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ORIGINAL INVESTIGATION

Exploring by whole exome sequencing patients with initial diagnosis of Rubinstein–Taybi syndrome: the interconnections of epigenetic machinery disorders

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Abstract

Rubinstein–Taybi syndrome (RSTS) is an autosomal-dominant neurodevelopmental disease affecting 1:125,000 newborns characterized by intellectual disability, growth retardation, facial dysmorphisms and skeletal abnormalities. RSTS is caused by mutations in genes encoding for writers of the epigenetic machinery: *CREBBP* (~ 60%) or its homologous *EP300* (~ 10%). No causative mutation is identified in up to 30% of patients. We performed whole-exome sequencing (WES) on eight RSTS-like individuals who had normal high-resolution array CGH testing and were *CREBBP*- and *EP300*-mutation -negative, to identify the molecular cause. In four cases, we identified putatively causal variants in three genes (*ASXL1, KMT2D* and *KMT2A*) encoding members of the epigenetic machinery known to be associated with the Bohring–Opitz, Kabuki and Wiedemann–Steiner syndromes. Each variant is novel, de novo, fulfills the ACMG criteria and is predicted to result in loss-of-function leading to haploinsufficiency of the epi-gene. In two of the remaining cases, homozygous/compound heterozygous variants in *XYLT2* and *PLCB4* genes, respectively, associated with spondyloocular and auriculocondylar 2 syndromes and in the latter an additional candidate variant in *XRN2*, a gene yet unrelated to any disease, were detected, but their pathogenicity remains uncertain. These results underscore the broad clinical spectrum of Mendelian disorders of the epigenetic apparatus and the high rate of WES disclosure of the genetic basis in cases which may pose a challenge for phenotype encompassing distinct syndromes. The overlapping features of distinct intellectual disability syndromes reflect common pathogenic molecular mechanisms affecting the complex regulation of balance between open and closed chromatin.

Introduction

Rubinstein–Taybi syndrome (RSTS, OMIM #180849, #613684) described in 1963 by Rubinstein, a pediatrician, and Taybi, a radiologist, is a rare neurodevelopmental multisystem malformation syndrome (Rubinstein and Taybi 1963) characterized by developmental delay and intellectual disability (DD/ID), growth retardation, skeletal anomalies including broad/short thumbs and/or big toes, and distinctive facial features (i.e. downslanting palpebral fissures, broad nasal bridge/convex nasal bridge, low hanging columella). A wide spectrum of other anomalies and malformations has also been reported in individuals with RSTS (Spena et al. 2015a, b). Phenotypic overlap between RSTS and other Mendelian conditions often makes the clinical diagnosis of RSTS difficult.

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Variants in two genes, *CREBBP* (16p13, OMIM #600140) and *EP300* (22q13, OMIM #602700), underlie RSTS. *CREBBP* and *EP300* encode two highly conserved, ubiquitously expressed and homologous lysine-acetyltransferases (KAT) that act as "writers" of the epigenetic machinery, named CBP and p300 (Fahrner and Bjornsson 2014; Bjornsson 2015). Up to 60% of RSTS cases harbor de novo mutations in *CREBBP*, which is the "major" gene (http://www.lovd.nl/CREBBP) (Spena et al. 2015a, b), while approximately 10% of affected individuals have de novo alterations in *EP300*, the "minor" gene (http://chromium.liacs.nl/LOVD2/home.php?select_db=EP300) (Masuda et al. 2015; Wincent et al. 2015; Fergelot et al. 2016; Hamilton et al. 2016; López et al. 2016; Negri et al. 2016; Sellars et al. 2016). Point mutations (i.e. frameshift, nonsense, missense and splicing in order of prevalence) represent the majority of genetic mutations found in both *CREBBP* and *EP300* patients followed by deletions (intragenic, whole gene or expanding to adjacent regions), translocations and inversions (Lacombe et al. 1992; Breuning et al. 1993; Masuda et al. 2015; Rusconi et al. 2015; Spena et al. 2015a, b; Negri et al. 2015, 2016). The genetic basis of ~30% of RSTS cases remains unknown.

Alterations in genes encoding proteins involved in the epigenetic regulation of chromatin dynamics, acting as "writers", "erasers", "readers" and "remodelers", are associated with other Mendelian disorders of the epigenetic machinery showing phenotypic overlap with RSTS such as Bohring–Opitz, Wiedemann–Steiner and Kabuki syndrome (Fahrner and Bjornsson 2014; Bjornsson 2015) or to other rare conditions (i.e. Floating–Harbor and genitopatellar syndromes) whose the underlying genes encode proteins that interact directly with CBP and/or p300 (Spena et al. 2015a, b).

We performed exome sequencing on eight cases with RSTS diagnosis in which mutations in *CREBBP* and *EP300* could not be identified. The rationale was that such novel genes would broaden our understanding of the epigenetic perturbations that lead to RSTS. We report the discovery of four novel de novo mutations within "epi-genes" associated with syndromic epigenetic disorders with clinical similarity to RSTS. We highlight the complex and unique phenotypes of these cases, accounting half of our WES-selected cohort, and comment on how epigenetic dynamics alterations could result in a wide spectrum of clinical features creating a continuum of overlapping syndromes. In two of the remaining four WES-processed cases, candidate variants were disclosed in genes associated with known syndromes not "tagged" as epigenetic disorders, but the pathogenicity of the variants remains unclear.

