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CREBBP Mutations in Individuals without Rubinstein–Taybi Syndrome Phenotype

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Mutations in *CREBBP* cause Rubinstein–Taybi syndrome. By using exome sequencing, and by using Sanger in one patient, *CREBBP* mutations were detected in 11 patients who did not, or only in a very limited manner, resemble Rubinstein–Taybi syndrome. The combined facial signs typical for Rubinstein–Taybi syndrome were absent, none had broad thumbs, and three had only somewhat broad halluces. All had apparent developmental delay (being the reason for molecular analysis); five had short stature and seven had microcephaly. The facial characteristics were variable; main characteristics were short palpebral fissures, telecanthi, depressed nasal ridge, short nose, anteverted nares, short columella, and long philtrum. Six patients had autistic behavior, and two had self-injurious behavior. Other symptoms were recurrent upper airway infections (n = 5), feeding problems (n = 7) and impaired hearing (n = 7). Major malformations occurred infrequently. All patients had a de novo missense mutation in the last part of

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exon 30 or beginning of exon 31 of *CREBBP*, between base pairs 5,128 and 5,614 (codons 1,710 and 1,872). No missense or truncating mutations in this region have been described to be associated with the classical Rubinstein–Taybi syndrome phenotype. No functional studies have (yet) been performed, but we hypothesize that the mutations disturb protein–protein interactions by altering zinc finger function. We conclude that patients with missense mutations in this specific *CREBBP* region show a phenotype that differs substantially from that in patients with Rubinstein–Taybi syndrome, and may prove to constitute one (or more) separate entities.

Key words: *CREBBP*; exon 30; exon 31; whole exome sequencing; intellectual disability; Rubinstein–Taybi syndrome; RSTS; syndrome; mutation; clinical features; case series; genotype–phenotype correlation

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INTRODUCTION

Rubinstein–Taybi syndrome (RSTS) is a well-known entity characterized by distinctive dysmorphic features, short stature, and moderate-to-severe intellectual disability [Hennekam, 2006]. The facial characteristics include highly arched eyebrows, downslanting palpebral fissures, a convex nasal ridge with a low hanging columella, and a characteristic grimacing smile. The main distal limb anomalies are the broad thumbs and broad halluces sometimes accompanied with broad distal phalanges of the fingers. Heterozygous de novo mutations affecting *CREBBP* [Petrij et al., 1995] or *EP300* [Roelfsema et al., 2005; van Belzen et al., 2011] can cause RSTS and are found in 65–70% of all individuals clinically diagnosed with RSTS. There is no reliable genotype–phenotype correlation known for mutations in either *CREBBP* and *EP300*, and the clinical specificity has been indicated to be nearly 100% [van Belzen et al., 2011].

With the advent of exome sequencing using panels that are targeted to detect variants in genes known to cause intellectual disability or by analyzing variants present in a patient but not in his/her parents ("trio approach"), individuals with phenotypes different from the RSTS phenotype are also being analyzed for variants in *CREBBP* and *EP300*. Here, we report on the results of exome sequencing in 10 patients with apparent intellectual disability in whom a missense mutation in a specific *CREBBP* region, coded by parts of exons 30 and 31 was detected. None of them had the typical characteristics of RSTS, although some had a (very) limited number of RSTS characteristics. In one additional patient with a doubtful clinical diagnosis, a mutation was found using Sanger sequencing. We report on their phenotypes and genotypes, and compare these to patients who have been molecularly proven to have the classical RSTS phenotype.

MATERIALS AND METHODS

Each of the patients reported here was referred to a clinical geneticist because of apparent intellectual disability. The suspicion of RSTS was raised in one patient (patient 3) initially, upon which Sanger sequencing of *CREBBP* was performed. Exome sequencing was performed in all other patients, as no clinical diagnosis was suggested. This was done by either using a targeted panel for genes known to be mutated in patients with intellectual disability, or untargeted analyses comparing the results of exome sequencing in parents and the affected child ("trio analysis"), assuming the occurrence of a Mendelian condition with autosomal dominant, autosomal recessive, or X-linked inheritance. The exact methods used in the various laboratories differed (see Supplementary data for full details). All mutations were confirmed by Sanger sequencing. We gathered clinical and molecular data, and two authors (LAM; RCMH) scored the facial and distal limb morphology of all patients as provided by the clinician, to achieve uniform scoring. The clinical data were compared with a series of 308 patients with a classical RSTS phenotype who had a molecularly proven mutation elsewhere in *CREBBP* [Fergelot et al., submitted].

Paternity was confirmed in all patients that were studied using exome sequencing but not in patient 3 who was studied using Sanger sequencing. The effect of the missense variants was predicted using three different in silico prediction programs: Polyphen2 [Adzhubei et al., 2010], SIFT [Ng and Henikoff, 2003], and MutationTaster [Schwarz et al., 2010]. We also determined the presence of the variants in population cohorts (Exome Sequencing Project [ESP] and Exome Aggregation Consortium [ExAc]). The Human Gene Mutation Database (HGMD) [Stenson et al., 2014], the Leiden Open Variation Database (LOVD) [Fokkema et al., 2011], and personal registries were searched for patients with classical manifestations of RSTS and a missense mutation in the same *CREBBP* region.

