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New insights into genetic variant spectrum and genotype– phenotype correlations of Rubinstein-Taybi syndrome in 39 *CREBBP*-positive patients

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Abstract

Background: Rubinstein-Taybi syndrome (RSTS) is a rare congenital disorder characterized by broad thumbs and halluces, intellectual disability, distinctive facial features, and growth retardation. Clinical manifestations of RSTS are varied and overlap with other syndromes' phenotype, which makes clinical diagnosis challenging. *CREBBP* is the major causative gene (55%–60% of the cases), whereas pathogenic variants found in *EP300* represent the molecular cause in 8% of RSTS patients. A wide range of *CREBBP* pathogenic variants have been reported so far, including point mutations (30%–50%) and large deletions (10%).

Methods: The aim of this study was to characterize the *CREBBP* genetic variant spectrum in 39 RSTS patients using Multiplex Ligation-dependent Probe Amplification and DNA sequencing techniques (Sanger and Trio-based whole-exome sequencing).

Results: We identified 15 intragenic deletions/duplications, ranging from one exon to the entire gene. As a whole, 25 de novo point variants were detected: 4 missense, 12 nonsense, 5 frameshift, and 4 splicing pathogenic variants. Three of them were classified as of uncertain significance and one of the patients carried two different variants.

Conclusion: Seventeen of the 40 genetic variants detected were reported for the first time in this work contributing, thus, to expand the molecular knowledge of this complex disorder.

KEYWORDS
CREBBP, epigenetics, genotype–phenotype correlation, Rubinstein-Taybi syndrome

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1 | INTRODUCTION

Rubinstein-Taybi syndrome (RSTS, OMIM #180849, #613684) is a rare genetic disorder characterized by post-natal growth retardation, moderate to severe intellectual disability (ID), and a wide range of typical dysmorphic features. Broad thumbs and halluces are a distinctive feature of the syndrome. Facial dysmorphology includes downslanting palpebral fissures, prominent beaked nose, and columella below the *alae nasi* Hennekam, 2006. The RSTS pattern of transmission is autosomal dominant with an estimated prevalence of 1:125.000 live births (Hennekam, 2006; Van Belzen, Bartsch, Lacombe, Peters, & Hennekam, 2011). Variants in two ubiquitously expressed and highly homologous genes, *CREBBP* (16p13, OMIM#600140), and *EP300* (22q13, OMIM #402700), underlie RSTS (Korzus, 2017). *CREBBP*, a 150kb gene with 31 exons, encodes the 2442 amino acid CREB-binding protein (CBP), whereas *EP300* (also consisting of 31 exons) encodes the 2414-amino-acid E1A-associated protein, p300. Both proteins act as transcriptional coactivators mediating the interaction between the RNA polymerase II complex and DNA-binding transcription factors. Additionally, CBP and p300 act as epigenetic factors modifying chromatin structure and regulating gene expression through their histone acetyltransferase (HAT) activity (Rusconi et al., 2015; Spena, Milani, et al., 2015).

Up to 60% of RSTS cases harbor de novo variants in *CREBBP*, which is the foremost gene associated with the syndrome (Spena, Gervasini, & Milani, 2015). To date, 235 unique causative variants have been described in *CREBBP* (http://www.LOVD.nl/CREBBP), whereas only 77 have been reported for *EP300* (http://www.LOVD.nl/EP300; Coupry et al., 2002; Rusconi et al., 2015; Spena, Gervasini, et al., 2015). Genetic modification of EP300 leads to milder RSTS phenotypes, being the majotity of the variants found frameshift type. Frameshift, nonsense, missense, and splicing variants, in order of prevalence and spread throughout the *CREBBP* gene, represent the majority (30%–50%) of genetic variants found in RSTS. In about 10% of the cases size-varying deletions (intragenic, whole gene or expanding to adjacent regions) were disease causative (Coupry et al., 2002; Roelfsema et al., 2005; Stef et al., 2007), followed by inversions and translocations, representing the well-documented allelic heterogeneity of the syndrome. However, despite the considerable knowledge gained since first description of RSTS, the molecular cause remains unknown in about 25%–30% of the probands with clinical suspicion of RSTS.

Furthermore, clinical diagnosis of RSTS is challenging due to the wide range of clinical presentations and phenotypic overlap with other syndromes. Some of these disorders are likewise associated with the epigenetic regulation machinery, such as Bohring-Opitz, Wiedemann-Steiner, and Kabuki syndromes (Bjornsson, 2015; Negri et al., 2019), or with other rare conditions as Floating-Harbor, in which the underlying genes encode proteins that directly interact with CBP and/or p300 (Spena, Gervasini, et al., 2015). Additionally, no clear genotype–phenotype correlation has been established in RSTS (Bentivegna et al., 2006; Coupry et al., 2002; Rusconi et al., 2015; Schorry et al., 2008; Spena, Milani, et al., 2015), although previous studies have reported an association between size (large deletions involving other genes) and location (particularly point mutations within HAT domain) of *CREBBP* alterations with disease severity (Bartsch et al., 2006; Kalkhoven et al., 2003).