Materials and methods

Subjects

All individuals were assessed by a clinical geneticist. Written informed consent was obtained from all subjects and this study was approved by the local institution ethical committee and review board (http://www.unimi.it/ateneo/normativa/50486.htm) and performed according to the Declaration of Helsinki protocol. This study includes a subset of patients and their healthy parents selected according to the following criteria: (i) genetic test found negative to *CREBBP*/*EP300* mutations, (ii) RSTS-like phenotype.

High-resolution array CGH

Array CGH experiments were performed with a commercially available 400 K 60-mer oligonucleotide microarray slide (Agilent Technologies Inc., Santa Clara, CA, USA) in accordance with the manufacturer's instructions. Commercially available sex-matched control genomic DNA (Promega) was used. Data were extracted and analyzed for copy number changes using Agilent CytoGenomics v.3.0.

Whole exome sequencing and data analysis

A total of 1 µg of genomic DNA was processed and enriched for exon sequences according to the protocol recommended for Roche/Nimblegen SeqCap EZ v3.0 (~62 MB target) (Roche, Basel, Switzerland). After quality check on the Agilent Bioanalyzer, libraries were sequenced on the Illumina HiSeq2000/2500 sequencers (Illumina, Applied Biosystems, Foster City, CA, USA). A standard bioinformatic pipeline was applied to analyze raw sequencing data, as previously described (Van der Auwera et al. 2013).

VarScan2 (http://www.dkoboldt.github.io/varscan) (Koboldt et al. 2012) and DeNovoGear (http://www.denovogear.weebly.com) (Ramu et al. 2013) algorithms were applied using standard parameters for de novo germ-line calling. To increase call specificity, only variants detected by both algorithms were considered as de novo candidates. Based on estimated disease prevalence of 1:125,000, we retained for further analysis de novo variants not reported in general population databases (ExAC: Exome Aggregation Consortium, http://exac.broadinstitute.org/ and gnomAD: Genome Aggregation Database, http://gnomad.broadinstitute.org/) and homozygous/compound heterozygous/hemizygous variants with allele frequency $\leq 0.3\%$ without homozygous/hemizygous individuals described in the above-mentioned databases. Only variants predicted to be likely protein

altering (nonsynonymous substitutions, splice-site and insertions/deletions) were examined further and variants with CADD v1.2 Phred score < 15 were filtered out to discard most likely benign nonsynonymous variants. We excluded variants in genes associated with non-neurodevelopmental disorders. We classified the significance of candidate variants according to ACMG criteria (Richards et al. 2015) using Varsome tool (https://varsome.com/).

Variant validation and segregation analysis

Variants of interest were confirmed by Sanger sequencing following PCR amplification and segregation analysis was always performed in each trio. PCR reactions were performed following the protocol for GoTaq Flexi DNA Polymerase (Promega) with specific primer sets. The same pairs of primers were used for Sanger sequencing. Sequence variants were described according to HGVS nomenclature guidelines (http://varnomen.hgvs.org/).

In silico analyses

The deleterious potential of missense substitutions was assessed by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), SIFT (http://sift.jcvi.org/), SNPs&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go/), MutPred v.1.2 (http://mutpred.mutdb.org/), PMut (http://mmb.pcb.ub.es/PMut/analyses/new/), SNAP2 (https://rostlab.org/owiki/index.php/Snap2), Mutation Taster (http://www.mutationtaster.org/), Panther (http://www.ngrl.org.uk/Manchester/page/missense-prediction-tools), PhD SNP (http://snps.biofold.org/phd-snp/phd-snp.html), MetaSNP (http://snps.biofold.org/meta-snp/pages/methods.html) and IMutant2 (http://folding.biofold.org/cgi-bin/i-mutant2.0.cgi) programs.

Multiple sequence alignments were performed by submitting protein sequences derived from UniGene (http://www.ncbi.nlm.nih.gov/unigene) to Clustal program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Variants detected by WES of *CREBBP*/*EP300* mutation-negative patients with initial RSTS clinical diagnosis

DNA samples from eight probands with RSTS initial clinical diagnosis but no detected pathogenic variant in either *CREBBP* or *EP300* by MLPA analysis, targeted sequencing and high-resolution array CGH were selected for WES. A first analysis considering only novel de novo variants with priority given to sequence changes in disease-associated genes belonging to the so-called epigenetic machinery allowed us to identify compelling candidate variants in four of eight patients (Table 1). Each variant was validated by Sanger sequencing and confirmed variants arose de novo. None of the identified variants is reported in ExAC and gnomAD databases.

