typically fall in the low-normal or mildly mentally retarded range [de Vries et al., 1996]. Skewing of X-inactivation toward the normal X chromosome is presumed to exacerbate the phenotype in females, whereas skewing of X-inactivation toward the mutant X chromosome is presumed to attenuate the phenotype.

Several patients with Fragile X Syndrome have been reported with a "Prader–Willi-like" phenotype, consisting of obesity; a full, round face; small, broad hands and feet; and developmental delay. This

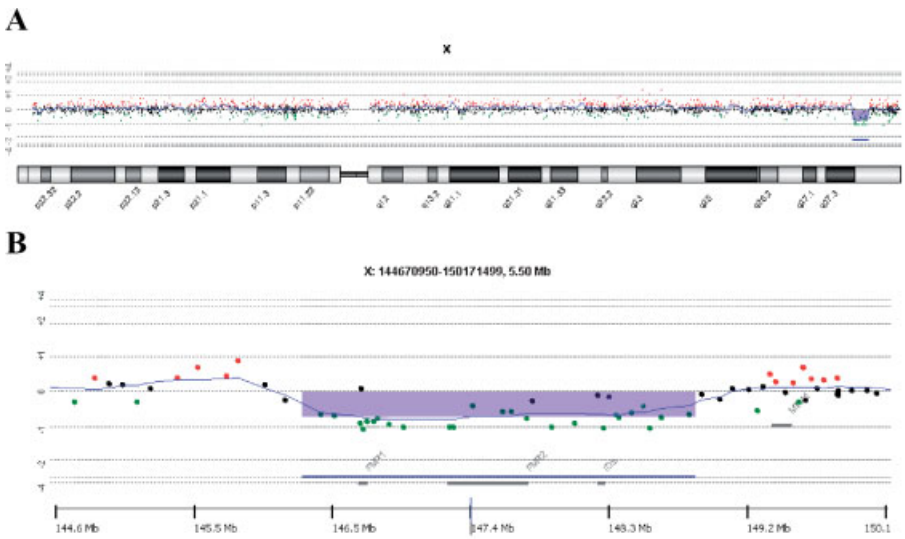

FIG. 5. Oligonucleotide microarray analysis results. **A**: The Agilent 44K microarray test/reference ratio data for the X chromosome are shown. The ideogram of X chromosome shows the location of each probe. With the Agilent aberration detection method, an algorithm finds intervals of consistent high or low log ratios within an ordered set of probes by measuring a set of genomic locations and considering their genomic order to make amplification or deletion calls. A ~2.7 megabase deletion at Xq27.3–Xq28 is observed. **B**: A high-resolution view of the Xq27.3–Xq28 deletion region is shown. The deletion encompasses the *FMR1*, *FMR2*, and *IDS* genes. The *MTM1* gene does not appear to be involved.

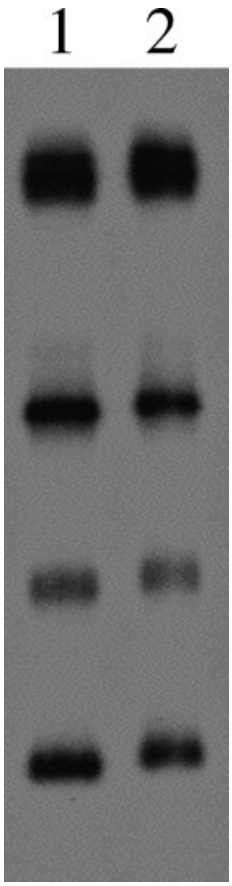

FIG. 6. X-inactivation analysis. PCR with primers for the *HUMARA* locus was performed on the patient's DNA before (**lane 1**) and after (**lane 2**) overnight digestion with the methylation-sensitive restriction endonuclease *Hpa*II, and the products were run on an SDS–PAGE gel. Each allele is represented by two major bands (one for each strand of DNA) due to internal α^{32}P-dCTP incorporation. There was no significant change in the banding pattern, indicating random X-inactivation.

phenotype can be seen in patients with either the classic trinucleotide expansion or a deletion of the *FMR1* gene [de Vries et al., 1993; Schrander-Stumpel et al., 1994; Hammond et al., 1997]. Thus, *FMR1* mutations should be considered in the differential diagnosis of patients who are being evaluated for Prader–Willi syndrome.

Like the *FMR1* gene, the *FMR2* gene (OMIM +309548) is mutated in males with mental retardation [Knight et al., 1993; Chakrabarti et al., 1996; Gecz et al., 1996; Gu et al., 1996]. Most authors report that patients with *FMR2* mutations have relatively mild mental retardation. Affected females have not been reported.

The final known disease gene that is deleted in our patient, *IDS*, codes for iduronate 2-sulfatase. This enzyme is deficient in patients with Hunter syndrome (Mucopolysaccharidosis Type II, OMIM +309900) [Bach et al., 1973; Wilson et al., 1990]. Fewer than a dozen affected females with this disease have been reported, and analysis has typically revealed a mutation in one copy of the *IDS* gene along with extremely skewed X-inactivation, such that the mutant allele is expressed and the normal *IDS* allele is transcriptionally silenced [Tuschl et al., 2005].

Since our patient had random X-inactivation and substantial IDS activity in her peripheral blood, the deletion of one of her two copies of *IDS* is unlikely to be a significant contributor to her phenotype. Thus, her developmental delay is most likely the result of the loss one copy each of the *FMR1* and *FMR2* genes, and perhaps one or more other genes in the region.

Deletions of both *FMR1* and *FMR2* are rare. In males, deletions of both *FMR1* and *FMR2* invariably lead to significant mental retardation. Epilepsy and joint hypermobility are common but are not reported in all cases [Tarleton et al., 1993; Albright et al., 1994; Wolff et al., 1997; Moore et al., 1999; Fengler et al., 2002]. If the *IDS* gene is involved, the child will also have Hunter syndrome [Birot et al., 1996]. In females, the phenotype is dependent not only on the extent of the deletion but also on the individual's X-inactivation status. A deletion of *FMR1*, *FMR2*, and *IDS*—when combined with extreme skewing of X-inactivation toward the normal X chromosome—yields a phenotype of mental retardation and Hunter syndrome [Clarke et al., 1990, 1991, 1992], while significant but incomplete skewing toward the normal X chromosome (i.e. the normal X is active in only ~20% of peripheral leukocytes) produces mental retardation but not Hunter syndrome [Dahl et al., 1995]. One reported individual with random X-inactivation had significant mental retardation [Birot et al., 1996], while two with significant but incomplete skewing toward the mutant X chromosome still had mild mental impairment [Birot et al., 1996; Wolff et al., 1997]. Only extreme skewing of X-inactivation toward the mutant X chromosome has been shown to yield normal intellect [Wolff et al., 1997].

In our patient, the identification of an *FMR1-FMR2-IDS* deletion, coupled with the finding of random X-inactivation, provides a complete explanation of this child's phenotype. Her sister did not have the deletion, suggesting that there may be a second genetic lesion in the pedigree that has not yet been identified. This case illustrates that CMA is a powerful assay not only for identifying the root cause of unexplained developmental delay in a child, but also for suggesting additional testing (in this case, X-inactivation studies and IDS enzyme activity) that may clarify the phenotype.

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