Here, we report the genetic variant spectrum found in a population of 39 clinically diagnosed RSTS patients, including 17 novel variants reported in this work for the first time. This study was aimed to broaden the molecular knowledge about the major RSTS gene, trying to identify a possible association between the pathogenic variants found and the clinical manifestations of the syndrome.

2 | MATERIAL AND METHODS

2.1 | Subjects

A cohort of 39 *CREBBP*-positive patients clinically diagnosed with RSTS was evaluated in this study. All patients' parents gave written informed consent for them to be included in the study and for their biological material to be sampled. Studies and procedures were in accordance with the ethical standards of the host institution and performed according to the Declaration of Helsinki protocol. RSTS clinical suspicion was referred by the corresponding patient's medical center, and phenotype description was harmonized when possible by the completion of a comprehensive questionnaire leaded by a clinical specialist. The presence of specific RSTS manifestations and medical complications were specifically confirmed or excluded by means of this structured questionnaire.

2.2 | Molecular diagnostic flowchart

All patients were subjected to a sequential molecular approach following the diagnostic algorithm proposed by Hennekam (2006). First, detection of large deletions or insertions in *CREBBP* was performed by Multiplex Ligation-dependent Probe Amplification (MLPA). All patients yielding negative MLPA results were subjected to Next Generation Sequencing (NGS) of *CREBBP*. Point variants detected with NGS technologies were further confirmed by direct sequencing, and their potential pathogenicity was elucidated using in silico prediction tools to establish them as RSTS disease-causing variants.

2.2.1 | DNA extraction

Blood samples from patients and their parents were collected using vacuum-EDTA tubes. DNA was isolated from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's protocol. Quantification of DNA concentration and purity assessment was carried out by spectrophotometric methods.

2.2.2 | MLPA

Presence of intragenic deletions or duplications in *CREBBP* was evaluated by MLPA, using the commercially available SALSA MLPA Kit P313 (MRC Holland) according to the manufacturer's standard protocol and reagents. The amplification products were separated by capillary electrophoresis using an ABI3130 Genetic Analyser (Applied Biosystems) and data were deeply analyzed with Coffalyser software (MRC Holland).

2.2.3 | NGS

Next Generation Sequencing methods were applied when looking for point mutations in those subjects without big *CREBBP* deletions/insertions. Standard procedures following manufacturer's instructions were applied. As a whole, library preparation was performed using Illumina kits, and sequencing was carried out in MiSeq Illumina platform, assuring quality criteria of 100% representativity in all the regions of interest (ROI), with a minimum depth of 100X reads. Bioinformatics analyses were performed for variant calling and interpretation enriching with fundamental databases as GnomAD, ExAC, and ClinVar.

2.2.4 | Sanger sequencing

Polymerase chain reaction (PCR) amplification was performed according to the standard procedures, followed by direct sequencing of the coding sequence and the corresponding exon-intron boundaries of *CREBBP*. Primers used for PCR amplification of *CREBBP* are available upon request. Sequencing was performed using Big Dye Termination cycle sequencing kit 3.1 (Applied Biosystems) following the manufacturer's standard protocol and sequenced on an ABI 3130 genetic analyzer. ABI SEQSCAPE software version 2.5 (Applied Biosystems) was used to perform sequence analysis.

2.2.5 | In silico analyses

Variant significance was classified following ACMG criteria (Richards et al., 2015), and their deleterious potential was further evaluated when possible using prediction tools as Provean, SNPs&GO, PolyPhen 2, SIFT, Mutation Taster, Human Splicing Finder, and MutPred. Sequence variants were described according to HGSV nomenclature guidelines (Den Dunnen et al., 2016). All variants were described in relation to the reference sequence NM_004380.2 (NP_004371.2 for protein level description). Databases such as public HGMD, Decipher, and LOVD, as well as the literature, were explored to look for previous reports on the variants found in this study.

3 | RESULTS

3.1 | Clinical description

An overview and summary of the main clinical findings of the 39 RSTS patients are provided in Table S1. The group consisted of 21 female and 18 male patients, aged 2 months to 42 years at the time samples were received in our laboratory. All patients presented with facial dysmorphic features characteristic of RSTS, as well as broad thumbs and/or halluces. Only four probands did not exhibit angulated thumbs. Other typical RSTS gestalt displayed by our patients included prominent forehead, grimacing smile and low anterior hairline.

Intellectual disability, ranging from mild to severe, was reported for all probands over 2 years of age except for two of them who did not show significant ID. In the vast majority of the cases (above 85%) psychomotor and language delay were reported, and growth retardation was present in 67.6% of informed patients. Gastrointestinal problems, with special prevalence of constipation, were referred in 27 of 32 reported cases and were particularly frequent in the first 2 years of age.