Two stop-gain variants, c.3856C>T, p.(Q1286*) and c.4243C>T, p.(R1415*), in exon 12 (Online Resource Fig. S1) of the Additional Sex Combs-Like 1 gene (*ASXL1*, OMIM *612990, NM_015338) were identified in cases #80 and #173, respectively. Heterozygous truncating variants in *ASXL1*, which encodes a protein belonging to the polycomb group (PcG) and trithorax complex family, underlie Bohring–Opitz syndrome (BOPS, OMIM #605039), a rare congenital malformation disorder, first described in 1999 by Bohring (Bohring et al. 1999). Neither variant has been reported previously in BOPS families (Online Resource Table S1 and Fig. S1) (Arunachal et al. 2016). According to ACMG guidelines, the clinical significance of the two variants is likely pathogenic and pathogenic, respectively. Both variants meet the same ACMG criteria (PM1, PM2, PM4, PM6, PP2) with p.(R1415*) additionally fulfilling PS3 criterion, being recently uploaded in ClinVar database and classified as pathogenic.

Case #95 was found to have a novel stop-gain variant, c.6040C>T, p.(Q2014*) in exon 28 (Online Resource Fig. S1) of lysine (K)-specific methyltransferase 2D (*KMT2D*, formerly *MLL2*, OMIM #602113, NM_003482), encoding

Table 1 Mutations detected in the four RSTS-like patients achieving molecular diagnosis

Patient	Gene	cDNA	Position	Protein	Type	Associated Syndrome	Role in the epigenetic machinery	ACMG classification (2015)
#80	*ASXL1*	c.3856C>T	Exon 12	p.(Q1286*)	Stop-gain	Bohring–Opitz	Reader	Likely pathogenic
#173	*ASXL1*	c.4243C>T	Exon 12	p.(R1415*)	Stop-gain	Bohring–Opitz	Reader	Pathogenic
#103	*KMT2A*	c.5363+1delG	IVS 18		Splicing	Wiedemann–Steiner	Writer	Pathogenic
#95	*KMT2D*	c.6040C>T	Exon 28	p.(Q2014*)	Stop-gain	Kabuki	Writer	Pathogenic

Position and type of the pathogenic variants, epigenetic role of the affected genes and associated syndromes are indicated

a methyltransferase responsible for histone 3 lysine 4 (H3K4) demethylation and trimethylation, an epigenetic mark of euchromatin and active transcription (Smith et al. 2011). *KMT2D* is the first identified and "major" gene for Kabuki syndrome (KS, OMIM #197420, #300867) a highly variable genetic condition characterized by growth deficiency, intellectual disability, minor skeletal anomalies and distinctive facial features (Niikawa et al. 1981). To date, more than 200 de novo mutations in *KMT2D* have been reported in about 56–75% of KS cases (Schott et al. 2016) (Online Resource Table S2 and Fig. S1). ACMG classification indicates the p.(Q2014*) variant as pathogenic fulfilling PVS1, PM2, PM4, PM6 and PP2 criteria.

In case #103, we found a c.5363+1delG variant at the donor site of intron 18 (Online Resource Fig. S1) of lysine-specific methyltransferase 2A (*KMT2A*, OMIM +159555, NM_001197104) encoding a writer of the epigenetic machinery. Truncating mutations of *KMT2A* underlie Wiedemann–Steiner syndrome (WDSTS, OMIM #605130), a heterogeneous disease described in 1989 by Wiedemann (Wiedemann et al. 1989), and characterized by hypertrichosis *cubiti*, intellectual disability and developmental delay together with a distinctive facial appearance and short stature (Aggarwal et al. 2017). To our knowledge, 26 mutations have been reported in clinically diagnosed WDSTS cases (Aggarwal et al. 2017) with the exception of one patient diagnosed with Cornelia de Lange syndrome (CdLS, OMIM #122470, #300590, #610759, #614701, #300882), a clinical and molecular heterogeneous plurimalformative syndrome with partial overlap to WDSTS (Yuan et al. 2015) (Online Resource Table S3 and Fig. S1). More recently, additional 29 *KMT2A* variants have been described in 33 patients selected as characterized by syndromic intellectual disability (Baer et al. 2018). The c.5363+1delG variant meets the PVS1, PM2 and PM6 ACMG criteria of pathogenicity, has a predicted null effect on a gene where loss-of-function is a known disease mechanism and is a previously unreported de novo variant.

Additional de novo variants in genes not associated with epigenetic machinery syndromes have been also evaluated (Online Resource Table S4).

The variant c.560G>A, p.(G187E) in 5-prime,3-prime-exoribonuclease 2 (*XRN2*, OMIM *608851, NM_001317960) gene, not yet associated with a specific syndrome, was found in patient #169. The deleteriousness potential of the variant was assessed by performing in silico prediction analyses. Eight out of 11 programs predicted a probable disease causing role for the identified variant. Furthermore, multiple sequence alignments (Clustal program) evidenced the broad conservation of the involved amino acid residue from yeast to human. According to ACMG guidelines, this variant has uncertain significance.

Subsequently, variants analysis was also performed under AR- and X-linked model of Mendelian inheritance (Online Resource Table S4). Homozygous missense variant c.209G>A, p.(R70Q) in xylosyltransferase-2 gene (*XYLT2*, OMIM *608125) was found in patient #76 with a family record of consanguinity (the grandparents are cousins of second grade). Frameshift mutations in this gene are reportedly associated with spondyloocular syndrome (SOS, OMIM #605822).

