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ARTICLE

DNA methylation episignature testing improves molecular diagnosis of Mendelian chromatinopathies

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ABSTRACT

Purpose: Chromatinopathies include more than 50 disorders caused by disease-causing variants of various components of chromatin structure and function. Many of these disorders exhibit unique genome-wide DNA methylation profiles, known as episignatures. In this study, the methylation profile of a large cohort of individuals with chromatinopathies was analyzed for episignature detection.

Methods: DNA methylation data was generated on extracted blood samples from 129 affected individuals with the Illumina Infinium EPIC arrays and analyzed using an established bioinformatic pipeline.

Results: The DNA methylation profiles matched and confirmed the sequence findings in both the discovery and validation cohorts. Twenty-five affected individuals carrying a variant of uncertain significance, did not show a methylation profile matching any of the known episignatures. Three additional variant of uncertain significance cases with an identified *KDM6A* variant were re-classified as likely pathogenic ($n = 2$) or re-assigned as Wolf-Hirschhorn syndrome ($n = 1$). Thirty of the 33 Next Generation Sequencing negative cases did not match a defined episignature while three matched Kabuki syndrome, Rubinstein-Taybi syndrome and BAFopathy respectively.

Conclusion: With the expanding clinical utility of the EpiSign assay, DNA methylation analysis should be considered part of the testing cascade for individuals presenting with clinical features of Mendelian chromatinopathy disorders.

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Introduction

Epigenetic chromatin dysregulation has emerged as a recurring mechanism in the etiology of a group of neurodevelopmental disorders known as chromatinopathies, caused by the genetic alterations of various components of the epigenetic machinery.[1] Currently, the molecular diagnosis for these disorders is principally based on Next Generation Sequencing (NGS) of exomes or targeted gene panels. Although both techniques are effective in identification of disease-causing variants, they possess significant limitations. For instance, variants of uncertain significance (VUS) are frequently encountered and result in considerable diagnostic uncertainty having impacts on the clinical management of patients. Assessment of a VUS to determine pathogenicity may require family segregation studies or functional assays if available. Moreover, NGS-based techniques are primarily focused on the coding sequence having minimal coverage of the flanking intronic DNA regions and limiting the detection of copy number variants, a known pathogenetic mechanism in chromatinopathies.[2]

There is a growing evidence that variants in genes that regulates chromatin remodelling show unique and disorder-specific DNA methylation patterns, known as episignatures.[3] We and others showed that several patients with rare disorders present with DNA methylation episignatures that are highly sensitive and specific for each disorder. Currently over 40 rare disorders exhibit these genome-wide DNA methylation profiles, detectable in peripheral blood. As such, DNA methylation testing has recently been implemented in clinical diagnosis of patients with rare disorders.[4]

Here we assessed the DNA methylation profiles in a cohort of 129 probands with a clinical presentation suggestive of a chromatinopathy, representing approximately 50 OMIM disorders caused by a gene involved in chromatin regulation. Our results demonstrate that episignature analysis is an effective diagnostic modality for this group of disorders. Detection of an episignature can help establish, as well as improve and define a molecular diagnosis achieved by the standard DNA sequencing approaches. This study also expands the utility of the clinically available EpiSign test as a molecular assay for individuals affected with chromatinopathy disorders.

Materials and Methods

Subjects dataset and cohorts

Our experimental dataset consisted of 129 patients grouped into four cohorts with analysis completed by a targeted NGS chromatinopathies panel (ChrPan)[2] and a genome-wide DNA methylation assay known as EpiSign.[4] The discovery cohort consisted of 48 patients with a well-defined clinical diagnosis associated with a pathogenic variant in a gene included in EpiSign (Table 1 and Supplemental Table 1).[4] The EpiSign assay was also used as the syndrome list in sample collection for the remaining three cohorts: (1) validation cohort consisted of 20 patients referred for the ChrPan without a clinical description of their phenotypic presentation and found to carry a pathogenic or likely pathogenic variant (Supplemental Table 2); (2) uncertain cohort consisted of 28 patients with a clinical diagnosis based on a well described phenotype and carrying a VUS (Supplemental Table 3), and (3) negative cohort consisted of 33 patients lacking the identification of any syndrome associated variant detected by ChrPan (Supplemental Table 4).

DNA samples preparation

Patients and their relatives were enrolled after obtaining appropriate consent from the physicians in charge, and approval from the local ethics committees. Genomic DNA was extracted from fresh and/or frozen peripheral blood leukocytes of patients and their available family members using different protocols.