Written informed consent for publication of clinical features and photographs were obtained from parents for all patients. The Medical Ethics Committee of the AMC in Amsterdam considered the work to fall in the realm of routine clinical care, and formal approval not being needed.

RESULTS

Phenotype

We provide the main information on the present 11 patients in Table I, in which facial and distal limb morphology are listed, and

TABLE I. Facial and Distal Limb Morphology of Currently Reported Patients

Patient	1	2	3	4	5	6	7	8	9	10	11	Total
Age (years)	8	1.5	10[a]	16	5	24	10	6	4	0.8	7	0.8–24
Gender (M/F)	M	M	M	M	M	F	M	F	F	F	F	6M/5F
CREBBP mutation	c.5128T>C	c.5240T>G	c.5357G>C	c.5456G>T	c.5478C>G	c.5513G>A	c.5599C>T	c.5600G>A	c.5602C>T	c.5602C>T	c.5614A>G	
Face, square (S)/flat (F)	–	S	?	–	–	–	–	S	S, F	F	F	3/10S, 3/10F
Telecanthi (T)/epicanthi (E)	T	E	?	–	E	–	T	T	T	T	T	6/10T, 2/10E
Palpebral fissures upslanted (U)/ downslanted (D)	–	U	D	–	D	U	–	–	U	U	U	5/11U, 2/11D
Palpebral fissures, short	–	+/–	–	–	–	–	–	+	+	+	+	4/11+, 1/11+/–
Ptosis	–	–	–	–	–	–	–	+	+	+	–	3/11
Squint	–	–	+	–	+	+	+	–	+	–	+	6/11
Depressed nasal ridge	–	+	–	–	+	–	–	–	+	+	+	5/11
Short nose	–	+	–	–	–	–	–	+	+	+	+	5/11
Broad nasal tip	–	–	+	–	–	–	–	+	–	+	+	4/11
Short columella	–	+	–	–	–	–	–	–	+	+	+	4/11
Anteverted nares	–	+	–	–	–	–	–	+	+	+	+	5/11
Full cheeks	–	+	–	–	–	–	+	–	–	+	–	3/11
Philtrum short (S)/long (L)/deep (D)	–	L	–	–	L	S	–	D	L, D	L, D	L	1/11S, 5/11L, 3/11D
Everted vermilion of upper lip	–	–	–	–	–	+	+	+	+	–	–	4/11
Thin vermilion of upper lip	+	+	–	–	+	–	–	–	–	–	–	3/11
High palate	+	–	+	–	–	?	–	–	+	–	–	3/10
Micro/retrognathia	+	–	+	–	+	+	+	+	–	+	–	7/11
Ears low-set (L)/short (S)	L	L,S	L	–	L	–	L	–	L	L, S	S	7/11L, 3/11S
Protruding ears (upper part)	+	–	+	–	+	–	+	–	+	+	+	7/11
Cupped ear	–	–	–	–	+	–	+	–	–	–	+	3/11
Overfolded helix	+	+	–	–	–	–	+	–	–	+	·	4/11
Ulnar deviation of finger (s)	+	–	?	–	–	+	–	–	–	–	+	3/10
Clinodactyly fifth finger	–	–	?	–	–	+	+	–	+	–	–	3/10
Prominent fetal tip pads	–	+	?	–	–	–	–	–	–	–	+	2/10
Sandal gap	+	+	–	–	–	+	+	+	–	–	–	5/11
Cutaneous partial syndactyly of toes	–	–	–	–	2+3	2+3+4+5[b]	–	–	4+5	–	–	3/11
Fibular deviation distal phalanx halluces	+	–	+	–	+	+	–	+	+	+	+	8/11
Halluces broad (B)/ narrow (N)	–	–	B+/–	B+/–	B	N	N	–	–	–	N	1/11B,2/11B+/– 3/11N
Other[3]	FU, LE, BE	DE	LE, EI, Cr, MC LC, CN		FU, DC, Cr	DE, LC, UT		TP	BE	Camp	TP, OT, AH, PT, AE	

3 FU, frontal upsweep of hair; LE, long eyelashes; BE, broad eyebrows; DE, deep-set eyes; EI, extra incisor; Cr, cryptorchidism; MC, megalocornea; LC, low hanging columella; CN, convex nasal ridge; DC, dolichocephaly; UT, unerupted teeth; TP, tapering fingers; Camp, camptodactyly, OT, overlapping toes, AH, anteriorly implanted hallux, PT, pointy canine teeth, AE, absent lobe of the ear.

[a]Died at 10 years of age.

[b]Toes also short.