Two variants in phospholipase C beta 4 gene (*PLCB4* OMIM *600810), the paternal frameshift c.2607_2609dup, p.(Ser870dup) and the maternal missense c.2653G>A, p.(V885M), were detected in patient #169 found also carrier of the *XRN2* variant. AR/AD mutations are reported in auriculocondylar syndrome 2 (ARCND2, OMIM #614669).

The clinical impact of all these variants was predicted as uncertain, following ACMG criteria.

The analysis of variants in the remaining cases #88 and #118 did not reveal any strong candidate variant.

Clinical phenotype of WES-processed cases with initial RSTS diagnosis

Case #80 (*ASXL1*)

Familial history is negative for genetic conditions and/or ID. Pregnancy was normal. The child was born at 41 weeks of gestational age by cesarean section; at birth, auxological parameters (not reported) were normal. He presented with sucking difficulties and hypotonia, and he developed a severe ID.

At the clinical evaluation (24 years), weight was 44 kg (< 3° centile), height was 166 cm (50°), and head circumference 57 cm (98°). He presented dolichocephaly, low hairline, low set and posteriorly rotated ears, synophrys, downslanting palpebral fissures, palpebral ptosis, hypertelorism, long eyelashes, high nasal bridge, low hanging columella, short philtrum, high and narrow palate, open mouth, malocclusion, thick vermilion of the lower lip (Fig. 1). Scoliosis and pectus excavatum were recognized, and low set and broad thumbs were evident. Corpus callosum hypoplasia, seizures and delayed bone age were also reported.

Case #173 (*ASXL1*)

Familial history is positive for macrocephaly and hydrocephalus in a cousin. Pregnancy was normal. The child was born at 37 weeks of gestational age by spontaneous delivery; at birth, weight was 2.400 kg (< 3°), length 45.5 cm (< 10°), OFC 31 cm (< 3°), and APGAR score was 6/8. Cleft lip was diagnosed.

He showed severe ID, autism spectrum disorder, increased levels of aggression and seizures.

