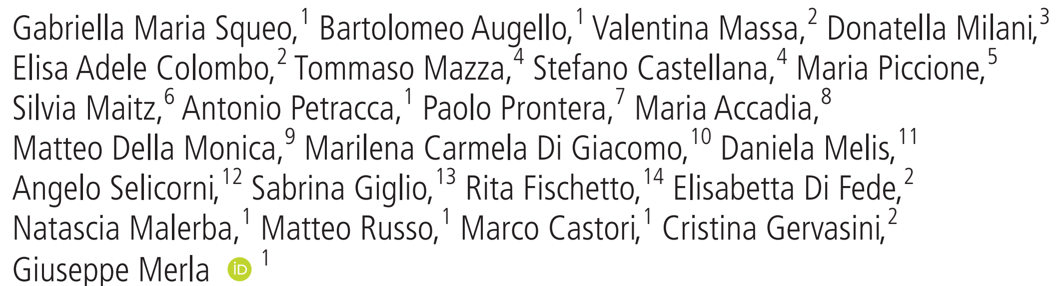
ORIGINAL RESEARCH

Customised next-generation sequencing multigene panel to screen a large cohort of individuals with chromatin-related disorder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ABSTRACT

Background The regulation of the chromatin state by epigenetic mechanisms plays a central role in gene expression, cell function, and maintenance of cell identity. Hereditary disorders of chromatin regulation are a group of conditions caused by abnormalities of the various components of the epigenetic machinery, namely writers, erasers, readers, and chromatin remodelers. Although neurological dysfunction is almost ubiquitous in these disorders, the constellation of additional features characterizing many of these genes and the emerging clinical overlap among them indicate the existence of a community of syndromes. The introduction of high-throughput next generation sequencing (NGS) methods for testing multiple genes simultaneously is a logical step for the implementation of diagnostics of these disorders.
Methods We screened a heterogeneous cohort of 263 index patients by an NGS-targeted panel, containing 68 genes associated with more than 40 OMIM entries affecting chromatin function.
Results This strategy allowed us to identify clinically relevant variants in 87 patients (32%), including 30 for which an alternative clinical diagnosis was proposed after sequencing analysis and clinical re-evaluation.
Conclusion Our findings indicate that this approach is effective not only in disorders with locus heterogeneity, but also in order to anticipate unexpected misdiagnoses due to clinical overlap among cognate disorders. Finally, this work highlights the utility of a prompt diagnosis in such a clinically and genetically heterogeneous group of disorders that we propose to group under the umbrella term of chromatinopathies.

INTRODUCTION

In the last decade, research in Mendelian disorders has witnessed an explosion in the discovery of genetic variants in the various components of the epigenetic machinery linked to a number of human genetic disorders.[1–5] Clinically relevant variants may occur in gene encoding for writers, erasers and readers of epigenetic marks as well as in chromatin remodellers. The writers place the appropriate modifications on particular regions of the genome based on the cell type, developmental stage and metabolic state. These marks 'highlight' individual regions for use or disuse depending on whether the mark favours a more open or more closed chromatin state.[6] The erasers remove these marks, thus favouring the opposite chromatin states. The readers recognise post-translational modifications within a single histone tail, within two tails on the same nucleosome or within two histone tails on different nucleosomes.[7] Finally, chromatin remodellers are multiprotein assemblies containing an ATPase subunit of the Snf2 subfamily that is capable of mobilising the nucleosomes by using the energy of ATP hydrolysis and thereby altering chromatin structure.[8]

To date, over 60 Mendelian disorders, such as Kabuki, Kleefstra, Coffin-Lowry, Wiedemann-Steiner, Rubinstein-Taybi, Floating-Harbor and Cornelia de Lange syndromes, are associated with genetic alterations of the various components of the epigenetic machinery (online supplementary table S1).[2 9] Most of them are characterised by intellectual and neurological dysfunctions, a fact demonstrating the pivotal role of chromatin remodelling in central nervous system development and function.[6] Although these conditions are often distinguished by the pattern of ancillary findings and/or facial dysmorphic features, clinical and molecular overlap is increasingly described among them, as an expected result of convergent pathogenesis in these disorders.

The recent introduction of high-throughput next-generation sequencing (NGS) methods facilitated the reduction of reporting time, costs and the rates of negative or inconsistent results of molecular testing in the clinical context. Here, we report our findings in a cohort of 263 index patients analysed with a customised NGS diagnostic panel of 68 selected genes associated with chromatin-related disorder. As the primary role of these genes concerns the structure and function of chromatin, we propose to group these disorders under the umbrella term of chromatinopathies.

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MATERIALS AND METHODS

Samples

We applied our customised NGS Chromatinopathy Panel (ChrPan; online supplementary table S1) to analyse 263 individuals with a suspected diagnosis of a chromatin-related disorder. In some cases, the clinical diagnosis was sharply defined. In others, considering the increasing awareness on the existence of an overlapping phenotypic spectrum caused by mutations of genes involved in the epigenetic machinery, samples were sent with a suspected diagnosis of 'Chromatinopathy' with features suggestive but not pathognomonic of a specific syndrome.

