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TECHNICAL ADVANCE

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Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability. It is most frequently caused by an abnormal expansion of the CGG trinucleotide repeat (>200 repeats) located in the promoter of the fragile X mental retardation gene (*FMR1*), resulting in promoter DNA hypermethylation and gene silencing. Current clinical tests for FXS are technically challenging and labor intensive, and may involve use of hazardous chemicals or radioisotopes. We clinically validated the Illumina Infinium HumanMethylation450 DNA methylation array for FXS screening. We assessed genome-wide and *FMR1*-specific DNA methylation in 32 males previously diagnosed with FXS, including nine with mosaicism, as well as five females with full mutation, and premutation carrier males (*n* = 11) and females (*n* = 11), who were compared to 300 normal control DNA samples. Our findings demonstrate 100% sensitivity and specificity for detection of FXS in male patients, as well as the ability to differentiate patients with mosaic methylation defects. Full mutation and premutation carrier females did not show *FMR1* methylation changes. We have clinically validated this genome-wide DNA methylation assay as a cost- and labor-effective alternative for sensitive and specific screening for FXS, while ruling out the most common differential diagnoses of FXS, Prader-Willi syndrome, and Sotos syndrome in the same assay. *(J Mol Diagn 2016, 18: 834—841; http://dx.doi.org/10.1016/j.jmoldx.2016.06.005)*

Fragile X syndrome [FXS; Online Mendelian Inheritance in Man (OMIM) 300624] is the most common inherited cause of intellectual disability, and is one of the most frequently ordered constitutional genetic tests.[1] The features of FXS include moderate to severe mental retardation, macroorchidism, and distinct facial features. The most common cause of FXS is an abnormal expansion in the number of trinucleotide CGG repeats located in the 5′ untranslated region of the fragile X mental retardation gene (*FMR1*) at Xq27.3. Large CGG expansions result in DNA hypermethylation and, consequently, inhibition of *FMR1* transcription. However, other mutational mechanisms, such as deletions of *FMR1*, can cause FXS.[2] Both mechanisms result in deficiency of the gene product, the fragile X mental retardation protein, which is

an RNA binding protein that regulates protein synthesis in dendrites.[3]

Depending on the number of CGG repeats, four main types of alleles are defined, which correlate with different clinical manifestations.[3,4] Normal alleles have up to 44 repeats, premutation (PM) alleles have 55 to 200 repeats, and full mutation (FM) alleles contain >200 repeats. In addition, alleles containing 45 to 54 repeats are commonly referred to as being in a gray zone, and are precursors for PM alleles. As expected for an X-linked disorder, full

mutations are penetrant in all males, who are also the index probands in families with fragile X. The physical and behavioral features seen in males with FXS have been reported in females heterozygous for the full mutation, but with low penetrance and mild expressivity. The lower penetrance in females is explained by two copies of X chromosome and/or nonrandom X inactivation.[5] Interestingly, mosaicism for the methylated FM and an unmethylated premutation account for >40% of affected males who demonstrate an atypical mild clinical manifestation of the disease.[6,7] Premutation alleles are not hypermethylated and do not result in FXS-related features, but can in some patients cause the fragile X—associated tremor/ataxia syndrome, which is characterized by late-onset progressive cerebellar ataxia and intention tremor.[8,9] Also, approximately 20% of premutation carrier females may develop primary ovarian insufficiency, which is defined as cessation of menses before age 40 years.[10] However, females carrying a premutation may transmit a full mutation allele to their children. The expansion of premutation to full mutation alleles occurs during transmission of the maternal X chromosome, but not the paternal X chromosome, to the offspring.[11]