Table II, in which the data are compared to those of 308 patients with RSTS and a mutation elsewhere in *CREBBP*. The phenotypes are illustrated in Figures 1 and 2. The genotypes are tabulated in Table III and shown in Figure 3. Below, we provide a short description of the clinical history of each patient in which only data are provided that are not available in Table I or II.

Patient 1 was the first child of healthy non-consanguineous parents. The pregnancy was induced by in vitro fertilization. He was born at term weighing 2,900 g (−1.4 SDS). He had apparent intellectual disability and severe speech delay; at the age of 9 he used four-word sentences. He had facial and limb dysmorphisms (Figs. 1 and 2), hypertrichosis, multiple ear infections, and a

TABLE II. Comparison of RSTS Characteristics of Currently Reported Patients to Those in RSTS Individuals With Mutations Elsewhere in *CREBBP* and Reported in Literature

Patient	1	2	3	4	5	6	7	8	9	10	11	All (n = 11) (%)	RSTS patients with other *CREBBP* mutations (n = 308) (%)
Prenatal growth retardation	−	−	−	−	+	−	−	−	−	+	−	27	25
Postnatal growth retardation	+	−	−	+	+	+	−	−	+	−	−	55	75
Microcephaly (OFC < 3rd centile)	+	−	−	+	+	+	−	+	+	+	−	64	54
Hypertrichosis	+	−	−	−	−	+	−	−	−	−	−	18	76
Highly arched eyebrows	−	−	−	−	−	−	−	−	+	−	−	9	85
Long eyelashes	+	−	+	−	−	−	−	−	−	−	−	18	89
Down-slanted palpebral fissures	−	−	+	−	+	−	−	−	−	−	−	18	79
Epicanthi	+	+	−	−	+	−	−	−	−	−	−	27	44
Convex nasal ridge	−	−	+	−	−	−	−	−	−	−	−	9	81
Low hanging columella	−	−	+	−	−	+	−	−	−	−	−	18	88
Grimacing smile	−	−	−	−	−	−	−	−	−	−	−	0	94
High palate	+	−	−	−	−	?	−	−	+	−	−	18	77
Micrognathia	+	−	+	−	+	+	−	−	−	−	−	36	61
Low-set ears	+	+	+	−	+	−	+	−	+	+	−	64	44
Broad thumbs	−	−	−	−	−	−	−	−	−	−	−	0	96
Angulated thumbs	−	−	−	−	−	−	−	−	−	−	−	0	49
Broad halluces	−	+/−	+/−	−	+	−	−	−	−	−	−	9	95
Apparent intellectual disability/ develop delay[a]	+	n.a.	+	+	+	+	+	+	+	n.a.	+	82–100	99
Severe		n.a.	+	+	+	+		+	+	n.a.		55–73	36
Moderate	+	n.a.								n.a.	+	18–36	48
Mild		n.a.					+			n.a.		9–27	14
Epilepsy	−	−	+/−	−	+	−	−	+	−	−	−	27	25
Autism/autism-like behavior	+	n.a.	+	+	+	+	−	n.a.	+	n.a.	−	55–82	49
Cardiovascular anomalies	−	−	−	−	−	−	−	−	−	−	+	9	35
Urinary tract anomalies	−	−	−	−	−	−	−	+	−	−	−	9	28
Scoliosis	−	−	−	−	−	+	−	−	−	−	+	18	18
Obesity	+	−	−	−	−	−	−	−	−	−	−	9	29

[a]Formal testing performed in only one patient; for all other patients, they are considered to have apparent intellectual disability; the degree of intellectual disability was indicated by the clinicians in charge of the patients, not by formal testing, and should only be used as indication. n.a. = parameters was not assessed, since the child was either too young (patients 2 and 10) to rule out intellectual disability or autism, or the intellectual disability was considered too profound to evaluate autistic features (in patient 8).