Common additional signs informed (over 74% frequency) were related to skin (mainly hirsutism and, to lesser extent, keloids), tooth, and eye problems. Major organ malformations affected heart (15 of 32) and limbs (21 of 32 cases with information). Cryptorchidism was referred in 13 males. Other common symptoms found in this population included hemangioma, nasolacrimal duct obstruction, micrognathia, and hypotonia. Additional clinical problems and/or malformations were reported in the majority of the cases (Table S1). Typical phenotypic features of our population, including patients harboring different types of variants and location, are depicted in Figure 1.

3.2 | *CREBBP* variation spectrum

Graphic representation of the *CREBBP* gene and the corresponding CBP domains is shown in Figure 2, including the whole spectrum of variants (all of them heterozygous de novo) identified in our RSTS population. Variants were depicted to illustrate type and exon location. As a whole, large

FIGURE 1 Dysmorphic and skeletal features of Rubinstein-Taybi syndrome patients. Patient's code (#) and CREBBP genetic variant is presented for every patient. Representative cases of every type of variant, location and deletion size are depicted for direct intra and intergroup comparison

intragenic deletions represented a 35% of the *CREBBP* genetic alterations. An intragenic duplication was present in one proband. Among the 25 pathogenic point variants detected, nonsense were the most frequent ones (30% of our population) followed by frameshift variants (12.5%), missense substitutions (10%), and splicing alterations (10%).

Fourteen patients were found to carry large intragenic *CREBBP* deletions, ranging from one exon to a deletion spanning the whole gene, as summarized in Table 1. In addition, a duplication of exons 14–19 was also detected by MLPA in one individual, who also carried a second relevant variant in exon 4 of *CREBBP*. Neither of them was present in the parents. In silico pathogenicity prediction of the missense variant in exon 4 provided contradictory results, ranging from neutral effect (Provean, SNPs&GO) to disease causing (SIFT, Poly-Phen, Mutation Taster). According to ACMG variant classification, it met PM2 criteria since the allele was not found in GnomAD exomes despite good coverage, and was classified as of uncertain significance.
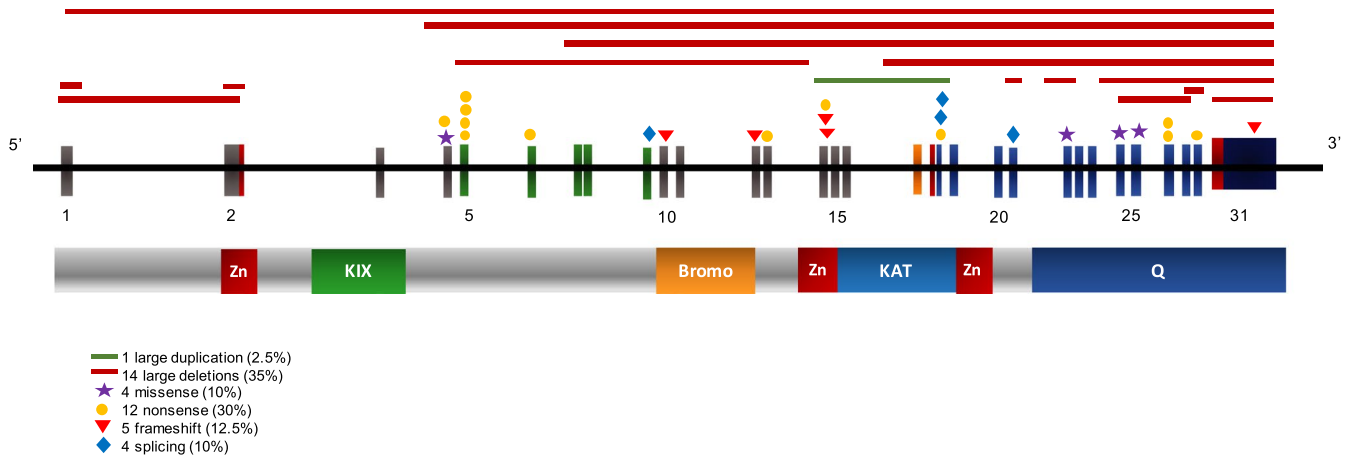
FIGURE 2 Distribution of pathogenic variants detected in this population along the CREBBP gene. Variant position is referred to the exon schematic representation. Types of variants are distinguished by different symbols