Genomic DNA (gDNA) was extracted from fresh and/or frozen peripheral blood leucocytes of patients and their available family members using an automated DNA extractor and commercial DNA extraction kits (EZ1 Advanced XL, Qiagen, Germany).

Targeted resequencing and NGS

A customised HaloPlex Target Enrichment NGS panel (Agilent Technologies, Santa Clara, California) was designed using Agilent's SureDesign tool available at www.agilent.com/genomics/suredesign (online supplementary table S1 and figure S1). Total genome target that spanned 384 kb was theoretically estimated to be completely covered (99.82%). A total of 17 470 amplicons were generated. The full design is available as a .bed file on request. The NGS panel was preliminarily validated using a cohort of 30 DNA samples with known variants in chromatinopathies genes or resulted negative to sequence analysis.[10–12]

Libraries were prepared according to the manufacturer's protocol using the human reference genome GRCh37/hg19. Fluorometry-based Qubit dsDNA BR Assay (Thermo Fisher Scientific) was used to determine the precise DNA concentration. DNA was fragmented using eight double-digests. Library of gDNA was hybridised to the probe set in the presence of indexing primer allowing for pooling, and the probe-fragment hybrids were captured on magnetic beads. The library was then amplified by PCR to produce a sequencing-ready, target-enriched sample. Fragmented gDNA and final libraries were evaluated using High Sensitivity D1000 ScreenTape System on TapeStation 2200 (Agilent Technologies) and Qubit dsDNA HS Assay Kits on Qubit 4 Fluorometer (Thermo Fisher Scientific) for quantification, according to the manufacturer's instructions. Sequencing was performed on Illumina MiSeq System, using the MiSeq Reagent Kit V2-500 cycles (Illumina, San Diego, California), yielding 151 bp-long, paired-end reads and achieving an average read depth of at least 400× and at least 20× per-base coverage in more than 98% of the targeted regions for 11–22 samples.

Data analysis and variants validation

The quality of sequences was preliminary checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmed using Trimmomatic[13] if the quality of at least half-read was lower than 10 (phred quality score). Residual adapter sequences were removed by cutadapt (https://cutadapt.readthedocs.io/en/stable/). Preprocessed reads were aligned against the GRCh37/hg19 reference genome sequence by the BWA-0.7.17 software. The depth of coverage statistics for the target regions was calculated by means of TEQC (https://bioconductor.org/packages/release/bioc/html/TEQC.html) and other custom scripts. Variants were identified by means of the HaplotypeCaller tool of GATK V.3.8, discarded if their calling quality did not exceed a phred threshold value of 20 or were not covered by at least 30 reads, otherwise annotated with ANNOVAR, using RefSeq gene and transcript annotations (updated December 2016). Variants were sought in the most important public collections, including dbSNP V.150,[14] ExAC V.0.3, Exome Variant Server (http://evs.gs.washington.edu/EVS, accessed in December 2016) and gnomAD.[15] Allelic frequencies were annotated using Haplotype Reference Consortium, Kaviar and ClinVar databases[16] (accessed in June 2018). Sequencing artefacts were controlled by matching individual-specific variants against an internal database of variants. Missense variants were fully annotated using dbNSFP V.3.5, from which we have retrieved precomputed pathogenicity predictions and evolutionary conservation predictions and measures. Potentially pathogenic variants were confirmed with PCR amplification and Sanger sequencing, as reported in refs.[17 18] When available, DNA from parents was analysed for variant inheritance.

The significance of candidate variants was classified according to the American College of Medical Genetics and Genomics criteria using InterVar (http://wintervar.wglab.org/),[19] Varsome (https://varsome.com/),[20] CAVA[21] and PMut prediction (http://mmb.pcb.ub.es/PMut/)[22] tools. Sequence variants were described according to the Human Genome Variation Society nomenclature guidelines (https://varnomen.hgvs.org/).[23]

RESULTS

Mutational screening of chromatinopathies cohort

We found variants of clinical relevance in 87 index patients (32%). The distribution of these variants is reported in tables 1 and 2, with 61 (70%) pathogenic or likely pathogenic variants and the remaining 26 (30%) of unknown significance (VOUS). Twenty-nine individuals (29 of 263; 10%) presented with features suggestive but not pathognomonic of a specific syndrome, referred to us as 'Chromatinopathy-affected' (table 1). Among them we found patient GDB1326 exhibited the c.5128T>C; p.(Cys1710Arg) pathogenic variant (table 1) located in a region between bp 5128 and 5614, which includes nine distinct codons of exon 30 of *CREBBP*. This exonic region has been associated with the recently described Menke-Hennekam syndrome-2 (MIM #618333), thus expanding the number of individuals affected by this newly described syndrome.[24–26]

Cases with an alternative clinical diagnosis after NGS analysis

In nine index patients molecular findings resulted in a pathogenic or likely pathogenic variant in a gene not associated with the original clinical suspicion. In four of them we have confirmed the clinical diagnosis after a clinical re-evaluation of the individual's phenotype (table 2). In 21 additional cases a VOUS was identified in a gene not associated with the referred clinical suspicion (table 2), a result that needs further and accurate investigations.