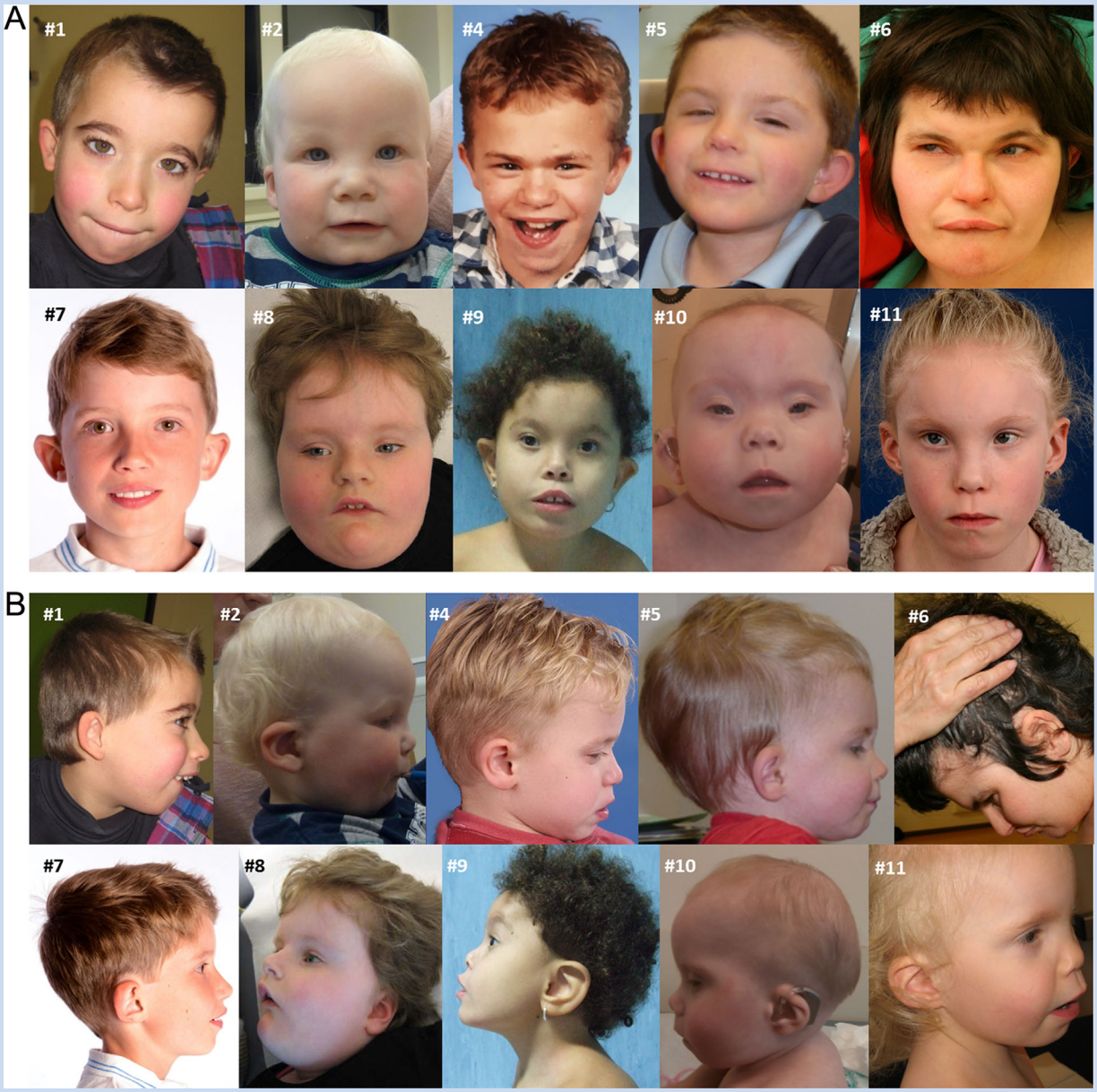

FIG. 1. Facial morphology of the presently described patients with a *CREBBP* mutation. Frontal (A) and lateral (B) views and evolution of the facial characteristics with age (C). Patient numbers correspond to those in text, tables, and other figures. Detailed description of facial and distal limb morphology can be found in Table I. Note that patient 8 also had cleidocranial dysplasia. Patients showed similarities but also differences. Especially patient 9, 10 (sharing the same mutation), and 11, and to some extent patients 2 and 7, resembled one another more than the other patients did. Especially telecanthi, short palpebral fissures, depressed nasal ridge, short nose, anteverted nares, short columella, and long philtrum characterized their faces. Patients 6–9 shared their mouth configuration. (C) Note that the depressed nasal bridge and ridge became less depressed and even high with age.

conductive hearing loss with malformation of the auditory ossicle chain on the right side. Hearing improved after surgical removal of granulation tissue restoring the normal mobility of the ossicle chain. At 8 years, his height was 119 cm (−3 SDS) and at 6 years his OFC was 48 cm (−3 SDS).

Patient 2 was born at term to unrelated parents weighing 3,190 g (−1 SDS) with an OFC of 36 cm (0 SDS). He had mild feeding problems in the first months of life. At 5 months, he was evaluated because of his unusual phenotype. Next to the facial and distal limb signs (Figs. 1 and 2), he was found to have hypermetropia. At