DNA Methylation Test and Data Analysis

Analysis of the DNA methylation array data was performed by the clinical bioinformatics laboratory (Verspeeten Clinical Genome Centre, London Health Sciences) using Illumina Infinium EPIC arrays. Methylation data for each sample were compared to the established DNA methylation episignatures for the 43 disorders (Table 1) which are part of the EpiSign clinical test. EpiSign analysis utilized the Episign Knowledge Databases, a clinical database with >5000 peripheral blood DNA methylation profiles including disorder-specific reference cohorts and population reference controls (general population samples with various age and racial backgrounds) housed at Verspeeten Clinical Genome Centre (https://www.lhsc.on.ca/palm/molecular.html). Individual DNA methylation data for each subject were compared to the Episign Knowledge Databases using the Support Vector Machine based classification algorithm for EpiSign disorders. Methylation Variant Pathogenicity (MVP) score is generated ranging between 0 and 1, representing the confidence of prediction for the specific class the Support Vector Machine was trained to detect. Conversion of Support Vector Machine decision values to these scores was carried out according to the Platt's scaling method.[5] Classification for a specific EpiSign disorder included MVP score assessment with a general threshold of >0.5 for positive, <0.1 negative, 0.1-0.5 for inconclusive or low confidence, in combination with hierarchical clustering and multidimensional scaling of the subject's methylation data relative to the disorder specific EpiSign probe sets and population controls. A detailed description of this analytics protocol was described previously.[3,6]

Table 1 Disorders detected by EpiSign v2 and NGS ChrPan

Syndrome	Causative Gene(s)/Region(s)	EpiSign v2	NGS ChrPan
Alpha-thalassemia/mental retardation syndrome, X-linked	*ATRX*	Episignature	+
Angelman syndrome	*SNRPN* promoter	Imprinting defect	-
Au-Kline syndrome	*HNRNPK*	-	+
Autism, susceptibility to, 18	*CHD8*	Episignature	+
BAFopathies: Coffin-Siris syndrome, types 1-4 & Nicolaides-Baraitser syndrome	*ARID1B, ARID1A, SMARCB1, SMARCA4, SMARCA2, SMARCE1, DPF2, ARID2, HUWE1, MBD5, HDAC4*	Episignature, excluding *HUWE1*, *HDAC4*, *MBD5*	+
Beckwith-Wiedemann syndrome	*ICR2*, *NSD1*, *CDKN1C*	Imprinting defect, excluding *NSD1* and *CDKN1C*	+
Baraitser-Winter syndrome	*ACTB, ACTG1*	-	+
Blepharophimosis Intellectual disability syndrome, SMARCA2 type	*SMARCA2*	Episignature	+
Bohring-Opitz syndrome	*ASXL1*	-	+
Borjeson-Forssman-Lehmann syndrome	*PHF6*	Episignature	+
Cerebellar ataxia, deafness, and narcolepsy, autosomal dominant	*DNMT1*	Episignature	+
CHARGE syndrome	*CHD7, SEMA3E*	Episignature, excluding *SEMA3E*	+
Chromosome 7q11.23 duplication syndrome	7q11.23 duplication	Episignature	-
Coffin-Lowry syndrome	*RPS6KA3*	-	+
Cornelia de Lange syndrome, types 1-4[b]	*NIPBL, SMC1A, SMC3, RAD21, HDAC8*[a], *SETD5, BRD4*	Episignature excluding *SETD5*, *BRD4*	+
Dystonia 28, childhood-onset	*KMT2B*	-	+
Down syndrome	Trisomy 21	Episignature	-
Epileptic encephelopathy, childhood-onset	*CHD2*	Episignature	+
Floating-Harbor syndrome	*SRCAP*	Episignature	+
Fragile X syndrome	*FMR1* promoter	Trinucleotide repeat expansion	-
Genitopatellar syndrome	*KAT6B*	Episignature	+
Growth retardation, developmental delay, facial dysmorphism	*FTO*	-	+
Holocarboxylase synthetase deficiency	*HLCS*	-	+
Helsmoortel-Van Der Aa syndrome	*ADNP*	Episignature	-
Hunter-McAlpine syndrome	5q35-qter duplication	Episignature	-
Imagawa-Matsumoto syndrome	*SUZ12*	-	-
Immunodeficiency-centromeric instability-facial anomalies syndrome, types 1-4	*DNMT3B, ZBTB24, CDCA7, HELLS*	Episignature	+, excluding *CDCA7, HELLS*
Intellectual developmental disorder 61	*MED13*	-	-
Intellectual developmental disorder with dysmorphic facies and ptosis	*BRPF1*	-	-
Kabuki syndrome 1 and 2	*KMT2D, KDM6A, RAP1A, RAP1B*	Episignature, excluding *RAP1A*, *RAP1B*	+
Kagami-Ogatta syndrome	*MEG3* promoter	Imprinting defect	
KBG syndrome	*ANKRD11*	-	-
Kleefstra syndrome 1	*EHMT1, KMT2C*[a]	Episignature	+
Koolen-De Vries syndrome	*KANSL1*	Episignature	+
Lujan-Fryns syndrome	*MED12*[a]	-	+