The following sections describe in detail the four patients with a confirmed clinical diagnosis after medical re-evaluation.

Case GDB1200

This patient, referred to us with a clinical suspicion of KS, is a 7-year-old boy born at term by caesarean section from consanguineous (first cousins) parents, by natural conception and with uneventful pregnancy. At birth he presented a weight of 3.420 g (50th centile), height 50 cm (50th centile) and head circumference of 36.5 cm (75th centile). Family history was mute. Transcranial ultrasound was normal at birth. On physical examination at the age of 7, his weight was 23 kg (25th–50th centile), height was 127 cm (50th–75th centile) and head circumference was 49.5 cm (<3rd centile). Hypertelorism, thick eyebrows, long

Table 1 Genetic variants in a subcohort of patients with a defined diagnosis

Gene	Patient identification	Clinical diagnosis	Inheritance	Type of mutation	Exon/intron	Variant	AA change	ACMG	CAVA variant classification system	Reference	ClinVar/LOVD	PMut prediction
KMT2D	GDB1112	KS	F*	Missense	10	c.1396C>T	p.(Arg466Cys)	VOUS	NSY	This study		Not reported
KMT2D	GDB1450	KS	NA	Frameshift	10	c.1914delA	p.(Pro639Leufs*291)	P	FS	This study		Not predictable
KMT2D	GDB1180	KS	M*	Missense	11	c.3193T>A	p.(Ser1065Thr)	VOUS	NSY	This study		Not reported
KMT2D	GDB1148	KS	NA	Nonsense	11	c.3532C>T	p.(Gln1178*)	P	SG	44	https://www.ncbi.nlm.nih.gov/clinvar/variation/94210/	Not predictable
KMT2D	GDB1056	KS	NA	Splice site	Intron 13–14	c.4132-2A>G	p.?	P	SS	18		Not predictable
KMT2D	GDB1066	KS	De novo	Frameshift	14	c.4135_4136delAT	p.(Met1379Valfs*52)	P	FS	11 45 46	https://www.ncbi.nlm.nih.gov/clinvar/variation/94216/	Not predictable
KMT2D	GDB1170	KS	De novo	Nonsense	22	c.5269C>T	p.(Arg1757*)	P	SG	47 48	https://www.ncbi.nlm.nih.gov/clinvar/variation/435669/	Not predictable
KMT2D	GDB1357	KS	NA	Nonsense	31	c.7228C>T	p.(Arg2410*)	P	SG	49–53	https://databases.lovd.nl/shared/variants/0000342377#00023885	Not predictable
KMT2D	GDB1197	KS	De novo	Nonsense	31	c.7891G>T	p.(Gly2631*)	P	SG	This study		Not predictable
KMT2D	GDB1142	KS	NA	Frameshift	34	c.9248delA	p.(Glu3083Glyfs*36)	P	FS	This study		Not predictable
KMT2D	GDB1146	KS	NA	Frameshift	39	c.11176delC	p.(Gln3726Asnfs*23)	P	FS	This study		Not predictable
KMT2D	GDB1116	KS	NA	Nonsense	39	c.11233C>T	p.(Gln3745*)	P	SG	This study		Not predictable
KMT2D	GDB1301	KS	NA	Nonsense	39	c.11272C>T	p.(Gln3758*)	P	SG	This study		Not predictable
KMT2D	GDB1173	KS	NA	Frameshift	39	c.11952dupA	p.(Leu3985Thrfs*27)	P	FS	This study		Not predictable
KMT2D	GDB1179	KS	De novo	Nonsense	39	c.13507C>T	p.(Gln4503*)	P	SG	11	https://databases.lovd.nl/shared/variants/0000040041#00023885	Not predictable
KMT2D	GDB1042	KS	De novo	Missense	45	c.14381A>G	p.(Lys4794Arg)	LP	NSY	18		Not reported
KMT2D	GDB1204	KS	NA	Nonsense	48	c.14710C>T	p.(Arg4904*)	P	SG	50 53 54	https://www.ncbi.nlm.nih.gov/clinvar/variation/94176/	Not predictable
KMT2D	GDB1199	KS	NA	Frameshift	51	c.16236delT	p.(Tyr5412*)	P	FS	This study		Not predictable
KMT2D	GDB1302	KS	NA	Missense	51	c.16232T>A	p.(Leu5411His)	LP	NSY	This study		Not reported
KMT2D	GDB1354	KS	De novo	Missense	51	c.16295G>A	p.(Arg5432Gln)	LP	NSY	45 55	https://www.ncbi.nlm.nih.gov/clinvar/variation/94191/	Not reported
KMT2D	GDB1114	KS	NA	Nonsense	52	c.16360C>T	p.(Arg5454*)	P	SG	10 45 50 54	https://www.ncbi.nlm.nih.gov/clinvar/variation/7538/	Not predictable
KMT2D	GDB1340	KS	De novo	Nonsense	52	c.16360C>T	p.(Arg5454*)	P	SG	10 45 50 54	https://www.ncbi.nlm.nih.gov/clinvar/variation/7538/	Not predictable
KMT2D	GDB1084	KS	NA	Frameshift	53	c.16469_16470delAA	p.(Lys5490Argfs*21)	P	FS	17	https://databases.lovd.nl/shared/variants/0000086865#00023885	Not predictable
KDM6A	GDB1363	KS	NA	Nonsense	10	c.968C>A	p.(Ser323*)	P	SG	This study		Not predictable
CREBBP	GDB1186	RSTS	NA	Nonsense	4	c.1108C>T	p.(Arg370*)	P	SG	56	https://databases.lovd.nl/shared/variants/0000178285#00001113	Not predictable
CREBBP	GDB1292	RSTS	De novo	Frameshift	14	c.2650_2663dup14	p.(Ser889Leufs*43)	P	FS	This study		Not predictable
CREBBP	GDB1295	RSTS	NA	Frameshift	14	c.2616dupG	p.(Thr873Aspfs*97)	P	FS	This study		Not predictable
CREBBP	GDB1182	RSTS	De novo	Missense	21	c.4627G>C	p.(Asp1543His)	LP	NSY	This study		Disease-causing
CREBBP	GDB1324	RSTS	NA	Missense	21	c.4627G>C	p.(Asp1543His)	LP	NSY	This study		Disease-causing
CREBBP	GDB1326	CHR	NA	Missense	30	c.5128T>C	p.(Cys1710Arg)	P	NSY	26	https://databases.lovd.nl/shared/variants/0000499559#00001113	Disease-causing
CREBBP	GDB1196	RSTS	De novo	Frameshift	31	c.6670_6671dupGG	p.(Met2225Alafs*78)	P	FS	This study		Not predictable
EP300	GDB1372	RSTS	NA	Missense	1	c.49A>G	p.(Lys17Glu)	LP	NSY	This study		Disease-causing
EP300	GDB1238	RSTS	De novo	Nonsense	12	c.2221C>T	p.(Gln741*)	P	SG	This study		Not predictable
EP300	18–0842	RSTS	NA	Frameshift	14	c.2525delC	p.(Pro842Leufs*8)	P	FS	This study		Not predictable
ARID1B	GDB1469	CSS	De novo	Frameshift	1	c.1202dupG	p.(Phe402Leufs*133)	P	FS	This study		Not predictable
ARID1B	GDB1409	CSS	NA	Nonsense	18	c.4864C>T	p.(Gln1622*)	P	SG	This study		Not predictable
ARID1B	GDB1381	CSS	De novo	Nonsense	20	c.5725C>T	p.(Gln1909*)	LP	SG	This study		Not predictable
ARID1B	GDB1201	CSS	NA	Nonsense	20	c.6164G>A	p.(Trp2055*)	LP	SG	This study		Not predictable
ARID1A	GDB1370	CSS	De novo	Frameshift	1	c.19_53del	p.(Pro7Alafs*92)	P	FS	This study		Not predictable
RPS6KA3	GDB1506	CLS	M	Missense	17	c.1784A>G	p.(Tyr595Cys)	LP	NSY	This study		Disease-causing
NSD1	GDB1203	Sotos	NA	Nonsense	7	c.3958C>T	p.(Arg1320*)	P	SG	57	https://www.ncbi.nlm.nih.gov/clinvar/variation/4143/	Not predictable
NSD1	GDB1285	Sotos	NA	Missense	23	c.6557A>G	p.(His2186Arg)	LP	NSY	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/159430/	Disease-causing
KMT2A	GDB1202	WDSTS	NA	Nonsense	4	c.3294G>A	p.(Trp1098*)	P	SG	This study		Not predictable