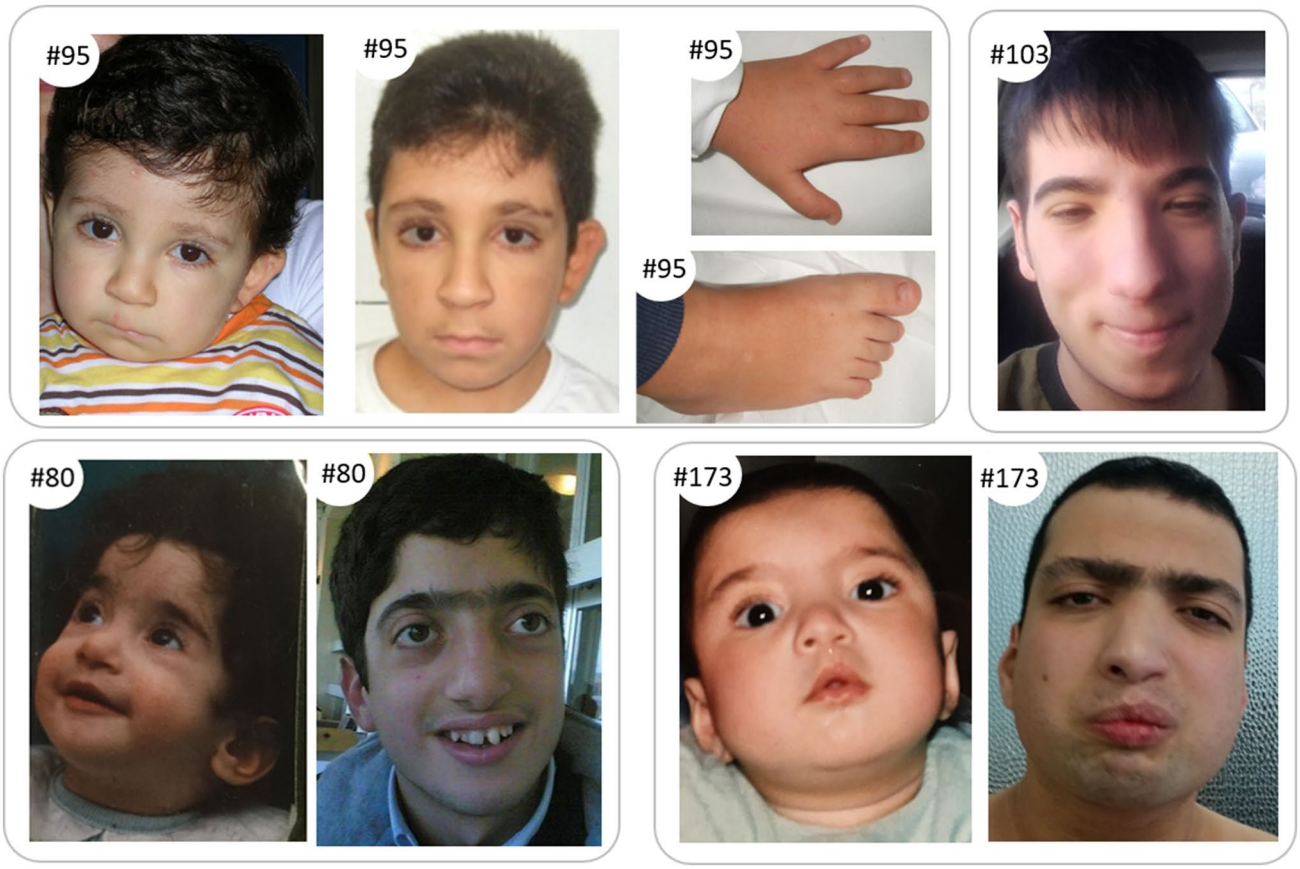

Fig. 1 Features of patients #95, #80, #103 and #173. Top left: features of pt #95 found carrier of *KMT2D* mutation at 1 year (left) and 10 years (right) with particular of foot and hand. Top right: features of patient #103 at 16 years. Bottom: features of pt #80 and pt #173 found carriers of *ASXL1* mutations. Faces are shown at 1 year (left) and in adulthood (right)

At the clinical evaluation (22 years), weight was 60 kg (10° centile), height was 163 cm (3°), and head circumference 55.8 cm (50°). He presented low hairline, arched eyebrows, long eyelashes, downslanting palpebral fissures, convex nasal bridge, thick vermilion of the lower lip, micro-retrognathia, facial grimacing, hypotonic facies with full cheeks and low set ears with increased posterior angulation (Fig. 1); first toe was broad.

Additional medical problems and malformations were reported: corpus callosum hypoplasia, strabismus, hypermetropic astigmatism, left superior lateral incisor agenesis, gastroesophageal reflux, hip dislocation, patellar subluxation, hypothyroidism, constipation, frequent airways infections.

Case #103 (*KMT2A*)

Familial history is negative for genetic conditions and/or ID. Pregnancy was normal. The child was born at 39 weeks of gestational age by spontaneous delivery. Birth parameters were weight > 10°, head circumference 50° and length 15°. He presented with sucking difficulties and hypotonia, and a glabellar nevus flammeus that fades with age; a postnatal growth retardation was evident. He showed a moderate ID.

At the clinical evaluation (6 years), weight was 22 kg (50° centile), height 112 cm (10°), and head circumference 52 cm (50°); he presented with narrow forehead, low anterior hairline, synophrys, hypertelorism, arched and thick eyebrows, narrow and downslanting palpebral fissures, convex nasal bridge with low hanging columella, short philtrum, thin lips, facial grimacing, high palate, micrognathia, and posteriorly angulated ears (Fig. 1). Thumb and first toe were broad; pectus excavatum and hirsutism (not specifically localized on the elbow) were evident.

Other medical problems subsequently reported were kyphosis and obesity.

Case #95 (*KMT2D*)

Familial history is positive for hypothyroidism, seizures, autism; a brother of the proband was diagnosed with moderate ID and autism. The child was born at 37 weeks of gestational age with cesarean section for breech presentation and

fetal distress. At birth, auxological parameters were: weight 25–50°, length 3–10°. During pregnancy, IUGR and weak fetal movements were referred; at birth, weight was 2.620 kg, length 44.6 cm, APGAR score was 9. In neonatal age, sucking difficulties are reported. Psychomotor retardation and hypotonia were evident.

At the age of 16 months, weight was 9 kg (3–10° centile), height 72 cm (< 3°), and head circumference 44 cm (3°).

At the clinical evaluation (9 years), weight was 36 kg (75° centile), height 127 cm (10°), and head circumference 53 cm (25°); the child had long eyelashes, low set ears, microstomia, thin lips, high palate, and micrognathia; thumb and first toe were broad, and brachydactyly was evident (Fig. 1).

Strabismus, myopia, lateral incisors agenesis, hypothyroidism, cryptorchidism (surgically treated), constipation and short stature (treated with growth hormone) were also reported.

Table 2 provides the detailed description of the four patients found carriers of de novo candidate variants with a definitive/strong role in disease. In particular, typical clinical signs of RSTS, KS, BOPS and WDSTS are detailed for comparison with features shown by patient #95 (carrier of *KMT2D* mutation), patients #80 and #173 (carriers of *ASXL1* mutations) and patient #103 (carrier of *KMT2A* mutation).

The typical RSTS heterogeneity in the number, type and severity of clinical findings is evident also in this cohort. Nevertheless, typical RSTS clinical signs, such as specific dysmorphic features (i.e. low hanging columella, etc.) or characteristic skeletal anomalies (i.e. broad thumb/hallux) are present in almost all patients (Table 2; Fig. 1).

In particular, craniofacial anomalies such as the typical nose with prominent columella, downslanting of the palpebral fissures, and long eyelashes are present in 3/4 or 4/4 described patients.

Micrognathia is highly prevalent (4/4 patients), synophrys is evident in 3/4 patients, while grimacing smile is detected in two patients [#173 (*ASXL1*), #103 (*KMT2A*)].