(continued)

Table 1 Continued

Syndrome	Causative Gene(s)/Region(s)	EpiSign v2	NGS ChrPan
Luscan-Lumish syndrome	*SETD2*	-	+
Mental retardation and distinctive facial features with or without cardiac defects	*MED13L*	-	-
Mental retardation, autosomal dominant 21	*CTCF*	-	+
Mental retardation, autosomal dominant 23	*SETD5*	Episignature	+
Mental retardation, autosomal dominant 51[b]	*KMT5B*	Episignature	-
Mental retardation, X-linked 3	*HCFC1*	-	+
Mental retardation, X-linked 93[b]	*BRWD3*	Episignature	-
Mental retardation, X-linked 97	*ZNF711*	Episignature	-
Mental retardation, FRA12A type	*DIP2B* promoter	Trinucleotide repeat expansion	
Mental retardation, X-linked syndromic, Claes-Jensen type[b]	*KDM5C*	Episignature	+
Mental retardation, X-linked syndromic, Nascimento-type	*UBE2A*	Episignature	-
Mental retardation syndrome, X-linked, Siderius type	*PHF8*	-	+
Mental retardation, X-linked, Snyder-Robinson type	*SMS*	Episignature	
Mental retardation, X-linked syndromic, Turner type	*HUWE1*	-	+
Microcephaly, postnatal progressive, with seizures and brain atrophy	*MED17*	-	+
Microphthalmia, syndromic 2	*BCOR*	-	+
Nicolaides-Baraitser syndrome	*SMARCA2*	-	+
O'Donnell-Luria-Rodan syndrome	*KMT2E*	-	-
Opitz-Kaveggia syndrome	*MED12*[a]	-	+
Ohdo Syndrome	*MED12*[a]	-	+
Pontocerebellar hypoplasia, type 8	*CHMP1A*	-	+
Prader-Willi syndrome	*SNRPN* promoter	Imprinting defect	
PRC2 complex disorders (Weaver and Cohen-Gibson syndromes)	*EZH2, EED, SUZ12*	Episignature, excluding *SUZ12*	+
Rahman syndrome	*HIST1H1E*	Episignature	-
Rubinstein-Taybi syndrome 1 and 2	*CREBBP, EP300*	Episignature	+
SBBYSS syndrome/Ohdo syndrome	*KAT6B*	Episignature	+
SETD1B-related syndrome	*SETD1B*	Episignature	-
Shprintzen-Goldberg syndrome	*SKI*	-	+
Smith-Magenis syndrome	*RAI1*[a]	-	+
Silver-Russell syndrome 1 and 2	ICR1, *MEST* promoter	Imprinting defect	
Sotos syndrome 1	*NSD1, NFIX, APC2*	Episignature, excluding *NFIX*, *APC2*	+
Tatton-Brown-Rahman syndrome	*DNMT3A*	Episignature	+
Townes-Brocks branchiootorenal-like syndrome	*SALL1*	-	+
Temple syndrome	*MEG3* promoter	Imprinting defect	-
White-Sutton syndrome	*POGZ*	-	+
Wiedemann-Steiner 2yndrome	*KMT2A*	Episignature	+
Williams-Beuren syndrome	7q11.23 deletion	Episignature	-
Wolf-Hirschhorn syndrome	4p16.13 deletion/*NSD2/KMT3F*	Episignature, excluding *NSD2*	-

NGS, Next Generation Sequencing.

[a]No evidence of a reproducible episignature; this is potentially due to small sample size.

[b]Healthy heterozygotes and those with incomplete penetrance are detectable.