The confirmation of a FXS diagnosis at a molecular level is technically complex. Current guidelines are based on the characterization of trinucleotide (CGG) repeat number related to allele size, as well as DNA methylation.[1] Two main approaches that are commonly used in clinical laboratories include the PCR and Southern blot analysis,[12−14] using the *FMR1* probe first characterized by Rousseau et al[13] in 1991. Amplification by PCR using primers flanking the CGG repeat regions allows the identification of the approximate number of repeats present in each allele. This PCR approach has several limitations. Most significantly, alleles with a large number of repeats can be difficult to amplify and may fail to be detected by a PCR-based approach. Furthermore, amplification may favor the smaller allele and skew the relative ratio of normal to abnormal alleles. For example, a female with one normal allele and one large nonamplifiable mutant allele could appear to only have the normal allele.[1] Similarly, male patients who are mosaics for a premutation along with a full mutation could appear to have only the premutation. Southern blotting is often used in parallel with PCR analysis to better characterize the patients with expanded alleles in the full mutation range. *FMR1* analysis by Southern blot enables the detection of all allele size ranges, but cannot be used for precise expansion sizing and assessment of the methylation status. Furthermore, Southern blot analysis requires a large amount of genomic DNA (10 μg), is labor intensive, is time-consuming, and may involve the use of hazardous and/or radioactive chemicals.[15]

Newer PCR-based methods have been developed to improve the detection of both large triplet repeat expansions, as well as the characterization of *FMR1* methylation status. Triplet-repeat primer PCR can reliably detect all full mutation alleles. The incorporation of capillary electrophoresis after triplet-repeat primer PCR facilitates the accurate repeat size determination up to approximately 200 repeats.[16,17] In addition, this technique enables the detection of mosaic and apparently homozygous females, because it ensures that normal allele does not outcompete the expanded allele during the amplification.[1] A more cost-effective screening platform using direct triplet-repeat primer PCR with melting curve analysis was recently proposed.[18] This method could be used as a first-tier screen to identify triplet repeat expansion carriers. In addition, methylation-specific PCR techniques have been used in the diagnosis of FXS, including quantitative methylation-sensitive PCR,[19] methylation-specific melting curve analysis,[20] and methylation-specific−quantitative melt analysis.[21]

Herein, we describe a highly sensitive, cost-effective, genome-wide DNA methylation screening technology that is capable of reliably screening for FXS. In addition to screening for FXS, this DNA methylation assay allows for comprehensive methylation screening across the entire genome, and can be useful as a supplement to copy number microarray analysis and gene/genome sequencing. Specifically, in addition to screening for FXS, this assay also enables simultaneous sensitive and specific screening for Prader-Willi syndrome (PWS) as well as for Sotos syndrome, which are the two conditions most commonly considered in the differential diagnosis of FXS.[22] The advantages of this method make it applicable for routine use in molecular diagnostic laboratories and screening programs, as part of the more comprehensive testing repertoire in families where FXS is ultimately identified.

Materials and Methods

Subjects and Blood Collection

Peripheral blood DNA samples were collected from patients referred for FXS genetic testing at the Greenwood Genetic Center. All patients were consented and counseled for FXS testing as part of their clinical referral. Genomic DNA was extracted from peripheral blood using standard techniques, and CGG repeat size was determined by the Molecular Diagnostic Laboratory at the Greenwood Genetic Center using the PCR protocol described by Fu et al,[23] with modifications based on the information reported by Houdayer et al.[24] In addition, a commercially available assay from Abbott Molecular (Des Plaines, IL) was used to detect the presence of *FMR1* expansion mutations of all sizes.[25] To confirm the presence of trinucleotide expansion within the *FMR1* locus, samples were also subjected to restriction enzyme digestion, followed by Southern blot analysis using the DNA probe, StB12.3. Based on CGG repeat size, our samples were grouped as follows: i) males with FM, ii) males with PM, iii) males with mosaicism

(mosaic-FM; FM+ normal/PM allele), iv) FM heterozygous females, and v) PM females.