TABLE 1 Large CREBBP deletions and duplications detected by MLPA

ID	Large deletion/ duplication	Databases (Decipher, public HGMD, LOVD) and references
#127	Ex1del	HGMD (Breuning et al., 1993; Udaka et al., 2005)
#76	Ex2del	HGMD (Breuning et al., 1993; López et al., 2018; Mogensen et al., 2011; Negri et al., 2019)
#196	Ex1-2del	HGMD (Breuning et al., 1993)
#256	Ex1-31del	HGMD (Bentivegna et al., 2006; Mogensen et al., 2011)
#230	Ex3-31del	This study
#97	Ex4-16del	HGMD (Bentivegna et al., 2006)
#73	Ex6-31del	HGMD (Rusconi et al., 2015)
#16	Ex17-31del	HGMD (López et al., 2018)
#58	Ex21del	This study
#7	Ex22-23del	HGMD (Bentivegna et al., 2006)
#4	Ex24-31del	This study
#157	Ex26-30del	This study
#163	Ex29-30del	This study
#160	Ex31del	HGMD (Bentivegna et al., 2006)
#118	Ex14-19Dup	This study

Note: Exons deleted or duplicated are specified for each patient, as well as previous description of the genetic alteration in literature and databases.

Nine of the 14 large deletions have already been reported in the literature (Table 1) related to RSTS phenotype. Deletion of *CREBBP* exon 2 has been previously described both at the DNA (Aradhya et al., 2012; Coupry et al., 2004) and the RNA level (Petrij et al., 2000).

Point variants were found throughout the *CREBBP* gene, from exon 4 to 31 (Table 2). According to ACMG pathogenicity classification, all the variants were predicted to be pathogenic or likely pathogenic, except for one missense variant and one splicing alteration (both in exon 22) and the already mentioned missense substitution in exon 4, which were classified as of uncertain significance. Nevertheless, all of them were predicted to be damaging in most of the other prediction tools

used. Single-base pair insertions were found in patients #25, #22, and #55 leading to a frameshift and premature stop codon 45, 101 and 137 amino acids further on, respectively. Patient #1 presented a single-nucleotide deletion introducing a stop codon four amino acids further on in exon 14. We found the pathogenic splicing variant in exon 18 of proband #142 to be in a mosaic condition (37%) in blood and corroborated in buccal swab with 49% of mutated cells. A deletion of exons 17–21 was observed by the RNA study (data not shown).

Two patients from our cohort harbored the same pathogenic nonsense variant in exon 5 (#172 and #37), which was also previously described (Coupry et al., 2002; Roelfsema et al., 2005; Schorry et al., 2008). Twelve of the 25 point

TABLE 2 Point pathogenic variants

ID	Exon	Coding sequence (GRCh38)	Protein-based sequence (GRCh38)	Variant type	ACMG significance	Human splicing finder	Provean	Mutation taster	SIFT	SNP&GO
#118	4	c.989C>A	p.(T330K)	Missense	Uncertain significance (PM2)	Dsp	N	D	D	N
#221	4	c.992C>G	p.(S331*)	Nonsense	Likely pathogenic (PVS1 PM2)	Dsp	N	D	n.a.	n.a.
#37	5	c.1237C>T	p.(R413*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3 PP5)	Dsp	D	D	n.a.	n.a.
#172	5	c.1237C>T	p.(R413*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3 PP5)	Dsp	D	D	n.a.	n.a.
#28	5	c.1270C>T	p.(R424*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3 PP5)	Dsp	D	D	n.a.	n.a.
#13	5	c.1318C>T	p.(R440*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3 PP5)	Dsp	D	D	n.a.	n.a.
#64	6	c.1447C>T	p.(R483*)	Nonsense	Pathogenic (PVS1, PM2, PP3, PP5)	Dsp	D	D	n.a.	n.a.
#67	9	c.1941+1_1941+5del	p.?	Splicing	Likely pathogenic (PVS1 PM2)	Psp	N.A.	D	n.a.	n.a.
#25	10	c.2041_2042insC	p.(N681Tfs*45)	Frameshift	Pathogenic (PVS1 PM2 PP3)	Dsp	N	D	D	n.a.
#22	12	c.2190dup	p.(N731Efs*101)	Frameshift	Likely pathogenic (PVS1 PM2 BP4)	Dsp	D	D	D	n.a.
#1	14	c.2621delC	p.(P874Hfs*4)	Frameshift	Likely pathogenic (PVS1 PM2)	Dsp	D	D	D	n.a.
#70	13	c.2461C>T	p.(Q821*)	Nonsense	Pathogenic (PVS1 PM2 PP3)	Psp	D	D	n.a.	n.a.
#43	14	c.2641C>T	p.(Q881*)	Nonsense	Pathogenic (PVS1, PM2, PP3)	Dsp	D	D	n.a.	n.a.
#55	14	c.2497dup	p.(L833Pfs*137)	Frameshift	Pathogenic (PVS1, PM2)	Dsp	D	D	D	n.a.
#19	18	c.3517C>T	p.(R1173*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3)	N	D	D	n.a.	n.a.
#142	18	c.3609+2_3609+5delTACA	p.?	Splicing	Likely pathogenic (PVS1 PM2)	Dsp	N.A.	D	n.a.	n.a.
#166	18	c.3609+1G>T	p.?	Splicing	Pathogenic (PVS1 PM2 PP3)	Psp	N.A.	D	n.a.	n.a.
#40	21	c.3836+5G>A	p.?	Splicing	Uncertain significance (PM2 PP3)	Psp	N.A.	D	n.a.	n.a.
#136	22	c.3847T>C	p.(C1283R)	Missense	Uncertain significance (PM2 PP3)	Dsp	D	D	D	D
#49	25	c.4244A>G	p.(Q1415R)	Missense	Likely pathogenic (PM1, PM2, PM5 PP3)	Dsp	D	D	D	D
#94	26	c.4394G>A	p.(G1465E)	Missense	Pathogenic (PM1 PM2 PP3 PP5)	Psp	D	D	D	D
#34	27	c.4492C>T	p.(R1498*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3)	Dsp	D	D	n.a.	n.a.
#151	27	c.4557C>G	p.(Y1519*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3)	Dsp	D	D	n.a.	n.a.
#103	30	c.5058G>A	p.(W1686*)	Nonsense	Pathogenic (PVS1 PM1 PM2 PP3)	Dsp	D	D	n.a.	n.a.
#226	31	c.6107_6116del	p.(P2036Rfs*36)	Frameshift	Pathogenic (PVS1 PM2 PP5)	Dsp	N	D	T	D