Continued

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Table 1 Continued

Gene	Patient identification	Clinical diagnosis	Inheritance	Type of mutation	Exon/intron	Variant	AA change	ACMG	CAVA variant classification system	Reference	ClinVar/LOVD	PMut prediction
ATRX	GDB1323	ATMDS, MRXHF1	NA	Nonsense	9	c.1727C>A	p.(Ser576*)	P	SG	58	https://databases.lovd.nl/shared/variants/0000349103#00000806	Not predictable
SRCAP	GDB1315	FLHS	De novo	Nonsense	34	c.7303C>T	p.(Arg2435*)	LP	SG	59–61	https://www.ncbi.nlm.nih.gov/clinvar/variation/30909/	Not predictable
RAI1	GDB1410	SMS	NA	Nonsense	3	c.3903C>A	p.(Cys1301*)	LP	SG	This study		Not predictable
KAT6B	GDB1547	CHR	NA	Missense	18	c.4225G>A	p.(Glu1409Lys)	VOUS	NSY	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/300889/	Neutral
KAT6B	GDB1383	SBBYSS	De novo	Frameshift	18	c.4831delG	p.(Val1611Tyrfs*39)	P	FS	This study		Not predictable
EZH2	GDB1362	WVS	NA	Frameshift	20	c.2211dupT	p.(Ala738Cysfs*25)	P	FS	This study		Not predictable
PHF6	GDB1321	BFLS	NA	Missense	7	c.860G>A	p.(Gly287Asp)	LP	NSY	This study		Disease-causing
NIPBL	GDB1379	CdLS	NA	Missense	36	c.6305A>G	p.(Asn2102Ser)	LP	NSY	This study		Neutral
NIPBL	GDB1490	CHR	NA	Frameshift	39	c.6741_6742insGC	p.(Met2248Alafs*18)	P	FS	This study		Not predictable
SKI	GDB1419	CHR	NA	Missense	3	c.1193C>T	p.(Pro398Leu)	VOUS	NSY	This study		Neutral
SMARCA2	GDB1455	CHR	De novo	Missense	25	c.3464A>C	p.(Gln1155Pro)	P	NSY	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/419659/	Disease-causing
SMARCA2	GDB1413	CHR	NA	Missense	26	c.3733C>G	p.(Arg1245Gly)	LP	NSY	This study		Disease-causing
SMARCA2	GDB1414	CHR	NA	Missense	26	c.3733C>G	p.(Arg1245Gly)	LP	NSY	This study		Disease-causing
SETD2	GDB1546	CHR	NA	Missense	14	c.6179C>T	p.(Ser2060Leu)	VOUS	NSY	This study		Neutral