Targeted sequencing Sanger, qPCR, and MS-MLPA methylation assay

The large majority of the samples included in this study had been previously reported elsewhere[7] or initially screened for variants through ChrPan (Agilent Technologies, Inc.).[1] The ChrPan includes all the genes assessed by the EpiSign genome-wide DNA methylation assay that are associated with chromatinopathies. Potentially disease-causing variants were confirmed with Sanger sequencing. When available, DNA from parents was analyzed for variant inheritance. All sequence variants were classified according to the American College of Medical Genetics (ACMG/AMP) guidelines for interpretation of genomic variants.[8] Sequence variants were described according to HGVS nomenclature guidelines (https://varnomen.hgvs.org/).[9] Real-time Quantitative PCR (qRT-PCR) was carried out as described[10] with oligo pairs based on the UCSC GRCh37/hg19 assembly and available upon request. Methylation assay for GD1323 was performed using the MS-MLPA Probemix ME030 BWS/RSS, version C3 (MRC-Holland) following the manufacturer's instructions.

Results

Identification of episignatures in discovery and validation cohorts

We first assessed the episignatures detected in the discovery cohort that consisted of 24 affected individuals with Rubinstein-Taybi type 1 (RSTS1, MIM #180849) and type 2 (RSTS2, MIM #613684), nine with Kabuki syndrome type 2 (KABUK2, MIM #300867), nine with a BAFopathy,[11] and one each with a pathogenic or likely pathogenic variant in *PHF6* (BFLS, MIM #301900), *SMC1A* (CDLS2, MIM #300590), *EHMT1* (KLEFS1, MIM #610253), *NSD2* (WHS, MIM #194190), and *EZH2* (WVS, MIM #277590) (Figure 1A and Supplemental Table 1). The resulting methylation profiles matched the defined episignature and confirmed the ChrPan findings. The affected individual GDB1323 carries a c.1727C>A pathogenic nonsense variant in *ATRX* and showed a methylation profile consistent with Alpha-Thalassemia/Mental Retardation syndrome (ATRX, MIM #301040). Interestingly, the methylation analysis also revealed hypomethylation of Imprinting Control Region 2 (ICR2) associated with Beckwith-Wiedemann syndrome (BWS, MIM# 130650),[12] and was confirmed by MS-MLPA analysis (data no shown).

The validation cohort consisted of 20 affected individuals carrying a pathogenic or likely pathogenic variant in 11 genes. Each subject had a suspected general diagnosis of chromatinopathy with features suggestive but not pathognomonic of a specific and unique syndrome. The DNA methylation profile confirmed the sequencing data in all cases (Supplemental Table 2 and Figure 1B).

Identification of episignatures in uncertain and negative cohorts

To support the pathogenicity and the clinical relevance of detected VUSs, 28 affected individuals with a well-defined clinical phenotype carrying a VUS were grouped as the uncertain cohort and underwent episignature analysis (Supplemental Table 3 and Figure 1C). Twenty-five samples did not show a methylation profile matching any of the known episignatures included in the EpiSign assay.

EpiSign analysis of two patients referred for a clinical presentation of Kabuki syndrome, and carrying a *KDM6A* VUS, showed the Kabuki syndrome-specific episignature. An additional individual referred as Kabuki syndrome and carrying a *KDM6A* VUS, presented with an unexpected methylation profile consistent with Wolf-Hirschhorn syndrome (WHS, MIM #194190)[4] (Figure 2) which was confirmed by qRT-PCR (data not shown).

Case GDB1406, referred as affected by CHARGE syndrome and presenting with a VUS in *CHD7*, did not show any of the defined episignatures, including CHARGE. Notably, exome sequencing revealed a pathogenic variant in *DYNC1H1*, which is associated with MRD13 syndrome (MIM #614563) (personal communication).

The affected individual GDB1321, a female carrying a X-linked *PHF6* likely pathogenic variant did not match the defined episignature for Borjeson-Forssman-Lehmann syndrome (BFLS, MIM #301900) (see Discussion).

Individual GDB1191 carries the null variant c.1243C>T in *SMARCA4,* inherited from a father with no apparent clinical signs of Coffin Siris syndrome 4 (CSS4, MIM #614609). *SMARCA4* truncating variants are reported in rhabdoid tumour predisposition syndrome-2 (RTPS2, MIM# 613325)[13] and have not yet been observed in CSS4.[14] The DNA methylation profile of the proband did not match the BAFopathy signature (Figure 2).