(Continues)

T A B L E 2 (Continued)

ID	PolyPhen—2	MutPred	Found in ExAC, 1000G, GenomAD	Databases (Decipher, public HGMD, LOVD) and references
#118	PossD	0.372	No	This study
#221	n.a.	n.a.	No	This study
#37	n.a.	n.a.	No	HGMD, ClinVar, LOVD (Roelfsema et al., 2005)
#172	n.a.	n.a.	No	HGMD, ClinVar, LOVD (Roelfsema et al., 2005; Stef et al., 2007)
#28	n.a.	n.a.	No	HGMD, ClinVar, LOVD (Sukalo et al., 2017)
#13	n.a.	n.a.	No	LOVD
#64	n.a.	n.a.	1000G	ClinVar
#67	n.a.	n.a.	No	HGMD. Same position but G>A (Schorry et al., 2008)
#25	PossD	0.270	No	Same position but 15bp+2 insertion (Roelfsema et al., 2005)
#22	ProbD	0.424	No	This study
#1	ProbD	0.158	No	This study
#70	n.a.	n.a.	No	HGMD, LOVD. (Thienpont et al., 2010)
#43	n.a.	n.a.	No	This study
#55	ProbD	0.557	No	This study
#19	n.a.	n.a.	No	HGMD, LOVD (Bentivegna et al., 2006)
#142	n.a.	n.a.	No	This study
#166	n.a.	n.a.	No	This study
#40	n.a.	n.a.	No	HGMD. Same position but C>G (Kalkhoven et al., 2003)
#136	ProbD	0.970	No	This study
#49	ProbD	0.875	No	HGMD (Thienpont et al., 2010)
#94	ProbD	0.931	No	This study
#34	n.a.	n.a.	No	Decipher, HGMD, LOVD (Roelfsema et al., 2005)
#151	n.a.	n.a.	No	HGMD (Schorry et al., 2008)
#103	n.a.	n.a.	No	This study
#226	B	0.202	No	ClinVar

Note: Summary of missense, nonsense, frameshift, and splicing variants detected in each patient from our cohort. Variant description, pathogenicity prediction, previous references, and database registration (public HGMD, LOVD or Decipher) are specified for each case.

Abbreviations: B, benign/tolerated; D, disease causing/damaging/deleterious; Dsp, potential alteration of splicing; N, neutral/not affecting splicing; n.a., not available; PossD, possibly damaging; ProbD, probably damaging; Psp, probable alteration of splicing.

sequence alterations were novel and, to the best of our knowledge, have been neither described in the literature, nor registered in specific databases.

3.3 | Genotype–phenotype correlation

The clinical description of each one of the patients included in this study was thoroughly examined to look for a potential association of genotype alterations with either severity or presence of specific clinical signs in the probands. Both variant type and location throughout the *CREBBP* gene, with special attention to the CBP domain affected, were specifically considered. However, no significant genotype–phenotype correlation was found. There is no clear association of the presence of large exonic deletions with more severe RSTS manifestations, since severity of clinical symptoms varied within the *CREBBP*-deleted individuals. They were neither systematically more affected than those harboring point variants. We did not find more severe phenotypes related to alterations in function-relevant CBP domains or leading to a truncated protein before HAT domain. Nonsense variant in exon 30 (further on the HAT domain) from patient #103 implies high frequency of RSTS clinical features. Moreover, proband #160, who harbors a deletion of the last exon, exhibits a severe form RSTS. Looking at the two patients with no ID reported one harbors a nonsense variant in exon 5 and the other one a frameshift variant in exon 10, both classified as pathogenic and producing a truncated protein.