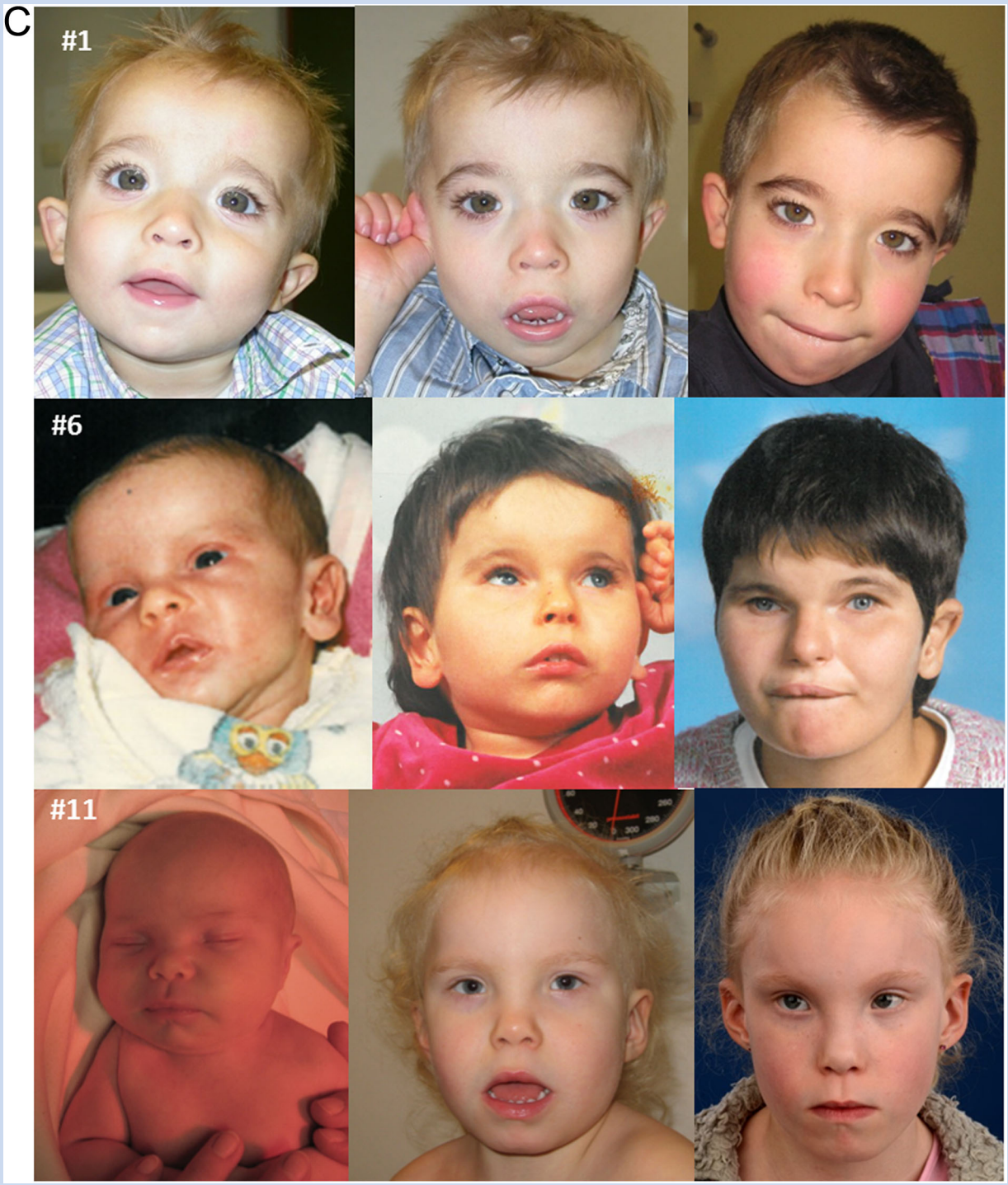

FIG. 1. Continued.

18 months, he could walk independently, understand simple assignments, and babble, but he did not use words. He could socially interact with others, but was rigid in his own way of playing and doing things. No formal behavioral testing has been done. Otherwise his general health was good. At 20 months, his height was 81 cm (−1 SDS) and his OFC was 46 cm (−1.6 SDS).

Patient 3 was born at term to consanguineous parents (first cousins) weighing 3,600 g (0 SDS) with an OFC of 36 cm (0 SDS). Except for a glabellar hemangioma and some feeding difficulties, no other problems were noticed. Ultrasonography of heart and kidneys, and a MRI of the brain was normal. At 4 years, his height was 100 cm (−1.5 SDS), and his OFC was 51 cm (0 SDS). His