Prenatal growth retardation is reported in two cases [pts #95 (*KMT2D*) and #173(*ASXL1*)] while postnatal growth retardation in 3/4 cases. Skeletal malformations, as expected, mainly concern thumbs and big toes, which are broad and short (3/4).

Intellectual disability (ranging from moderate to severe) is always present, and language is absent or very limited except for patient #103 (*KMT2A*).

Other RSTS-like signs recorded in our cases are dental anomalies [pts #80 (*ASXL1*), #95 (*KMT2D*), #173(*ASXL1*)], and behavioral problems [pts #80 (*ASXL1*), #95 (*KMT2D*), #173 (*ASXL1*)].

However, clinical features atypical for RSTS but described in the different syndromes associated with the identified pathogenic variants are also present: out of them buphthalmos [pt #80 (*ASXL1*)], contractures [pts #80 (*ASXL1*), #173 (*ASXL1*)], described in BOPS or eversion of the lateral third of the lower eyelids [pt #95 (*KMT2D*)], present in KS (Table 2; Fig. 1).

The clinical phenotype of the remaining patients #76, #88, #118, and #169 was also re-evaluated and supported the initial RSTS/RSTS-like diagnosis. Supplemental Table S5 summarizes the clinical features of these patients, compared to major typical signs of RSTS.

Discussion

We identified putatively causal variants in four patients with an initial RSTS diagnosis in genes known to underlie other multiple malformation syndromes with phenotypic features that overlap RSTS: the involved genes, namely *ASXL1* (patients #80 and #173), *KMT2D* (patient #95) and *KMT2A* (patient #103), are known to be responsible for Bohring–Opitz, Kabuki and Wiedemann–Steiner syndromes, respectively.

The identified variants meet the pathogenicity criteria recommended by ACMG guidelines, including de novo occurrence, absence in general population and predicted LOF effect in genes (*ASXL1, KMT2A* and *KMT2D*) causing diseases through this proved pathogenetic mechanism. Type and gene localization of mutations are indiscernible from those described in BOPS, KS and WDSTS patients, respectively (Online Resource Tables S1–S3 and Fig. S1).

We also identified a novel likely pathogenic variant in *XRN2* gene: the de novo missense mutation identified in patient #169 is predicted to cause a change of a highly evolutionary conserved aminoacid, p.(G187E) and is considered damaging by 8 out of 11 interrogated prediction softwares. *XRN2* is not yet associated with a known human disease. It codes for a 5′ → 3′ exoribonuclease belonging to a large family of conserved enzymes in eukaryotes, ubiquitously expressed and acting in the nucleus. Studies performed in the yeast *Schizosaccharomyces pombe* (Tucker et al. 2016) demonstrate the activity of the protein in RNA silencing, suggesting a role in the epigenetic machinery, now expanded to include enzymes reversibly modifying mRNA (Koboldt et al. 2012; Van der Auwera et al. 2013). Further studies are needed to assess the potential pathogenic role of the *XRN2* c.560G>A, p.(G187E) variant, although a different epigenetic mechanism involving RNA modification might be hypothesized to justify the phenotype of the carrier patient.

Finally, following the autosomal recessive inheritance model we found homozygous/composite heterozygous variants in *XYLT2* (pt #76) and *PLCB4* (pt #169) genes. *XYLT2* and *PLCB4* genes are associated with spondyloocular and auriculocondylar 2 syndromes, two diseases showing no clinical signs overlapping to RSTS. Clinical re-evaluation of patients #76 and #169 did not highlight any sign resembling

Table 2 Clinical signs of patients #95, #80, #173 and #103 compared to typical features of RSTS, KS, BOPS and WDSTS

	RSTS	#95	KS	#80	#173	BOPS	#103	WDSTS
Gene with pathogenetic variant		*KMT2D* c.6040C > T, p.(Q2014*)		*ASXL1* c.3856C > T, p.(Q1286*)	*ASXL1* c.4243C > T, p.(R1415*)		*KMT2A* c.5363 + 1delG	
Date of birth		2006		1993	1994		2001	
Time of clinical assessment		2007		2006	2011		2008	
Sex		male		male	male		male	
Dysmorphisms								
Low anterior hairline	+	−	−	−	+	+	−	−
Long eyelashes	+	+	−	+	+	−	+	+
Synophrys	+/−	−	−	+	+	−	+	+
Ptosis	+	+	+	−	−	−	−	−
Downslanting palpebral fissures	+	−	−	+	+	−	+	+
Upslanting palpebral fissures	−	−	−	−	−	+	−	−
Eversion of the lateral third of lower eyelid	−	+	+	−	−	−	−	−
Thick eyebrows	+/−	−	−	+	+	−	+	+
Narrow palpebral fissures	−	−	−	−	+	−	+	+
Hypertelorism	+	−	−	+	+	+	+	+
Prominent eyes	−	−	−	+	−	+	−	−
Low hanging columella	+	−	−	+	+	−	+	−
Broad nasal tip	−	+	−	+	+	−	+	−
Convex and wide nasal bridge	+	−	+	−	−	+	−	+
Anteverted nares	−	−	−	−	−	+	−	−
Thin lips	−	+	+	−	−	−	+	+
Facial grimacing	+	−	+	−	+	−	+	−
Microstomia	−	+	−	−	−	−	+	+
High-arched palate	+	−	+	+	na	−	+	+
Cleft lip/palate	−	−	+	−	+ (cleft lip)	+	−	−
Micrognathia	+	+	+	+	+	+	+	+
Low set ears	+	+	−	+	+ (with increased posterior angulation)	+	+	+
Strabismus	+	+	−	+	+	+	−	+
Flammeus nevus/angioma	+/−	−	−	−	−	+	+	−
Hypotonic facies with full cheeks	−	−	−	−	+	+	−	−
Growth failure								
IUGR	−	+	−	−	+	+	+	+