Methylation Array and Data Analysis

DNA methylation analysis was performed using the Illumina HumanMethylation450 microarray (San Diego, CA), according to the standard protocol at the Genetic and Molecular Epidemiology Laboratory at McMaster University. In addition to the *FMR1* promoter, this array covers approximately 485,000 human genomic methylation CpG sites, including 99% of RefSeq genes and 96% of CpG islands. Methylation data were converted to .idat files using Genome Studio software version 2011.1 (Illumina, Victoria, BC, Canada), and imported into Partek Genomic Suite software version 6.6 (St. Louis, MO) for analysis. Initial analyses were generated by comparing the following groups: i) all FXS males (FM and FM-mosaic; $n = 32$) versus male controls ($n = 210$), ii) mosaic-FM males ($n = 9$) versus male controls, iii) mosaic-FM males versus FM males ($n = 23$), iv) FM females ($n = 5$) versus female controls ($n = 151$), and v) PM males ($n = 11$) and PM females ($n = 11$) versus sex-matched controls. Analysis of variance test was performed to generate a probe-level statistical analysis, including *P* value (*t*-test), F value (signal to noise), and estimate (net methylation difference). Genomic regions with significant DNA methylation patterns were identified that met the following statistical criteria: i) a minimum of three consecutive probes with probe-level differential methylation $P < 0.01$, ii) a mean F value across the region >50, and iii) an estimate value >0.15 (±15% increase or decrease in methylation). Significant regions were mapped against the CpG islands and gene promoter regions. Last, regions with most significant methylation changes, including the *FMR1* locus, were filtered and annotated in respect to their distance to CpG islands and gene promoters. Data were visualized using Partek Genomic Suite genomic browser, and individual probes at the *FMR1* promoter region were tested for statistical differences between FXS FM, mosaics, and controls.

Results

FMR1 Methylation Analysis in the FXS Patients

To assess the ability to discern differential methylation levels at the *FMR1* gene promoter, we first compared DNA methylation levels in control, non-FXS males and females (Figure 1). Control males showed hypomethylation at the promoter CpG island of the *FMR1* gene, whereas females, with one inactive X chromosome, showed hemimethylation (approximately 50% methylation) at this locus ($P < 0.01$, estimate = 0.31, and F = 1310). Trinucleotide CGG repeat size from the 32 males with FXS ranged from 200 to 2000 repeats, nine (28%) of whom also showed mosaicism with the expanded triplet repeat allele and a premutation or a normal allele (Supplemental Table S1). Eleven males with

premutation had repeats ranging from 55 to 160 (Supplemental Table S1). Female patients included five with FM and 11 premutation carriers (Supplemental Table S2). Genome-wide DNA methylation analysis of all males with FXS revealed hypermethylation at the *FMR1* promoter site within the CpG island (Figure 1, A−C). This included significant hypermethylation at a 1482-bp region containing 13 probes within the *FMR1* promoter ($P = 0.0000446$, estimate = 0.66, and F = 1883). Average methylation level across this region was 0.75 (SD = 0.19) and 0.09 (SD = 0.01) for males with FXS and male controls, respectively (Supplemental Table S3). At a methylation level cutoff of 0.2 (20%), the sensitivity and specificity of the methylation array to detect FXS patients in this study was 100% (Figure 2A). All FM males had a robust hypermethylation (0.84; SD = 0.10), whereas males with mosaicism exhibited a relative attenuation of methylation signal (0.54; SD = 0.21) (Figure 1, A−C, and Supplemental Table S3). The size of the repeat in nonmosaic FM males did not appear to correlate with the extent of methylation (Supplemental Figure S1), but was consistent with near complete methylation across the CpG island. Statistically significant hypermethylation in FXS male patients was evident across all 13 probes ($P < 0.01$) (Supplemental Figure S2). In patients with mosaic-FM *FMR1* mutation, statistically significant hypermethylation at a single-probe level was also evident, albeit at lower levels than in the FM patients ($P < 0.01$) (Supplemental Figure S2). Females with one FM allele, including one female with mild intellectual disability and one female with behavioral problems, did not show differential methylation at the *FMR1* locus compared to control females (Figure 1, D and E, Figure 2B, and Supplemental Table S4). Similarly, the presence of a premutation at *FMR1* was not associated with changes in *FMR1* methylation in both males and females (Figure 1, A and D). These results demonstrate the ability of genome-wide methylation array to identify FM FXS males with 100% sensitivity and specificity, as well as to effectively differentiate mosaic-FM patients from controls or FM patients, as visualized either across the *FMR1* promoter as a whole (Figure 1, B and D) or at a single probe resolution (Supplemental Figure S2). Significant methylation changes were not observed in female with full mutation (Figure 1E).