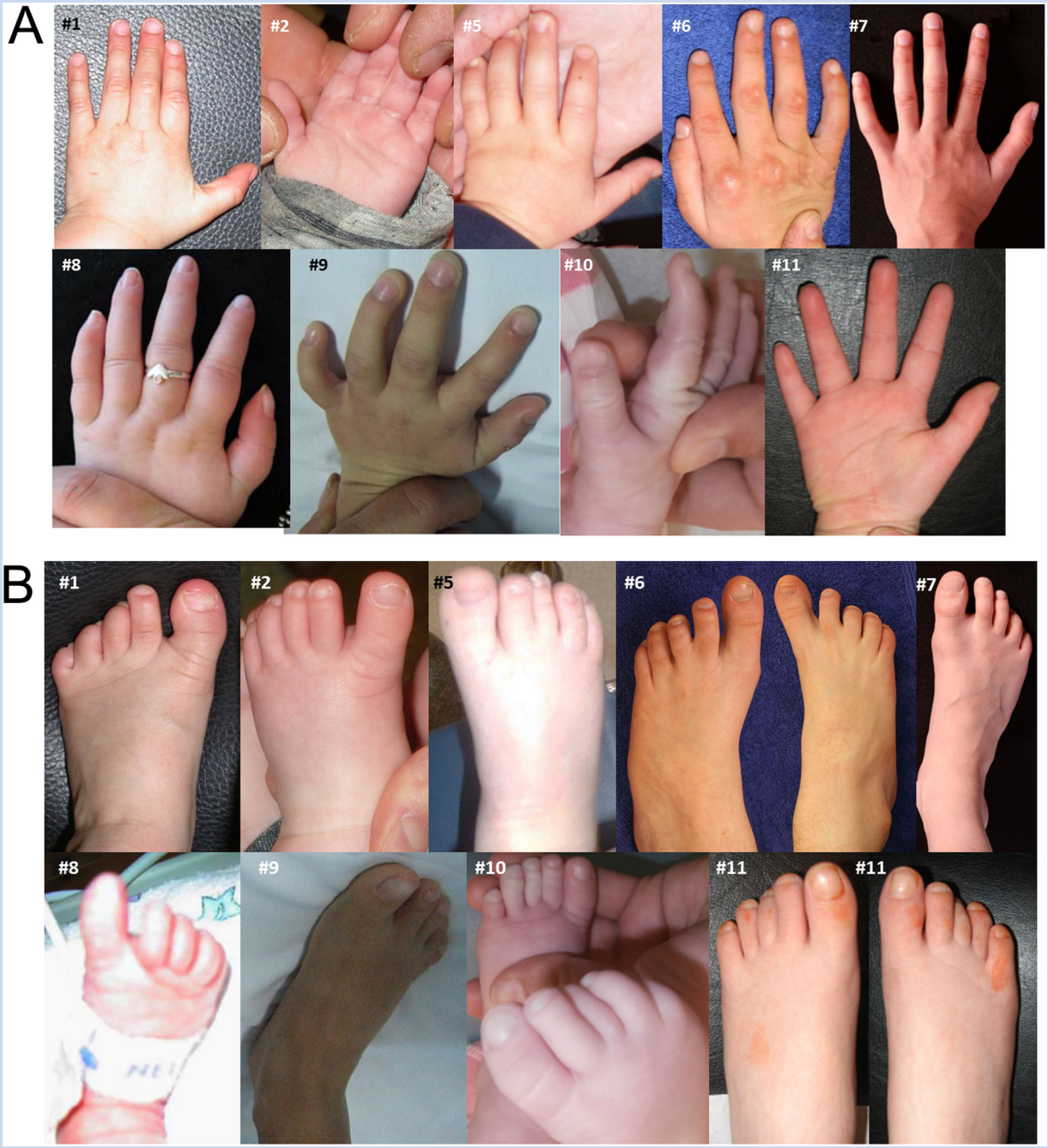

FIG. 2. Distal limb morphology of the presently described patients with a *CREBBP* mutation. Patient numbers correspond to text, tables, and other figures. Detailed description of the signs can be found in Table I. (A) Hands. (B) Feet. Note that none of the patients had broad or angulated thumbs, nor broad distal phalanges of the fingers, as seen in patients with classical Rubinstein–Taybi syndrome. The halluces of patients 5 and, to a lesser extent patient 2, were broad, but not angulated, and some other halluces were narrow. Patient 1, 6, and 8–11 showed fibular deviation of the distal phalanx of the hallux; patients 1, 2, and 6–8 had sandal gaps; and patients 5, 6, and 9 had partial cutaneous syndactyly.

unusual morphology was noted (Table I). He was able to walk, though unsteadily, and had no speech. He was friendly and pleasant but also had autistic features. A marked inferior limb spasticity developed as well as seizures (without EEG anomalies). When evaluated locally the differential diagnosis of the clinical geneticist included several disorders, including RSTS because of a convex nasal ridge and low hanging columella. Sanger sequencing involved several genes including both *CREBBP* and *EP300*. This

TABLE III. Genotypes of the Patients Reported Herein With a *CREBBP* Mutation (NM_004380.2) in the Last Part of Exon 30 or the First Part of Exon 31*

Patient	Exon	DNA variant	Predicted protein change	Protein domain[a]	Inheritance	ESP or ExAc[b]	Conservation[c]	SIFT	Mutation taster	Polyphen2
1	30	c.5128T>C	p.Cys1710Arg	ZNF2	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
2	31	c.5240T>G	p.Leu1747Arg	None	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
3	31	c.5357G>C	p.Arg1786Pro	ZNF3	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
4	31	c.5456G>T	p.Cys1819Phe	ZNF3	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
5	31	c.5478C>G	p.Cys1826Trp	ZNF3	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
6	31	c.5513G>A	p.Cys1838Tyr	ZNF3	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
7	31	c.5599C>T	p.Arg1867Trp	None	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
8	31	c.5600G>A	p.Arg1867Gln	None	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
9 + 10	31	c.5602C>T	p.Arg1868Trp	None	De novo	No	Drosophila	Deleterious	Disease causing	Probably damaging
11	31	c.5614A>G	p.Met1872Val	None	De novo	No	Drosophila	Deleterious	Disease causing	Possibly damaging