Table 3 summarizes the prevalence of typical RSTS features in our population contrasted with previous phenotypic description by Schorry et al. (2008) in *CREBBP*-positive patients and Hennekam et al. (1993), Dutch population and literature. Frequency of typical RSTS dysmorphic features is comparable to what was previously reported, considering the variability in the manifestations and age of diagnosis. It could be worth mentioning the higher prevalence of angulated thumbs reported in our population (88.6%) compared to the others (33%–48%). Urinary tract anomalies are slightly more frequently reported in our population, although low set ears were informed in fewer cases.

We compared the prevalence of clinical characteristics within our patients grouped by the nature of *CREBBP* variants: large intragenic deletions, missense variants and truncating variants (nonsense, frameshift and splicing variants). Phenotype of patient #118 was not considered for this comparison since he harbors a duplication and missense variant in exon 4, complicating the interpretation of the effect of each alteration. There was no clear association of the type of variation and the prevalence of RSTS clinical features in our cohort, other than less frequent psychomotor delay reported and no heart anomalies in the missense group (Table 3). Microcephaly was more frequent in those carrying large *CREBBP* deletions.

Finally, the two patients harboring the same pathogenic nonsense variant showed significantly different phenotypes, with distinct dysmorphology although similar cognitive impairment. Both of them are able to carry out easy tasks by their own and live at a certain independent status under supervision.

4 | DISCUSSION

Rubinstein-Taybi syndrome is a rare genetic condition caused by alterations of *CREBBP* gene in up to 60% of the cases. This works aimed to provide new insights in the *CREBBP* genetic variant spectrum leading to RSTS phenotype, as well as to delve into the genotype–phenotype correlation in a population of 39 *CREBBP*-positive RSTS patients. We have followed a sequential step methodology by which gene dosage alteration was firstly assessed by MLPA and, secondly, presence of point variants was further evaluated by NGS and confirmed by Sanger sequencing. Following this approach, we have found 14 intragenic deletions, one duplication and 25 pathogenic point variants.

CREBBP deletions accounted for 35% of the sequence alterations found in this cohort, which represents a bigger percentage compared with about 10% of large deletions in *CREBBP* detectable by FISH in other populations (Breuning et al., 1993; Petrij et al., 2000; Schorry et al., 2008). The higher deletion frequency detected in our population could be explained by the sequential diagnosis approach in which MLPA was used as the first-level technique. MLPA, together with aCGH analysis, has greatly improved detection of *CREBBP* deletions (Aradhya et al., 2012; Rusconi et al., 2015). We have found one of the highest deletion rates reported in *CREBBP* so far, more in line with previous results obtained using other complementary methodologies to FISH (Lee et al., 2015; Roelfsema et al., 2005; Rusconi et al., 2015; Stef et al., 2007). The relevant contribution of *CREBBP* dosage anomalies in this cohort highlights the importance of evaluating *CREBBP* rearrangements before undertaking an extensive search for other variants in *CREBBP* or *EP300*.

CREBBP deletions were spread along the gene ranging in size from one exon to the entire gene. Half of the patients carried deletions of five exons or more. One of the probands showed a deletion of the whole *CREBBP* gene from exons 1 to 31. Assessment of a potential deletion beyond *CREBBP* involving adjacent genes needs to be further explored in this patient. A more severe phenotype associated with deletion of *CREBBP* and contiguous genes was previously observed (Bartsch et al., 2006), although not confirmed in other populations (Rusconi et al., 2015). Clinical evaluation of this *CREBBP*-deleted patient was limited since he is a newborn. Comprehensive clinical follow-up

TABLE 3 Rubinstein–Taybi syndrome (RSTS) clinical features by variant type

	Hennekam et al. (1993)		Schorry et al. (2008)	This work				
	Dutch population	Literature	All CREBBP mutations	Whole population	Large deletions	Truncating	Missense	Inside HAT
N	45	571	52	39	14	21	3	5
Growth retardation	75 (<P3)	78	21	65.7	66.7	63.2	66.7	60.0
Intellectual disability	75	74 (IQ < 50)	100	84.2	100.0	80.0	100.0	80.0
Psychomotor delay	n.a.	n.a.	n.a.	88.6	91.7	89.5	66.7	80.0
Language delay	n.a.	n.a.	n.a.	93.8	100.0	88.2	100.0	100.0
Behavioral problems Anxiety, autism	n.a.	n.a.	12–33	56.0	50.0	60.0	50.0	50.0
Typical facial dysmorphims Thick arched eyebrows	74	68	n.a.	91.4	92.3	88.9	100.0	80.0
Long eyelashes	87	51	n.a.	86.1	84.6	84.2	100.0	80.0
Microcephaly	35 (<P3)	95 (<P2)	25	77.1	91.7	68.4	66.7	60.0
Downslanting palpebral fissures	88	90	85	86.8	92.9	85.0	66.7	50.0
Columnella below the alae nasi	93	78	n.a.	85.7	100.0	66.7	100.0	75.0
Beaked nose	94	93	88	82.9	100.0	73.7	100.0	60.0
Narrow palate	89	93	n.a.	94.1	100.0	88.9	100.0	80.0
Narrow mouth	84	56	n.a.	91.2	100.0	83.3	100.0	80.0
Low set/posteriorly rotated ears	82	81	n.a.	60.6	58.3	70.6	33.3	40.0
Skeletal abnormalities Broad thumbs/halluces	100	100	96	100.0	100.0	100.0	100.0	100.0
Angulated thumbs	33	48	33	88.6	84.6	88.9	100.0	80.0
Skin abnormalities Hirsutism	n.a.	n.a.	n.a.	78.1	90.9	70.6	66.7	40.0
Heart anomalies	24	34	44	44.1	58.3	42.1	0.0	50.0
Urinary tract anomalies	17	52	n.a.	65.6	72.7	55.6	66.7	60.0
GI problems Infant feeding problems/ Constipation	71	77	88	84.8	81.8	88.9	66.7	50.0
Teeth malformations	n.a.	n.a.	n.a.	85.7	87.5	77.8	100.0	100.0
Eye anomalies	n.a.	n.a.	6–65	75.0	80.0	72.2	66.7	40.0