Genomic DNA Methylation Analysis in the FXS Patients

In addition to assessment of the *FMR1* locus, genome-wide DNA methylation screening allows for the concurrent identification of additional aberrant methylation loci across the genome, including >99% of gene promoters and all known imprinted loci. Genomic methylation analysis of each FXS male also showed some locus-specific methylation changes (Supplemental Table S5). As an example, one FXS male patient demonstrated hypermethylation of a CpG island overlapping the retinitis pigmentosa GTPase regulator-interacting protein (*RPGRIP1*) gene (OMIM 605446) (Supplemental Table S5). *RPGRIP1* is associated with cone-rod dystrophy
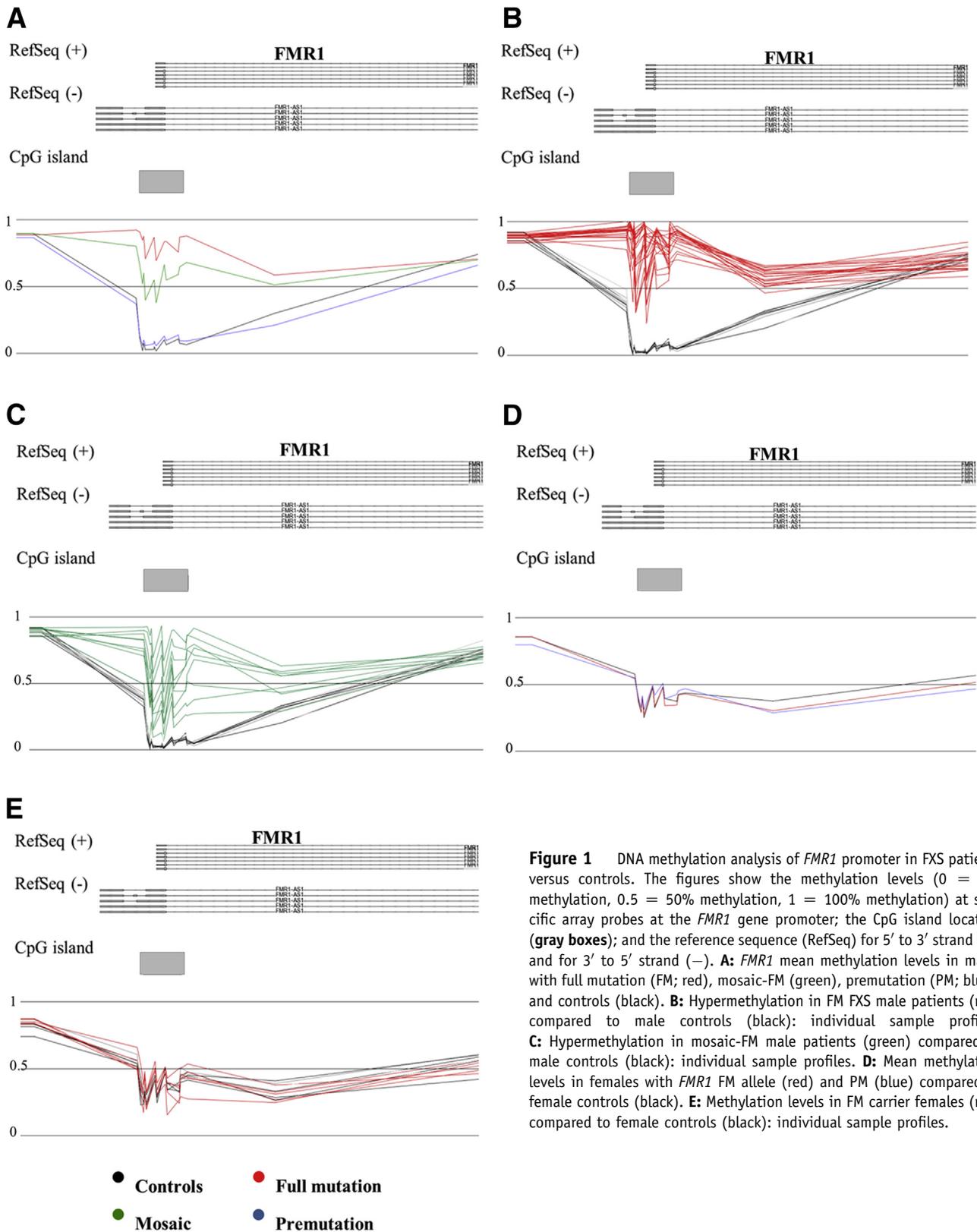
Figure 1 DNA methylation analysis of *FMR1* promoter in FXS patients versus controls. The figures show the methylation levels (0 = 0% methylation, 0.5 = 50% methylation, 1 = 100% methylation) at specific array probes at the *FMR1* gene promoter; the CpG island location (**gray boxes**); and the reference sequence (RefSeq) for 5′ to 3′ strand (+) and for 3′ to 5′ strand (−). **A:** *FMR1* mean methylation levels in males with full mutation (FM; red), mosaic-FM (green), premutation (PM; blue), and controls (black). **B:** Hypermethylation in FM FXS male patients (red) compared to male controls (black): individual sample profiles. **C:** Hypermethylation in mosaic-FM male patients (green) compared to male controls (black): individual sample profiles. **D:** Mean methylation levels in females with *FMR1* FM allele (red) and PM (blue) compared to female controls (black). **E:** Methylation levels in FM carrier females (red) compared to female controls (black): individual sample profiles.