SG: stop-gain (nonsense) variant caused by base substitution.
SS: any variant that alters splice site base within the first eight intronic bases flanking exon (ie, +8 to −8) but not an ESS or SS5 base.
FS: frameshifting insertion and/or deletion. It alters the length and frame of the coding sequence.
NSY: non-synonymous variant. It alters the amino acid(s) but not the coding sequence length.
*Clinical status unknown.
AA, amino acid; ACMG, American College of Medical Genetics; ATMDS, alpha-thalassaemia myelodysplasia syndrome; BFLS, Borjeson-Forssman-Lehmann syndrome; CdLS, Cornelia de Lange syndrome; CHR, chromatinopathy; CLS, Coffin-Lowry syndrome; CSS, Coffin-Siris syndrome; ESS, exonic splicing silencer; F, father; FLHS, Floating-Harbor syndrome; KS, Kabuki syndrome; LOVD, Leiden Open Variation Database; LP, likely pathogenic; M, mother; MRXHF1, mental retardation-hypotonic facies syndrome, X linked; NA, not available; P, pathogenic; RSTS, Rubinstein-Taybi syndrome; SBBYSS, Say-Barber-Biesecker-Young-Simpson syndrome; SMS, Smith Magenis syndrome; SS5, +5 Splice-Site; VOUS, variant of unknown significance; WDSTS, Wiedemann-Steiner syndrome; WVS, Weaver syndrome.

palpebral fissures, epicanthic folds, large and prominent ears, short philtrum, and thin lips were also noted. In addition to the global developmental delay, the parents also reported repetitive and compulsive-like behaviours. Kidney/abdominal ultrasound, ECG and echocardiography were normal. ChrPan revealed the likely pathogenic de novo variant c.1033C>T; p.(His345Tyr) in *CTCF*, which causes the autosomal dominant intellectual disability type 21 (MIM #615502)[27]; this variant was never reported in gnomAD, dbSNP, ExAC and ClinVar.

Case GDB1054

The proband is the only child of an unaffected mother; the father was not available for genetic analysis. Antenatal ultrasound showed hydroureteronephrosis (third grade), tricuspid valve insufficiency and mild pericardial effusion. Birth parameters were in the normal range. At birth, patent foramen ovale, mitral insufficiency, pyelectasis of the right kidney and a choroid plexus cyst were diagnosed. He underwent surgery for pyloric stenosis and vesicoureteral reflux at 1 and 9 months of age, respectively. At the age of 2, the patient had osteomyelitis of the right femur. Other medical problems include astigmatism, advanced bone age and overweight. Psychomotor development was delayed, and he received the diagnosis of borderline intellectual disability (IQ=70). The mother described him as very insecure and sometimes aggressive. He tends to isolate himself from peers. At the age of 10, his weight was 41.5 kg (90th–97th centile), height was 135 cm (25th centile) and head circumference was 52.5 cm (25th–50th centile). He showed overweight, hypertrichosis, high and narrow palate, a flat profile, synophrys, hypertelorism, epicanthus of the right eye, upslanting palpebral fissures, broad nose with bulbous tip and tapering fingers. A provisional diagnosis of KS was proposed by the referring clinician. ChrPan revealed the pathogenic de novo variant c.4727dupA; p.(Tyr1576*) in the *KMT2A*, which is causative of the Wiedemann-Steiner syndrome (MIM #605130)[28]; this variant was never reported in gnomAD, dbSNP, ExAC and ClinVar.

Case 4075-17

This 7-year-old girl was the second child of healthy non-consanguineous parents. Pregnancy and delivery were uneventful. At birth hypotonia and mild dysmorphic features were noted. She had a history of global developmental delay, moderate/severe intellectual disability (according to the Vineland Scale), bilateral neurosensorial deafness, recurrent otitis media and unilateral myopia. At the age of 7, her weight was 23 kg (25th–50th centile), height was 120.3 cm (25th–50th centile) and head circumference was 50 cm (10th centile). Dysmorphic features included sparse hair, long face, bushy eyebrows, upslanting palpebral fissures, flat nasal root, anteverted nares, macrostomia and thick lower lip. For this case, the referring clinician proposed a provisional diagnosis of KS. ChrPan detected the pathogenic de novo variant c.4536G>A; p.(Trp1512*) in the *ARID1B*, which is one of the gene causative of the Coffin-Siris syndrome (MIM #135900)[29]; this variant was absent in gnomAD, dbSNP and ExAC.