Note: Frequency (in percentage) of RSTS characteristic features in our whole population and regarding the variant type, including direct comparison with other populations previously described by Hennekam et al. (1993) and Schorry et al. (2008). n.a.: not available.

should be completed to ascertain the severity of RSTS over the course of the disease and evaluate the effect of other genes on the phenotype. In line with previous results, we did not find an association of larger deletions with disease severity in our cohort (Lee et al., 2015). A correlation between location of affected exons and phenotype was neither observed, which agrees with previous studies (Rusconi et al., 2015; Stef et al., 2007). In fact, deletion of exon 31 was sufficient to be disease causing in a severe form of RSTS, as was also described in one case from an Italian population (Rusconi et al., 2015). Phenotypic comparison of both cases did not show specific shared features. Indeed, our proband presented with genitalia, ocular, and urinary anomalies that were not reported in Rusconi's patient, who did present hypotonia. Neither of both patients exhibited heart problems.

Regarding point pathogenic variants, the 25 *CREBBP* alterations found in our population are evenly distributed throughout the 31 coding exons, although an 81-nt long region of exon 5 clusters 4 of the 10 nonsense variants, being one of them identical in two of the patients (Figure 1). We have extensively compared the phenotype of these patients resulting in no evident-specific similarities among all of them. In fact, patient #13 showed no ID, although exhibited the vast majority of RSTS dysmorphic features. Proband #25, who also lacked ID, carried a frameshift insertion in exon 10, preventing a specific *CREBBP* region from being potentially discarded as ID causative. No more patients from our study showed a normal cognition level. The nonsense variant affecting amino acid 413 (c.1237C>T) has been previously reported in different RSTS patients (Coupry et al., 2002; Roelfsema et al., 2005; Schorry et al., 2008), resulting in a recurrent amino acid variation in RSTS. Schorry et al. (2008) found a pathogenic variant involving the same amino acid but implying a frameshift deletion of one nucleotide that leads to a stop codon after 20 amino acids. Detailed examination of the two patients from our population carrying the same nonsense variant showed a similar clinical involvement for both of them, although distinct phenotype, suggesting a relevant role of other modulating factors or compensatory mechanisms, which need to be further studied.

In silico pathogenic prediction confirmed all the genetic alterations to be likely causative of the disease. One splicing and one missense variant in patients #40 and #136 were classified as of uncertain significance according to ACGM guidelines. Both patients showed the characteristic RSTS phenotype and the other prediction tools available confirmed pathogenicity of the variants found, leading to their disease causative consideration. Additionally, mosaic condition of the splicing variant found in patient #142 deserves special consideration as well. This could represent another example of the previously reported dosage sensitivity of the *CREBBP* gene (Gervasini et al., 2007),

determining the repercussion on the phenotype by mosaic variants and leading in this case to a severe form of the disease.

Another missense variant of uncertain significance was detected in exon 4 of one patient (#118) who also carried duplication of exons 14–19, being both de novo and in heterozygosis. Intragenic duplication of *CREBBP* was found in other RSTS populations. A patient carrying duplication of exon 16 was reported in a previous study (Stef et al., 2007), which was confirmed to produce a truncated protein. Roelfsema et al. (2005) described exon 1 duplication, although it was not clear how this leaded to the inactivation of the allele. Partial gene duplication has been related to disease in several syndromes as Duchenne, Menkes, Optiz, Johansson-Blizzard or Mowat-Wilson syndromes (Baxter et al., 2017; Mogensen et al., 2011; Schwaibold et al., 2014; Sukalo et al., 2017), although with minor weight in the global variant spectrum. The specific role of the missense variant in exon 4 and duplication of *CREBBP* exons 14–19 as causative of the clinical manifestations in #118 needs to be further elucidated. Phenotypic characteristics of this patient were typical of RSTS and clinical manifestations did not stand out from the rest of our cohort.