13 (OMIM 608194) and Leber congenital amaurosis 6 (OMIM 613826). Another FXS male individual showed hypermethylation at the CpG island in the promoter of the *AKR7L* gene (OMIM 608478) (Supplemental Table S5). This gene encodes an aldo-keto reductase, which is involved in the detoxification of aldehydes and ketones. The clinical significance of these locus-specific hypermethylation/hypomethylation variants in these patients is unclear at this time.
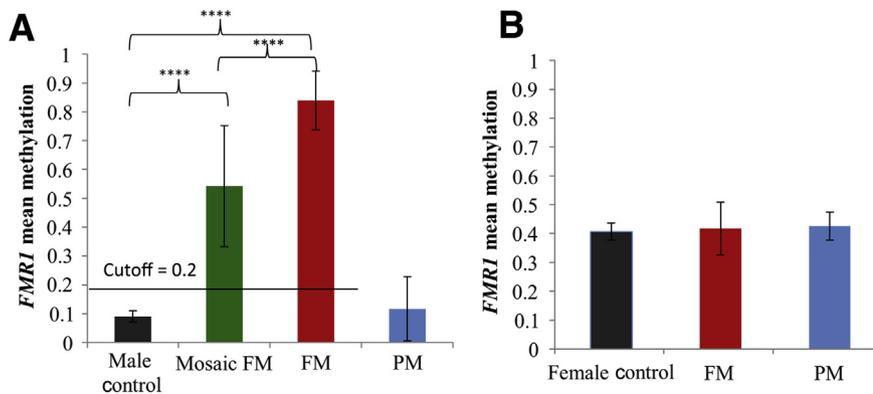
Figure 2 **A:** Mean methylation for all 13 probes at *FMR1* gene promoter in male controls, premutation males (PM), mosaic—full mutation (FM), and FM FXS patients. A methylation value cutoff of 0.2 enables accurate identification of FXS patients. **B:** Mean methylation for all 13 probes at *FMR1* gene promoter in female controls, PM, and FM female carriers. ****$P < 0.0001$ (methylation level is significantly different).