Methylation profile assessment of the sequencing negative cohort did not detect a defined episignature in 30/33 cases (Supplemental Table 4 and Figure 1D). Case KB444, referred as Kabuki syndrome, and case RSTS222, with a strong clinical phenotype of Rubinstein-Taybi syndrome, were both negative by sequencing and MLPA analysis but matched the defined Kabuki syndrome and RSTS episignatures, respectively. Finally, case GDB1430 which was also negative by ChrPan analysis matched the BAFopathy episignature.

Discussion

Genetic alterations in various components of the epigenetic machinery have emerged as a recurring mechanism in the aetiology of a group of neurodevelopmental disorders called chromatinopathies. Currently, molecular diagnosis of these conditions is mainly based on targeted gene panel

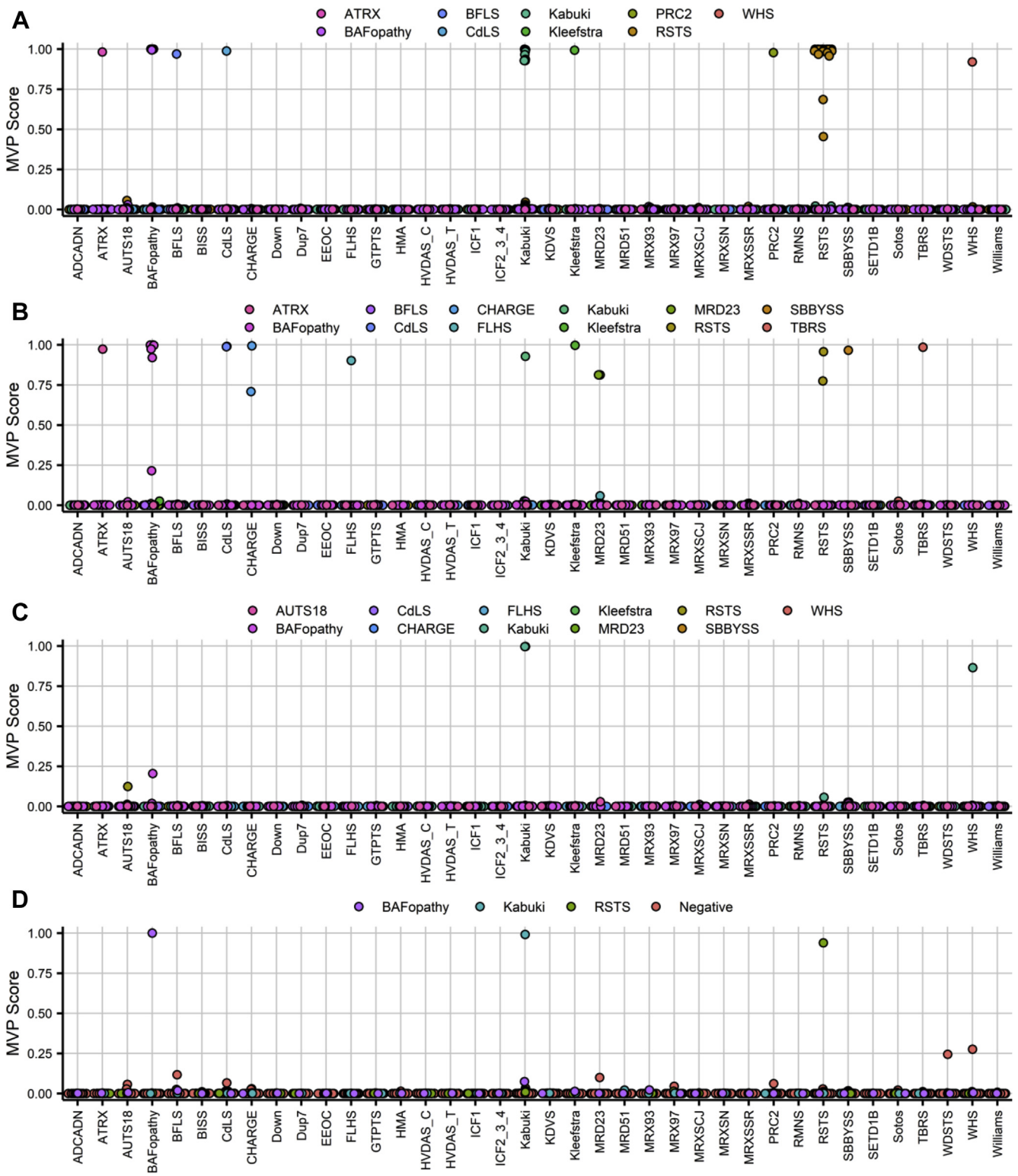

Figure 1 Methylation variant pathogenicity (MVP) probability scores for cohorts of samples. Our support vector machine-based classifier can assign a MVP probability score to a sample for each of 38 episignatures representing 43 neurodevelopmental syndromes. A score near one indicates that the sample has a methylation signature similar to other samples with the given episignature. For each plot, the colours indicate the sample type, and the X axis label indicates the episignature being tested. For example, in (A) the orange RSTS samples have high scores when evaluated using the RSTS episignature but low scores for all other signatures. The two positive Kabuki cases in the Uncertain cohort (C) both predict an MVP score of 1. A. Discovery cohort. B. Validation cohort. C. Uncertain cohort. D. Negative cohort.