Case GDB1401

This patient is a 29-year-old man, the second child of healthy and unrelated parents. Family history was negative. He was born at term after an uneventful pregnancy. Delivery was dystocic with perinatal respiratory distress. His birth weight was 3700 g (50th centile), length was 51 cm (–50th centile) and head circumference was 36 cm (50th centile). At birth, perianal fistula, cryptorchidism and epigastric hernia were noted and surgically

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Table 2 A subcohort of patients with variants in genes not associated with the original clinical suspicion

Patient identification	Clinical diagnosis	Mutated gene	Inheritance	Exon/ intron	Variant	AA change	ACMG	CAVA variant classification system	Associated phenotype	Reference	ClinVar/LOVD	PMut prediction
Nonsense												
4075-17*	KS	*ARID1B*	De novo	18	c.4536G>A	p.(Trp1512*)	P	SG	CSS	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/560952/	Not predictable
GDB1241	RSTS	*ARID1B*	NA	2	c.1720C>T	p.(Gln574*)	P	SG	CSS	This study		Not predictable
GDB1401*	CLS	*ATRX*	M	2	c.109C>T	p.(Arg37*)	P	SG	ATMDS, MRXHF1	62 63	https://www.ncbi.nlm.nih.gov/clinvar/variation/11742/	Not predictable
Frameshift												
GDB1427	RSTS	*HDAC4*	NA	7	c.722_723delTT	p.(Leu241Glnfs*6)	P	FS	CSS	This study		Not predictable
GDB1054*	KS	*KMT2A*	De novo	14	c.4727dupA	p.(Tyr1576*)	P	FS	WDSTS	This study		Not predictable
GDB1405	KS	*EHMT1*	NA	23	c.3358_3359delinsACCTGAG	p.(Val1120Thrfs*2)	P	FS	KLEFS	This study		Not predictable
Missense												
GDB1239	RSTS	*ACTB*	NA	4	c.587G>A	p.(Arg196His)	VOUS	NSY	BRWS	64	https://www.ncbi.nlm.nih.gov/clinvar/variation/29599/	Disease-causing
18-2798	RSTS	*SETD2*	NA	14	c.6179C>T	p.(Ser2060Leu)	VOUS	NSY	LLS	This study		Neutral
GDB1430	RSTS	*BCOR*	NA	7	c.3331C>T	p.(Pro1111Ser)	VOUS	NSY	MCOPS2	This study		Neutral
GDB1052	KS	*ACTB*	NA	4	c.547C>T	p.(Arg183Trp)	VOUS	NSY	BRWS	65	https://www.ncbi.nlm.nih.gov/clinvar/variation/18275/	Disease-causing
GDB1185	KS	*SRCAP*	F	7	c.800G>T	p.(Gly267Val)	VOUS	NSY	FLHS	This study		Neutral
GDB1311	KS	*SRCAP*	NA	25	c.5206C>G	p.(Pro1736Ala)	VOUS	NSY	FLHS	This study		Neutral
GDB1154	KS	*CHD7*	NA	26	c.5426T>C	p.(Met1809Thr)	VOUS	NSY	CHARGE	This study		Disease-causing
GDB1200*	KS	*CTCF*	De novo	5	c.1033C>T	p.(His345Tyr)	LP	NSY	MRD21	This study		Disease-causing
GDB1166	KS	*SMARCB1*	M	6	c.646G>A	p.(Glu216Lys)	VOUS	NSY	CSS	This study		Disease-causing
GDB1128	KS	*SMARCA4*	F	27	c.3850G>A	p.(Asp1284Asn)	VOUS	NSY	CSS	This study		Neutral
GDB1400	KS	*SETD5*	NA	18	c.3107T>C	p.(Tyr885His)	VOUS	NSY	CdLS	This study		Neutral
GDB1405	KS	*SRCAP*	NA	34	c.9361C>G	p.(Arg3121Gly)	VOUS	NSY	FLHS	This study		Disease-causing
GDB1406	KS	*CHD7*	NA	15	c.3613A>G	p.(Ile1205Val)	VOUS	NSY	CHARGE	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/191225/	Neutral
18-2261	KS	*KAT6B*	NA	18	c.4225G>A	p.(Glu1409Lys)	VOUS	NSY	SBBYSS-genitopatellar syndrome	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/300889/	Neutral
GDB1433	KS	*SETD2*	NA	3	c.2000G>A	p.(Cys667Tyr)	VOUS	NSY	LLS	This study		Neutral
GDB1422	KS	*SRCAP*	NA	34	c.9368G>A	p.(Arg3123His)	VOUS	NSY	FLHS	This study		Neutral
GDB1428	KS	*BCOR*	NA	4	c.455C>T	p.(Pro152Leu)	VOUS	NSY	MCOPS2	This study		Disease-causing
GDB1355	CLS	*ATRX*	NA	28	c.6254G>A	p.(Arg2085His)	P	NSY	ATMDS, MRXHF1	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/372728/	Disease-causing
GDB1404	CLS	*SMC1A*	NA	7	c.1235G>A	p.(Arg412Gln)	VOUS	NSY	CdLS	This study		Neutral
GDB1418	CdLS	*EP300*	NA	31	c.6911C>A	p.(Ser2304Tyr)	VOUS	NSY	RSTS	This study		Disease-causing
GDB1281	BCOR	*CHD8*	NA	22	c.3379C>T	p.(Arg1127Cys)	VOUS	NSY	AUTS18	This study		Disease-causing
GDB1431	MED12	*EHMT1*	NA	5	c.905A>G	p.(Lys302Arg)	VOUS	NSY	KLEFS	This study	https://www.ncbi.nlm.nih.gov/clinvar/variation/210930/	Neutral
GDB1407	Lujan-Fryns syndrome	*SETD2*	NA	3	c.3776G>A	p.(Gly1259Asp)	VOUS	NSY	LLS	This study		Neutral
GDB1268	CdLS	*ARID1A*	NA	20	c.5192T>C	p.(Leu1731Ser)	P	NSY	CSS	This study		Disease-causing