Discussion

In this study, we have clinically validated a novel FXS screening approach. We have demonstrated 100% sensitivity of detecting affected males with full mutation and full mutation mosaicism. Although we did not observe methylation differences in relation to the size of the FM expansion, this is consistent with previous findings that show that only full mutation alleles undergo promoter methylation with subsequent *FMR1* gene silencing and loss of fragile X mental retardation protein, and that the severity of FXS does not appear to be correlated to the absolute size of the mutant allele.[12] Alternatively, affected mosaic-FM males tend to have a less marked clinical phenotype, as well as higher intelligence quotient scores than those with fully methylated alleles, which is attributed to residual expression of the fragile X mental retardation protein.[26] Mosaicism may occur both in terms of repeat length and methylation status (methylation mosaics). However, it is the level of DNA methylation at the *FMR1* promoter that equates to the percentage of cells with loss of fragile X mental retardation protein expression. Our findings demonstrate that this assay effectively distinguishes mosaic-FM males from males with nonmosaic FXS. As anticipated, this assay did not detect methylation differences at the *FMR1* promoter in females with a full mutation allele, even though some of these females presented a mild phenotype of intellectual disability or behavior problems. This corroborates previous findings that X chromosome inactivation, and consequently *FMR1* promoter methylation, occurs preferentially in the chromosome with the *FMR1* mutated allele.[27] It was previously shown that in FM females, where some elevation of the *FMR1* methylation can be demonstrated, methylation status generally does not correlate with clinical manifestation of the disease symptoms.[19] In addition, this assay is not able to detect FXS in the rare cases (<0.05%) where the cause of FXS is deletion of the *FMR1* gene in male patients. However, in females, FMR1 deletion would be detectable because of the loss of the hemimethylated profile, resulting in either hypermethylation or hypomethylation at that locus, depending on whether the deletion is on the active or the inactive X.

One clear advantage of this DNA methylation array approach over PCR-based methods for FXS screening is the genome-wide coverage of the assay, which enables the identification of additional locus-specific methylation changes that may be associated with the patient phenotype by acting as modifiers of the disease. A previous study that compared nine FM FXS males with 53 controls using this same methylation array has confirmed the ability to detect *FMR1*-specific methylation changes, but was unable to find common methylation changes beyond the *FMR1* gene locus.[28] The group analysis on our larger cohort of 23 FM males and nine mosaic FM males versus 210 controls has corroborated the *FMR1* methylation findings. In addition, in some patients, we were able to demonstrate the existence of unique patient-specific methylation changes in genes such as *AKR7L* and *RPGRIP1* (Supplemental Table S5) that may have additional phenotypic consequences independent of the *FMR1* defects in each patient. A large reference cohort enables more sensitive assessment for such patient-specific changes, as we have previously demonstrated.[29,30] Currently, there is little known about such gene-specific DNA methylation changes, and how they may affect and/or modify clinical presentation in individual patients. Expanding the databases of DNA methylation profiles in patients with defined and distinct clinical phenotypes will enable us to differentiate recurrent clinically significant DNA methylation defects from nonpathogenic epigenetic polymorphisms in a similar manner to which DNA copy number assessment evolved using microarrays over the years.

In contrast, some genomic DNA methylation changes, including imprinting defects and genetically caused episignatures, have a clearly defined clinical association. Two disorders that are most commonly considered in the differential diagnosis of FXS because of the similar clinical presentation, Sotos syndrome and PWS,[22] possess unique DNA methylation signatures. Diagnosis of PWS involves DNA-based methylation testing to detect abnormal parent-specific loss of imprinting within the Prader-Willi critical region at the *SNRPN* gene locus on chromosome 15.[31] Sotos syndrome is caused by loss-of-function mutations of the

NSD1 gene, which has recently been shown to result in a highly sensitive and specific DNA methylation signature across multiple genomic loci.[32] In Figure 3, we demonstrate the ability of this DNA methylation array to detect methylation defects at the *SNRPN* locus associated with PWS and its reciprocal imprinting condition Angelman syndrome (Figure 3A), as well as defining two representative DNA methylation signature loci associated with Sotos syndrome (Figure 3B). These data demonstrate that in addition to FXS screening, the DNA methylation assay we have described can be used as a concurrent test for the differential diagnoses of PWS and Sotos syndrome in these patients.