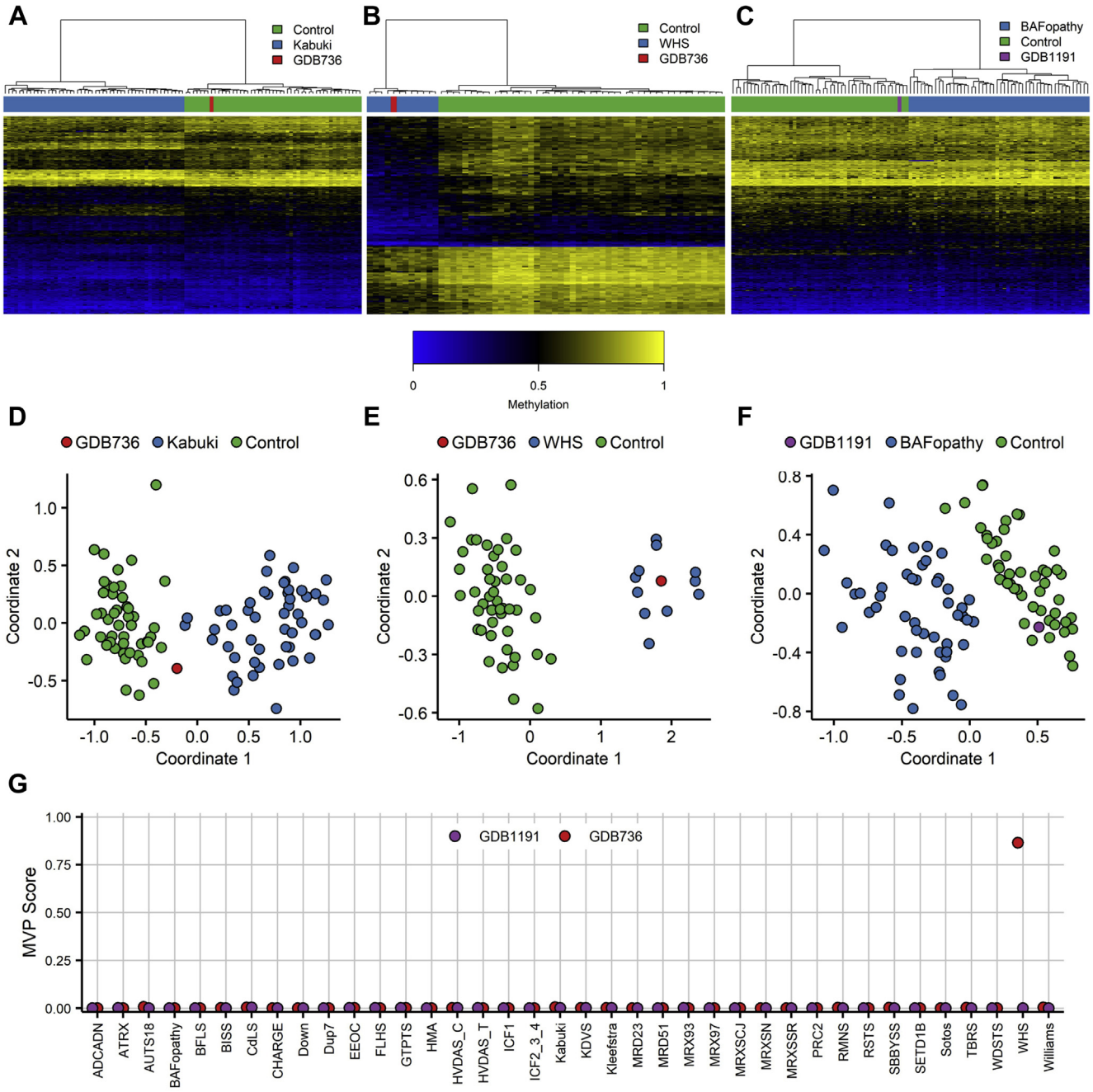

Figure 2 **EpiSign analysis of GDB736 and GDB1191 samples.** GDB736 sample with a variant in *KDM6A* was suspected of having Kabuki syndrome however unsupervised clustering to compare the sample with other samples from patients with Kabuki syndrome showed the sample did not cluster with other Kabuki samples by either hierarchical clustering (A) or multidimensional scaling (D). The sample clustered with samples from patients with Wolf-Hirschhorn syndrome (WHS) (B,E). A score near one indicates that the sample has a methylation signature similar to other samples with the given episignature. Sample GDB736 had a high score for WHS and low scores for all other episignatures (G). Patient sample GDB1191 with a variant in *SMARCA4* was suspected of having a BAFopathy (Coffin-Siris Syndrome 4), however, the sample did not cluster with other BAFopathy samples (C,F) and had low MVP scores for all episignatures tested (G). *MVP*, Methylation variant pathogenicity.

sequencing, and less frequently, by exome sequencing.[1] However, these methods can be limited by sequence coverage, inability to detect complex genomic rearrangements and structural variations, and exclusion of deep intronic and noncoding sequences. Moreover, the diagnostic uncertainty of a VUS adds further challenges. One emerging way to address these limitations is DNA methylation analysis of peripheral blood DNA samples. The so called episignature is a highly sensitive and specific diagnostic biomarker in an increasing number of chromatinopathies, allowing us to distinguish affected from unaffected individuals, disease-causing from non-disease-causing

variants, as well as assess the functional significance of VUSs, leading to reclassification where applicable.[15,16] Moreover, EpiSign is a clinically validated molecular test[4] that can enable diagnosis of cases showing uncertain clinical or molecular findings, or where the disease-causing variant cannot be identified through standard sequencing approaches.

This manuscript describes the implementation of genome-wide DNA methylation analysis in a large cohort of affected individuals with clinical and molecular diagnosis of chromatinopathies. Episignature analysis confirmed DNA sequencing results and, thus, substantiated the diagnosis using the discovery and validation cohorts. Analysis of these two cohorts established the utility of episignatures as a supporting diagnostic modality.