SG: stop-gain (nonsense) variant caused by base substitution.
FS: frameshifting insertion and/or deletion. It alters the length and frame of the coding sequence.
NSY: non-synonymous variant. It alters the amino acid(s) but not the coding sequence length.
*Molecular diagnosis confirmed by clinician after patient phenotype re-evaluation.
AA, amino acid; ACMG, American College of Medical Genetics and Genomics; ATMDS, alpha-thalassaemia myelodysplasia syndrome; AUTS18, Autism, susceptibility to, 18; BCOR, BCL6 corepressor; BRWS, Baraitser-Winter syndrome; CdLS, Cornelia de Lange syndrome; CHARGE, Charge association-coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies syndrome; CLS, Coffin-Lowry syndrome; CSS, Coffin-Siris syndrome; F, father; FLHS, Floating-Harbor syndrome; KLEFS, Kleefstra syndrome; KS, Kabuki syndrome; LLS, Luscan-Lumish syndrome; LOVD, Leiden Open Variation Database; LP, likely pathogenic; M, mother; MCOPS2, microphthalmia syndromic 2, OFCD syndrome; MED12, Mediator Complex Subunit 12; MRD21, mental retardation, autosomal dominant 21; MRXHF1, mental retardation-hypotonic facies syndrome, X linked; NA, not available; OFCD, oculofaciocardiodental syndrome; P, pathogenic; RSTS, Rubinstein-Taybi syndrome; SBBYSS, Say-Barber-Biesecker-Young-Simpson syndrome; VOUS, variant of unknown significance; WDSTS, Wiedemann-Steiner syndrome.

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treated. Psychomotor development was delayed: he walked with a gait on tiptoe and spoke his first words at 2 years. Growth parameters were in the normal range. In childhood, he reported sleep disorder, which has gradually resolved. No critical events were observed and EEG was always normal. A brain MRI performed at 6 years of age which showed areas of non-specific parietal leucoencephalopathy was reported, while at 20 years a single focal area of 12 mm in diameter with a gliotic aspect on a repeated brain MRI was reported. On examination, facial features included long face, bushy eyebrows, hypertelorism, anteverted nares, broad nose with bulbous tip, macrostomia, thick lower lip and agenesis of two lower incisors. No haematological alterations or alpha-thalassaemic trait is present. A diagnostic hypothesis of Coffin-Lowry syndrome was proposed. Constitutional karyotype, array-comparative genomic hybridisation, sequence analysis for Coffin-Lowry syndrome and *FRAXA* were all negative. ChrPan revealed the pathogenic, maternally inherited variant c.109C>T p.(Arg37*) in *ATR-X*, associated with the X linked recessive disorder 'alpha thalassemia-mental retardation/intellectual disability syndrome' (MIM #301040)[30]; this variant has been reported as pathogenic in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/11742/).

DISCUSSION

In this work, we presented our experience of molecular screening of a cohort of 263 index patients with an initial suspicion of various hereditary disorders of chromatin regulation by application of a custom-made NGS panel of 68 causative and/or candidate genes (online supplementary table S1). We found variants of potential clinical relevance in 32% (87 patients) of them, with a predominance of pathogenic and likely pathogenic variants (tables 1 and 2). In families with positive results, our findings impacted the establishment of a final diagnosis, particularly in those with pathogenic and likely pathogenic variants, and contributed to reallocating the diagnosis within a wider nosology of partially overlapping disorders in a proportion of them. In the cohort of patients analysed, we were able to detect potentially clinically relevant variants in 32% of the cases. These data are somehow surprising as we expected a higher number of positive patients according to known rates of positive genetic testing available for at least the most common conditions included in the panel, such as Kabuki syndrome and Rubinstein-Taybi syndrome.[26 31] This may be due to the involvement of other causative genes, not included in the ChrPan, epigenetic mechanisms, or yet unrecognised promoter or deep intronic variants affecting normal splicing. Moreover, although we did not explore in detail the positive rates by referring centres or professionals, it is possible that the overall rate on positive laboratory results is not representative of all involved centres due to heterogeneity in patient selection. Finally, our current bioinformatics pipeline did not include a diagnostically valid algorithm for the identification of CNVs, which is a known mechanism causing disease in some chromatinopathies.[18 32]