Table 2 (continued)

	RSTS	#95	KS	#80	#173	BOPS	#103	WDSTS
Birth parameters: weight–length–OFC (percentiles)		25° to 3° to na		na (referred as normal)	< 3° to <3° to 10°		< 10° to 15° to 50°	
PNGR	+	+	+	−	+	+	+	+
PN (age) parameters: weight–height–OFC (percentiles)		75°–10°–25° (9 y)		25°–50°–98° (23 y)	10°–3°–50° (21 y)		nk	
Intellectual disability	+	+ (moderate)	+	+ (severe)	+ (severe)	+	+ (moderate)	+
Speech delay/absence	+/−	+	+/−	+	+	+	−	+
Behavioral problems	+/−	−	+/−	+ (hyperkinesis, agitation)	+ (aggressivity, agitation)	+	−	+
Vision problems								
Myopia	+	+ (and hypermetropic astigmatism)		−	+ (and hypermetropic astigmatism)	+	−	−
Hearing loss		−	+/−		na	−	−	−
Teeth anomalies	+	+ (absence of superior lateral incisors)	+	+ (enamel hypoplasia, hypodontia)	+ (absence of superior lateral incisors)	−	−	+
Musculoskeletal anomalies								
Broad thumbs	+	+	−	−	−	−	+	−
Angulated thumbs	+/−	−	−	−	−	−	−	−
Broad halluces	+	+	−	−	+	−	+	−
Clinodactyly	+	+	+	+	−	−	+	+/−
Brachydactyly	+	+	−	−	−	+	+	−
Camptodactyly		−	−	−	+	+	−	−
Microcephaly	+	−	+	−	+	+	+	-
Trigonocephaly	−	−	−	−	−	+	−	+
Delayed bone age	+	−	−	+	na	−	−	+
Fetal fingertip pads	−	+	+	+	−	−	+	−
BOPS posture	−	−	−	−	−	+	−	−
Fixed contractures	−	−	−	+	−	+	−	−
Hypotonia	+	+	−	+	−	+	+	+
Organ anomalies								
Genitourinary anomalies	+	+ (CO)	+	−	−	−	+ (CO)	−
Heart defect	+	−	+	−	−	+	−	+
Brain anomalies	+	−	−	+ (HCC)	+ (ACC)	+	−	+
Seizures	+	+	−	+	+	+	+	−
Hypertrichosis	+	+	+	−	+	+	+	+
Keloids/naevi	+	−	−	−	−	+/−	−	−
Pilomatricoma	+	−	−	−	−	−	−	−
Frequent infections	+	−	+	−	−	+	−	−

Table 2 (continued)

	RSTS	#95	KS	#80	#173	BOPS	#103	WDSTS
Feeding problems	+	+	–	+	–	+	+	+/–
Gastroesophageal reflux	+	–	+	+	–	+	–	–
Others		Hypomobility, absence of superior lateral incisors, hypothyroidism, GH therapy for short stature		Buphthalmos; dolichocephaly; hyperreflexia; joint hypermobility, enamel hypolasia; abnormal tooth number	Acanthosis nigricans, striae distensae and erythema (chest); hepatomegaly, compulsive food research and eating; astigmatism, hypermetria		Kyphosis, pectus excavatum, obesity	

Sign: + means present, sign – absent and sign +/– present in a few cases

ACC agenesis of corpus callosum, *CO* cryptorchidism, *HCC* hypoplasia of corpus callosum, *na* not assessed, *nk* not known

those of spondyloocular and auriculocondylar 2 syndromes. Moreover, patient #169 is found carrier of both *XRN2* and *PLCB4* variants, making difficult to decipher the role of each gene in pathogenesis. In these cases, no clear pathogenetic role can be defined.

Provision of a definite molecular diagnosis to half of the WES-processed patients represents a main goal of this work combined to the disclosure of known genes implicated in epigenetic syndromes with partial phenotypic overlap with RSTS. Besides highlighting common molecular mechanisms underlying these syndromes, this result highlights the challenge of diagnosis of syndromic disorders spanning a broad phenotypic spectrum.