This study showed a wide range of point *CREBBP* variant types, as was also reported by others (Bartsch et al., 2005; Bentivegna et al., 2006; Coupry et al., 2002; Schorry et al., 2008). Although *CREBBP* variants have already been deeply studied as causative of RSTS, the comprehensive description of the type and pathogenicity of the *CREBBP* variants found in this large Spanish population, together with the corresponding phenotypic evaluation, adds valuable information to RSTS knowledge and provides new data to establish general epidemiology of this disorder. Nonsense variants were predominant in our population, implying a 30% of the overall *CREBBP* variation found (12 of 40), although frameshift variants were the most prevalent ones in the general characterization of RSTS (López et al., 2018; Spena, Gervasini, et al., 2015). Additionally, five frameshift (12.5%), four missense (10%), and four splicing (10%) variants were detected. Distribution of variant types differs among the different RSTS cohorts studied so far (Bentivegna et al., 2006; Lee et al., 2015; Roelfsema et al., 2005; Schorry et al., 2008; Spena, Gervasini, et al., 2015; Thienpont et al., 2010; Udaka et al., 2005; Wincent et al., 2015). Comparing clinical manifestations among patients carrying different variant types, we did not find a characteristic pattern for any of them, although microcephaly was more frequent in patients carrying large *CREBBP* deletions. Exon location, even focusing on HAT-domain affecting variants, did not support evidence for more severe forms of RSTS prediction, although variants in this highly conserved domain have been shown to be sufficient to cause the full RSTS phenotype (Kalkhoven et al., 2003). After grouping variants in truncating or single amino acid substitutions, no striking differences were found regarding prevalence and severity of clinical signs, although the number

of missense variants available for this evaluation ($N = 3$) was too limited to draw conclusions. In fact, a slight lower frequency of RSTS features could be noticed in the truncating variants group. However, this could be a consequence of the already considerable phenotypic variability in the syndrome, better represented in this larger group.

Most prior studies have not shown significant genotype–phenotype correlations (Schorry et al., 2008; Spena, Milani, et al., 2015). Our cohort of *CREBBP*-positive patients does not support the existence of decisive phenotypic difference between patients with large or small deletions, although certain RSTS signs, especially those related to organ malformations, may be more prevalent in *CREBBP*-deleted patients compared to point genetic variants. We observed no evident correlation of variant type, location or involvement of the crucial HAT domain with disease severity, confirming the absence of a genotype–phenotype correlation, as was previously reported in other cohorts (Bentivegna et al., 2006; Rusconi et al., 2015; Stef et al., 2007; Suzuki et al., 2013). Heterogeneity of *CREBBP* alterations and phenotypic features found in this study is also in accordance with other populations previously characterized (Hennekam et al., 1993; Schorry et al., 2008), establishing the variability both in genotype and phenotype representative of RSTS. Regarding tumor incidence, which was associated with RSTS (Boot et al., 2018), only hemangioma was reported in this population, affecting 23% of the patients. However, long-term follow-up could provide further information, especially considering the young age of most of the participants of the study.

Rubinstein-Taybi syndrome is a multisystem dysmorphic syndrome with many nonspecific features and phenotypic overlap with other syndromes, which makes accurate and early diagnosis challenging. The lack of standardized clinical criteria also hampers gathering comprehensive clinical data and interstudy comparison. Indeed, one limitation faced in this study is the limited phenotypic information that could be compiled in some of the cases, even though a standardized questionnaire was asked to be filled-in. ID could not be quantitatively compared since a standardized quantification method was not available for the whole cohort. In fact, it was not possible to assess ID in 6 of the cases, all but one due to being under 2 years of age, which hampers accurate performance evaluation. Additionally, the detection of a borderline cognitive disability could be challenging and blurred by early extensive stimulation of the patient. The availability of a molecular test would greatly aid the clinician in the confirmation of the diagnosis. A genotype–phenotype correlation could also aid in disease prognosis. Nevertheless, the genetic cause of RSTS remains unknown in 25%–30% of the patients, which highlights the need to continue deepening in the etiology and molecular mechanisms of the syndrome. Almost half of the genetic variants found in this cohort were reported in this work for the first time, hence, contributing to the RSTS molecular knowledge and expanding the *CREBBP* genetic variant repertoire of this complex disorder.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

AUTHOR CONTRIBUTION

AO-G, JA, SG-M, JMM-L, MPM, MAR-A, FS-S, and VS performed the clinical diagnosis and recruited the patients. AO-G evaluated the patients according to standardized guidelines and contributed to the genotype–phenotype study. VP-G, ML, and ED-G carried out bioinformatics and data analysis. VP-G, ED-G, and AG-O wrote and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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