Because most families with FXS are ascertained through the diagnosis in the affected male probands, this DNA methylation array can be implemented as a primary screen, thereby replacing costly, potentially hazardous, and labor-intensive Southern blots and long-range PCR

assays. In the small percentage of families in which FXS is detected (<1%), the more complex trinucleotide analysis assays can be used for further testing and risk assessment in matrilineal relatives. Similarly, in patients who are shown to have a DNA methylation signal consistent with PWS or Sotos syndrome, follow-up molecular and cytogenetic testing may be warranted. Therefore, this assay is meant to be used as a screening tool to identify families with FXS, where male probands are identified with the clinical features. Because it is the males who present with more severe phenotypes, they are more commonly to be identified first in these families. The assay, however, is not meant to replace further molecular workup, including the assessment of the repeat size and the identification of possible premutation. Also, the assay is not meant to identify the full mutation carrier females, affected or not. Because of the preferential skewing of the X-inactivation to the abnormal
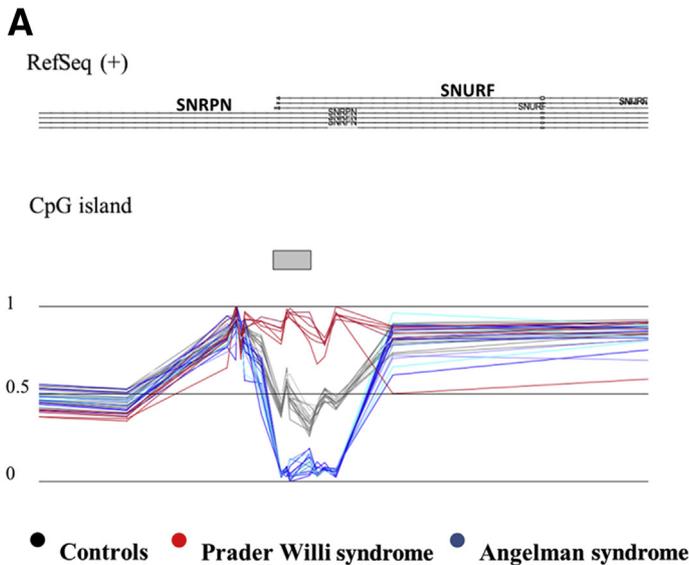

Figure 3 Differential DNA methylation analysis using genome-wide methylation array. The figures show the methylation levels (0 = 0% methylation, 0.5 = 50% methylation, 1 = 100% methylation); the CpG island location (**gray boxes**); and the reference sequence (RefSeq) for 5′ to 3′ strand (+) and for 3′ to 5′ strand (−). **A:** DNA methylation pattern at *SNRPN* locus on chromosome 15 in patients with Prader-Willi syndrome (red) and Angelman syndrome (blue). **B:** Episignature in patients with Sotos syndrome; two patients (blue and red) show similar DNA methylation changes at two loci on chromosome 6 when compared with controls (black). Both genes have recently been described as part of the unique Sotos episignature.[32]
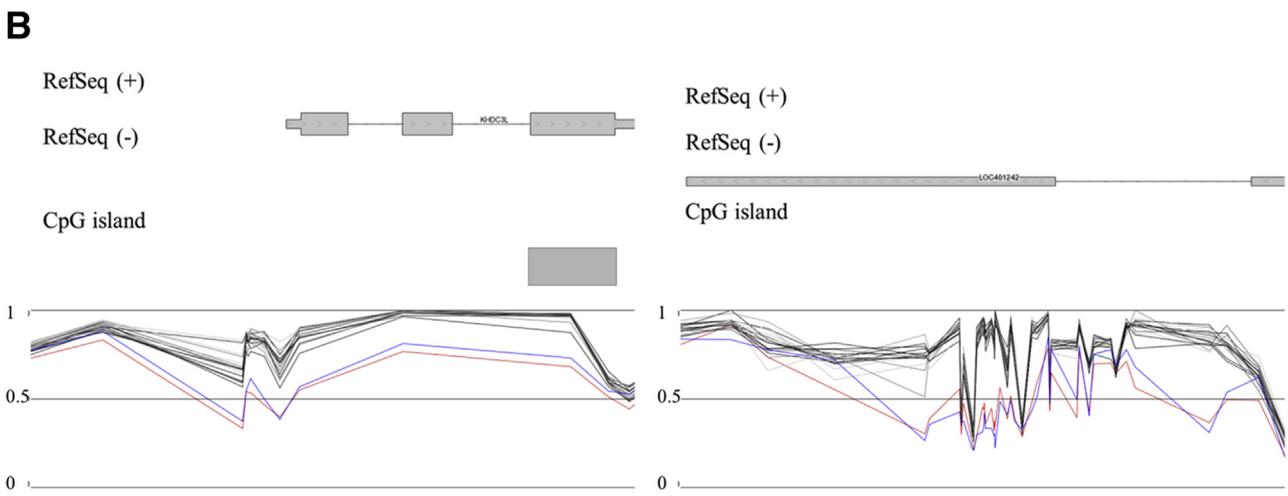
X chromosome, and possibility of differential skewing patterns in the different tissues, full mutation females may not show differential methylation at the *FMR1* locus in peripheral blood. Two of the females tested in our cohort have been confirmed to have mild cognitive and behavioral abnormalities, and did not show any abnormal DNA methylation pattern, including at the *FMR1* locus.