Genotype–phenotype correlations are very complex in the molecularly solved cases. All the eight patients were enrolled in this study as they received an initial diagnosis of Rubinstein–Taybi syndrome, supported by the presence of several typical signs (Table 2 and Table S5). In particular, broad thumb/hallux, typical supraorbital region features and low hanging columella are signs present in almost all the described patients that probably induced the clinician to formulate the RSTS or RSTS-like diagnosis. However, focusing on the four patients found carriers of likely pathogenetic variants, the patients also display additional signs, atypical in RSTS, as exemplified by the distinctive Kabuki sign of eversion of the lateral third of the lower eyelids in patient #95, who turned out to be carrier of a *KMT2D* mutation (Fig. 1).

To reconcile the initial clinical to the final molecular diagnosis, one need to recall that the RSTS diagnosis has been formulated during infancy of our patients and phenotypic changes manifested during growth were only monitored at clinical re-evaluation upon WES results. In addition, clinical features of syndromes such as BOPS have been defined only in the last years, hampering the referring clinician to raise this diagnostic hypothesis. Indeed, a specific alternative diagnosis (i.e. BOPS for patients #80 and #173, KS for #95 and WDSTS for #103) has been taken into account only after the molecular findings. However, the phenotype of patient #103, found carrier of *KMT2A* mutation associated to WDSTS, remains RSTS-matching also in his adolescence. Moreover, he does not show the generalized or localized (i.e. hypertricosis *cubiti*) hypertrichosis which is a typical sign of WDSTS (Aggarwal et al. 2017) and conversely maintains the distinctive RSTS facies (Fig. 1). Similarly, patients found carriers of *ASXL1* mutations (#80 and #173) display an atypical RSTS phenotype, but their clinical signs are only in part reminiscent of BOPS features and in a milder form (e.g. exophthalmos in one out of two patients, camptodactyly, mild growth delay). In the literature, the BOPS patients show a severe overall clinical presentation that may compromise the survival in the childhood; despite the mutations identified in these patients are similar to those of BOPS

patients in term of type and gene localization, our patients show very limited BOPS features (Table 2; Fig. 1).

Furthermore, we do not exclude that mutations in the same gene give rise to different or composite phenotypes which may be attributed to stochastic progression during development, to differences in the genetic background between patients and to different strength mutations. Several *CREBBP* and *EP300* mutations were identified in the last years by NGS approaches in patients who had not, or only in a very limited way, the RSTS features (Woods et al. 2014; Masuda et al. 2015; Dauwerse et al. 2016; Menke et al. 2016, 2018; Sellars et al. 2016). Conversely, the same applies to BOPS, KS and WDSTS syndromes and patients featuring with WDSTS were shown mutated in genes underlying the main form of Cornelia de Lange syndrome (Yuan et al. 2015).

Clinical genomics is increasingly revealing molecular pathogenesis and promises to foster remarkable advances in the molecular diagnosis of epigenetic machinery syndromes, as already shown for chromatin-related disorders. Examples from the recent literature are mutations of the gene for ankyrin repeat domain-containing protein 11 (*ANKRD11*, OMIM *611192) which are usually associated with KBG syndrome which is characterized by macrodontia of the upper central incisors, distinctive craniofacial features, short stature, skeletal anomalies and intellectual disability (Sirmaci et al. 2011). Recently, few patients with features consistent with Cornelia de Lange syndrome (partially overlapping with KBG) sorted out from a large CdLS cohort were found carriers of *ANKRD11* mutations (Parenti et al. 2016).

Despite the wide presentation of our patients' phenotype, which may be defined peculiar or unique, we can grasp a slight overlapping feature between the patients, attested by their initial RSTS diagnosis. Though the identified genetic causes are distinct, all the involved genes belong to the epigenetic apparatus: KMT2A and KMT2D are known writers (just like CBP and p300), ASXL1 is classified as reader. Alteration in one of the component of the epigenetic machinery can perturb the equilibrium of opening/closing chromatin, but also the crosstalk between the different players of this vast and complicated interconnected network may be imbalanced (Allis and Jenuwein 2016). Information flow is bi-directional forming a feedback loop among individual epigenetic machinery. Nevertheless, disruption of histone marks leads to disruption of DNA methylation levels and vice versa (Jin et al. 2008; Sobreira et al. 2017) but also one histone mark alteration can influence the global histone modification. In this view, different overlapping disorders can develop from similar shared epigenetic modification and the specific cell population sensitive to loss of epigenetic machinery component at specific time can induce the mild differences underlying the different features of epigenetic syndromes.

These findings highlight then on one hand the known overlap between RSTS and other conditions and on the other underline the expansion of the phenotypic spectrum of variants in genes encoding epigenetic machinery.

In summary, mutations in genes other than *CREBBP* or *EP300* such as *ASXL1, KMT2A*, and *KMT2D*, known to be causative of the epigenetic syndromes BOPS, WDSTS and KS, respectively, are found associated with a specific, different and composite phenotype which seems to be more RSTS-typical in early childhood.

The patients herein described might be the top of a platform of cases that are overlooked by conventional workflow and could be easily detected by NGS targeted to multigene panel. Results from this work support the view that different molecular causes, all belonging to the same epigenetic interplay, underpin different/unique overlapping phenotypes: these multiple causes should be considered in order to perform the appropriate diagnosis and management of patients with epigenetic syndromes.

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