*The reference genome of Hg19 was used for characterizing the DNA variants.
[a]ZNF2 = zinc finger, ZZ-type; ZNF3 = zinc finger, TAZ-type.
[b]ESP/ExAc "no" = mutation not found in population cohorts Exome Sequencing Project and/or Exome Aggregation Consortium.
[c]See also Figure 3.

demonstrated a *CREBBP* variant, of which the significance was concluded at that time to be uncertain. At follow-up at 5 years of age, one of us (DL) evaluated the patient himself and concluded the patient did not have RSTS. The patient was subsequently lost to follow-up. He died at the age of 10 years for reasons unknown to us.

Patient 4 was born at term to unrelated parents weighing 2,940 g (−0.5 SDS). At 6 weeks of age, he had a viral meningitis from which he recovered completely. From early on he liked chewing on objects, produced excessive saliva, and showed mild gastroesophageal regurgitation and obstipation. Between 1.5 and 6 years, he had recurrent otitis media with hearing loss for which grommets were placed and prophylactic antibiotics were prescribed repeatedly. He started walking at 2 years of age, and spoke a few words at the same age but lost this capacity later on. He displayed autistic behavior and was subsequently diagnosed as having autism. He had short stature (at 12 years 131 cm; −3.4 SDS) and microcephaly (at 11 years 50 cm; −2.3 SDS) and limited dysmorphisms (Figs. 1 and 2). At 15 years, he had no speech and an ataxic gait, but was otherwise healthy.

Patient 5 was the first child born to unrelated parents. The father was said to have learning difficulties, dyslexia, and growth deficiency as a child but not as an adult. The boy was delivered at full term, weighing 2,150 g (<−2 SDS), and having a small OFC (32 cm; <−2 SDS). He had prolonged feeding difficulties and significant nasal regurgitation. Further evaluation showed facial and limb dysmorphisms (Figs. 1 and 2), hypermetropia, moderate conductive hearing loss, bilateral cryptorchidism, and chronic constipation. He had a tendency to sort objects, he disliked changes, and had poor social interaction with children, but had not been diagnosed as having autism by a psychiatrist. He had one grand mal seizure and possibly some absences. He started walking at 2 years, and did not speak at 5 years of age. At 5 years, his height was 93 cm (−3.5 SDS) and his OFC was 48 cm (−3.5 SDS).

Patient 6 was born at term to non-consanguineous parents weighing 2,500 g (−1.5 SDS). Due to severe feeding difficulties, nasogastric tube feeding was necessary until the age of 4 years. She had also recurrent bronchopulmonary infections. She could sit at the age of 2 years and started walking at the age of 7 years. She had no speech and a very limited speech comprehension. A cranial MRI showed a cystic pineal body and a median arachnoidal cyst. Several mandibular unerupted teeth were extracted in her teens, and dysplasia of her left hip was diagnosed at the age of 21 years. An abdominal ultrasound, performed because of irregular menstruation, was normal. Ophthalmologic examination demonstrated hypermetropia and nystagmus. Her adult height was 145 cm with an OFC of 50 cm (both −3.5 SDS). She had hirsutism and several dysmorphisms of the face and limbs (Figs. 1 and 2). Her breasts, nipples, and legs were markedly asymmetric. There was no asymmetry elsewhere and no skin pigmentation anomaly. She had hypermobility of her large joints, muscle hypertonia, and an ataxic