In addition to demonstrating technical validity of the assay, EpiSign analysis has resulted in some interesting and unexpected findings with significant clinical implications in a number of patients. The methylation analysis of GDB1323 identified the expected ATRX syndrome episignature and additionally a previously unrecognized, hypomethylation of ICR2 locus, a finding consistent with the BWS,[12] which was confirmed by methylation-specific MLPA. Based on methylation data, the clinical parameters were re-evaluated. Along with the classical phenotype of ATRX syndrome, the individual presented with visceromegaly, a suggestive BWS feature as well as two cardinal features, macroglossia and lateralized overgrowth, which have not been described as clinical features of ATRX. According to the BWS scoring proposed by Brioude *et al.*,[17] this individual has a BWS score of 5, sufficient to confirm a clinical diagnosis of BWS. In this case, DNA methylation analysis has been a powerful tool in detecting the co-presence of these two syndromes, which would have been difficult to identify and differentiate by clinical examination only and ultimately improved clinical management.

Cases with previously identified genetic VUS and patients with no negative genetic testing also showed some interesting clinical findings. The first group included 28 affected individuals carrying a VUS associated with a well-defined clinical phenotype (Supplemental Table 3). For 25/28 cases (89%) the DNA methylation profile did not match with any syndromes included in EpiSign, providing evidence against significance, albeit not conclusively, of the identified VUS and suggesting that those individuals need to be clinically and molecularly reconsidered. Where parental DNA was available, inheritance was assessed and determined to be inherited in all cases ($n = 9$) adding further evidence to support both the EpiSign result and a likely benign variant. In the negative cohort, 30/33 cases (91%) did not match a defined episignature, consistent with the sequencing results. The negative cases in both cohorts may fall under the hypothesis that episignatures/variants in other unassessed genes, not included in both EpiSign and ChrPan, could be the cause of the observed clinical phenotype.