Our study revealed that in a number of patients, the molecular findings were not in line with the original clinical diagnosis, and for some of them the clinical reassessment after molecular testing allowed the attribution of the alternate diagnoses (table 2). Specifically, we found (1) heterozygous pathogenic variants in *CTCF*, *KMT2A*, *SRCAP*, *ARID1B* and *CHD7* in five patients with a clinical suspicion of KS, respectively; (2) a heterozygous *ARID1A* pathogenic variant in a case of Cornelia de Lange syndrome; and (3) an *ATR-X* null variant in a man with a previous diagnosis of Coffin-Lowry syndrome.

So far only seven patients have been reported with de novo CTCF mutations (MIM #615502).[27 33–35] Consistent clinical features among these patients included developmental delay/intellectual disability, hypotonia, early feeding difficulty and microcephaly. Moreover a unique facial dysmorphism has been reported, which includes upslanting palpebral fissures, micrognathia, flat malar bone, long eyelashes and unique eyebrows (wider on the medial sides and thinner on the lateral sides).[34] We were, thus, able to identify the eighth patient with a de novo *CTCF* missense variant with matching clinical phenotype, expanding the mutation spectrum of *CTCF* gene and reinforcing the set of clinical signs that define this emerging syndrome. This patient supports the need of a pathway approach in patient selection and laboratory study design for optimising diagnostic resources.

Advances in genome high-throughput NGS techniques have significantly accelerated the research into the genetic basis of rare monogenic diseases. Also diseases' nosology is changing and patients' classification reflecting commonalities in pathway perturbations based on laboratory evidence is overwhelming classification according recognisable patterns on physical examination. Over recent years, a growing number of genes encoding different regulators of gene transcription and chromatin organisation have been linked to a spectrum of neurodevelopmental disorders often with overlapping clinical features, including growth retardation, intellectual disability, developmental delay and a combination of similar facial features. As the primary role of this overall group of genes concerns the structure and function of chromatin, we think that the widest term of 'Chromatinopathies' may be suitable for grouping together most, if not all, hereditary disorders of chromatin regulation due to variants in writers, erasers, readers and chromatin remodellers.[2 36]

Recently a number of studies strongly supported the notion that causative variants within these genes generate unique DNA methylation episignatures with, however, some overlaps across the different conditions in terms of shared molecular targets and biological pathways.[37–40] It has been postulated, indeed, that the clinical overlapping observed in a large number of studies[4 5 40–43] is thought to be caused by the downstream events orchestrated by the primary functional defect in genes encoding the epigenetic protein machinery.[2 6]

Our data support the indication that seldom a clear correlation of the genetic variant with the clinical manifestation of the phenotype is possible, likely due to the broad variability of clinical features seen in patients with chromatinopathies. Our results highlight how diagnosing the aetiology of this group of genetic diseases can be a challenge; the presenting conditions may have a plethora of differential diagnoses with subtle or absent phenotypes that may exclude a condition within the initial clinical assessment, and false-negative molecular results may have been previously reported leading the referring clinician to consider other causes. This study highlights the importance of simultaneously screening genes associated with the suspected condition as well as the differential diagnoses. Therefore, we strongly recommend the application of NGS technologies, exome sequencing and/or gene panel sequencing to allow the most efficient molecular analysis of patients with a clinical diagnosis of chromatinopathy. Moreover, since our understanding of this group of Mendelian disorders evolves over time, any panel should be regularly updated to capture the most clinically and molecularly relevant alterations in order to stay constantly up-to-date.

This work demonstrates that a pathway-based NGS approach may offer a unique opportunity to learn about the role of epigenetics in health and disease in humans and to provide

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additional evidence of common molecular gene network(s) for this group of diseases. Our findings suggest that variants in different and functionally related chromatin-associated factors might result in strongly overlapping clinical pictures. Also, these findings emphasise the necessity to further identify and molecularly characterise the disease-relevant mechanisms involved in the regulation of function and structure of chromatin. This is of paramount importance for making accurate diagnoses, understanding the pathogenesis and developing therapies.

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Contributors GM and CG conceived and designed the study. GM and BA designed the NGS panel. GMS, BA, NM and MR performed the NGS experiments and Sanger validation. GM, GMS and VM revised the literature. TM and SC performed the bioinformatics analysis. CG, DoM, PP, MC, AP, MP, MA, SM, MDM, MCDG, DaM, AS, SG, RF, EAC and EDF provided samples and clinical data. GM wrote the manuscript, with contributions from GMS, MC, VM and CG. GM, CG and VM provided funding. All authors contributed to and approved the final version of the manuscript.

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