In a similar manner to the way in which cytogenetic microarray testing has largely replaced single copy number analyses, such as fluorescence *in situ* hybridization, for diagnosis of constitutional genetic disorders in the past decade, genome-wide DNA methylation screening has a potential to displace many of the known epigenetic single analyte tests. Our laboratory has validated this assay for sensitive and specific detection of all known imprinting disorders, including PWS, Angelman syndrome, Beckwith-Wiedemann syndrome, and Russell-Silver syndrome (data not shown). Furthermore, genome-wide DNA methylation screening will enable parallel screening of genetic conditions associated with DNA methylation episignatures. In addition to the previously described episignatures Sotos syndrome[32] and the X-linked intellectual disability caused by the *KDM5C* gene,[33] our laboratory has recently demonstrated unique DNA methylation signatures in patients with three other conditions, including Floating-Harbor syndrome (R.L. Hood et al, unpublished data), caused by mutation in the *SRCAP* gene, cerebellar ataxia, and deafness, narcolepsy syndrome (K.D. Kernohan et al, unpublished data), caused by mutations in the *DNMT1* gene, and X-linked α-thalassemia/mental retardation, caused by mutation in the *ATRX* gene (L.C. Schenkel et al, unpublished data). Analogous to the clinical cytogenetic microarray screening, as new epigenetic signatures become defined, utility of genome-wide DNA methylation screening is expected to evolve beyond single-disorder testing to encompass multidisorder screening. Utilization of this technology in routine clinical practice will enable the discovery of new epigenetic biomarkers and will enhance our understanding of human disease etiology, but will also potentially yield many findings that we currently are not able to clinically interpret. Identification of such epigenetic variants of unknown clinical significance will require delivery of testing to be performed in regulated clinical laboratories, the development and implementation of clinical and laboratory testing guidelines, and availability and integration with pretest and post-test genetic counseling.

In conclusion, we have demonstrated a genome-wide DNA methylation approach for sensitive and specific screening of patients with clinical features of FXS, as a cost- and labor-effective alternative to the currently used monogenic testing approaches. Moreover, this assay enables concurrent assessment of disorders commonly considered as the differential diagnosis for FXS, including Sotos syndrome and PWS, along with other imprinting syndromes and constitutional genetic disorders associated with episignatures. Therefore, the use of genome-wide DNA methylation array as a first-line testing will not only allow the identification of a broad range of known genetic conditions, but will also provide genome-wide information on the DNA methylation patterns in patients with complex phenotypes.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2016.06.005*.

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