In the uncertain cohort, two individuals carrying *KDM6A* variants, classified as VUSs at the time of NGS analysis, showed a DNA methylation profile consistent to Kabuki syndrome. Based on the episignature analysis, and recent availability of parental DNA, inheritance assessment and clinical parameters of both individuals were performed following the Kabuki international consensus diagnostic criteria.[18] Both individuals presented with a history of infantile hypotonia, developmental delay, intellectual disability, and typical Kabuki syndrome dysmorphic features, leading to a definite clinical diagnosis. From a molecular point of view, GDB502 carries a *de novo* hemizygous c.2933A>T; p.(Asp980Val) variant located outside any known *KDM6A* domain[19] and is reported as a VUS based on ACMG/AMP guidelines. GDB836 is heterozygous for a *de novo* c.3743A>G; p.(Gln1248Arg) variant located within the JmjC functional domain of *KDM6A*.[19] and with confirmed inheritance, is reported as likely pathogenetic to further support the EpiSign result.

One additional suspected Kabuki affected individual, GDB736, matched the WHS episignature, a finding that was confirmed by qRT-PCR. WHS is a contiguous gene syndrome associated with a heterozygous 4p16.3 deletion and characterized by distinct facial features, prenatal and postnatal growth retardation, intellectual disability, and seizures.[20] Of note, our custom ChrPan does not include any of the genes included in the 4p16.3 region and therefore, the use of episignatures was effective in diagnosing this patient and highlights the importance of supporting NGS with additional diagnostic methods.

GDB1321 is a female carrying an X-linked *PHF6* likely pathogenic variant that did not show the typical DNA methylation profile for BFLS. The BFLS episignature is based on a cohort of positive male samples and therefore confidently detects males with causative variants in *PHF6*.[3] Most affected heterozygote females show skewed X-inactivation of the chromosome carrying the *PHF6* variant, suggesting that a differentially methylated profile could be observed in female patients. At this time, a specific and distinctive episignature for *PHF6* affected heterozygote females has not yet been discovered and a larger cohort of affected females, with an ascertained X-inactivation skewing, would be needed to determine if such an episignature exists.

The last case of the uncertain cohort, GDB1191, confirmed the power of the episignature approach. This patient carries a c.1243C>T null variant in *SMARCA4*, inherited from an unaffected father and neither individual matched the BAFopathy signature (Figure 2F). Interestingly, *SMARCA4* truncating variants are reported in RTPS2, while all reported variants in CSS4 patients are non-truncating, implying a gain-of-function or dominant-negative effect.[21] Therefore, episignature analysis has helped to discriminate between two distinct allelic conditions and support the model that loss-of-function of *SMARCA4* may not be causative for CSS4.

The negative cohort found three patients matching a specific episignature reported in EpiSign. Specifically, two cases, KB444, referred as Kabuki syndrome, and RSTS222, with a strong clinical phenotype of Rubinstein-Taybi syndrome were both negative for sequence and copy number

variants by NGS and MLPA analysis but showed a specific Kabuki and RSTS episignature respectively. The methylation profile of a third case, GDB1430, was consistent with the BAFopathy episignature which is generated from five distinct genes and associated to multiple syndromes (Table 1). This suggests that the BAFopathy episignature may also identify defects in other BAFopathy genes that have not yet been confirmed experimentally.[11] The inconsistency between sequencing and DNA methylation analyses may be due to several putative reasons: (1) our NGS analysis is restricted to ± 20 bp flanking intronic DNA regions and therefore more deep splicing sites may not be detected, (2) regulatory gene regions are not covered by our ChrPan, (3) MLPA does not guarantee full gene coverage, as is the case for Kabuki syndrome, and (4) we cannot exclude the presence of a unidentified gene(s) that would also match the current episignatures. These three cases require further molecular investigations to identify the underlying molecular variant that is likely beyond current detection techniques such as complex rearrangements, structural variant or deep intronic variants.

In summary, episignature screening on a large group of affected individuals, as well as the description of several specific cases, highlighted the importance of adopting EpiSign testing as a diagnostic tool in the clinical setting for chromatinopathies. The presence of a disease-specific DNA methylation episignature can be supportive for resolving complex diagnostics, which can impact patient care and healthcare costs. With these benefits, we also recognize the current limitations in that episignature analysis is restricted to known, defined methylation profiles and specific to blood tissue types only.

This study describes the implementation of genomic DNA methylation testing in patients with chromatinopathies. Expanding clinical utility of the EpiSign test on larger-scale studies with both cost-benefit and clinical-benefit analysis may support its application as a priority diagnostic assay.

Declarations

Data availability

The datasets generated by the current study are available upon request.

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Ethics Declaration

Patients and their relatives were enrolled after obtaining appropriate informed consent by the physicians in charge, and approval obtained by the local ethics committees. The study was approved by the Western University Research Ethics Board (REB 106302).

Conflict of Interest

The authors declare that they have no competing interests

Additional Information

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