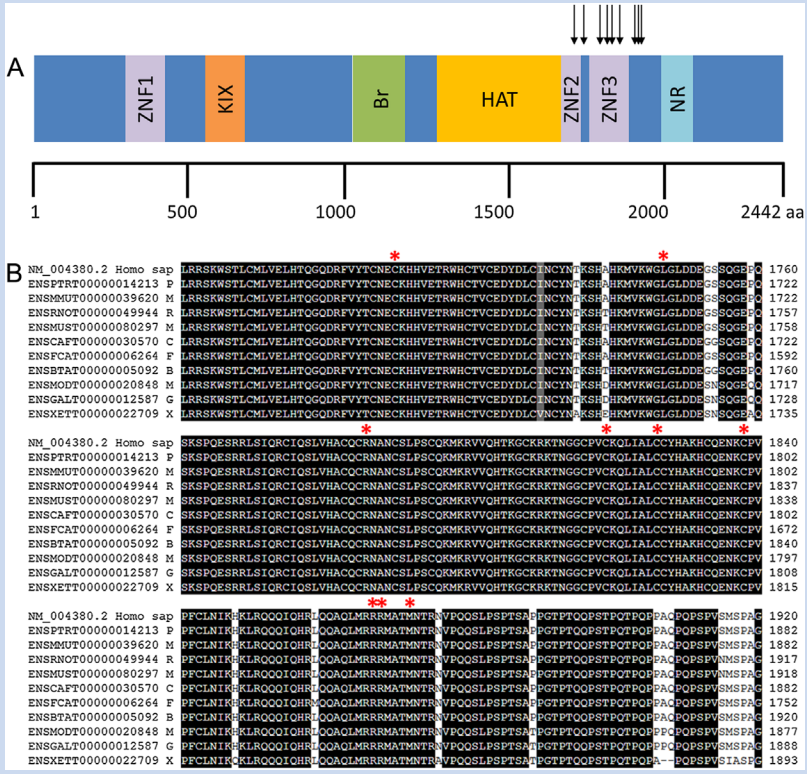

FIG. 3. Distribution of *CREBBP* Domains and Mutations in Present Patient Cohort. (A) Schematic representation of the CREBBP protein and its functional domains. The location of the variants is depicted with an arrow. ZNF1 = zinc finger, TAZ-type (344–439); KIX = CREB-binding domain (587–672); Br = bromodomain (1066–1201); HAT = histone acetyltransferase domain (1323–1700 de novo); ZNF2 = zinc finger, ZZ-type (1701–1744); ZNF3 = zinc finger, TAZ-type (1764–1853); NR = nuclear receptor coactivator (2019–2115). Deducted from http://www.ebi.ac.uk/interpro/protein/Q92793. (B) Alignment of *CREBBP* orthologues, from human to Drosophila. The location of the variants is marked with an asterisk.

gait. She easily choked and produced excessive saliva. She showed self-injurious and autistic behavior with stereotypic hand movements and temper tantrums.

Patient 7 was born at term to non-consanguineous parents weighing 3,260 g (−1 SDS). He had a very large anterior fontanelle, crumpled ears, metatarsus adductus, and prolonged, though mild, feeding problems. He presented with speech delay, using his first word at the age of 2, and putting two words together at the age of 4. He developed somewhat challenging behavior, and later on demonstrated anxieties. At 10 years (Figs. 1 and 2), his height was 146.5 cm (0.7 SDS), and OFC was 54 cm (0 SDS). He had learning difficulties with his reading abilities being much better than his mathematics. He enjoyed repetitive activities but generally was a sociable and happy child. His main medical issue was recurrent chest infections for which he received prophylactic antibiotics. A CT-scan of the chest, immunological function tests, and sweat test yielded normal results.

Patient 8 was the first-born of a dizygotic twin of non-consanguineous parents. Both parents had a apparently below average intelligence and the girl's elder sister was healthy but experienced learning problems. Her twin brother was known with a mild developmental delay. The twins were born at a gestational age of 31 weeks and the patient had a birth weight of 1,580 g (−0.7 SDS). At birth, a right-sided hydronephrosis was detected. She also had small clavicles and a poorly ossified skull with partial absence of the parietal bone on X-rays, resulting in a clinical diagnosis of cleidocranial dysplasia. An oligo-array analysis demonstrated a de novo 392 kb deletion on 6p12.3 including *RUNX2*, confirming the clinical diagnosis of cleidocranial dysplasia. Health issues included hypotonia, severe hip dysplasia, and epilepsy (West syndrome) that was difficult to treat. At the age of 5 years, all her primary teeth were extracted because of severe caries. An orthopantomogram could not be performed. She had developmental delay with absent speech and ambulation, absence of meaningful use of her hands, epilepsy, microcephaly, mild hearing loss, and cortical visual impairment that could not be explained by the *RUNX2* deletion. At 6 years (Figs. 1 and 2), her height was 134 cm (+2.5 SDS), and OFC was 47 cm (−2.5 SDS).

Patient 9 was born to unrelated parents at 37 weeks of gestation weighing 2,500 g (−1 SDS). She developed gastro-intestinal difficulties, consisting of vomiting, diarrhea, and abdominal pain associated with bowel dilatation. A malrotation and recto-vaginal

fistula were diagnosed. Because of persisting feeding problems, a gastrostomy was placed at 1 year of age. She developed a gastrocolic fistula, possibly due to the gastrostomy, which was surgically closed. Evaluation because of developmental delay at 14 months of age demonstrated short stature, microcephaly, and several dysmorphisms (Figs. 1 and 2). She had increased subcutaneous fat tissue around the hips, and a partial cutaneous syndactyly of the fourth and fifth toes. In later years, developmental delay became obvious, she had no development of speech, and she showed a self-injurious and autistic behavior. She could walk at 7 years of age. Brain MRI showed thinning of corpus callosum, subarachnoid widening and enlarged fourth ventricle, and a repeat MRI demonstrated progressive reduction of the white matter volume with progressive cerebral atrophy. At 5 years, her height was 102 cm (−2 SDS) and OFC was 46 cm (−2.6 SDS). Additional studies showed her to have retinal pigment dystrophy with altered results at the Visual Evoked Potential, and sensorineural deafness.

Patient 10 was the second child of healthy non-consanguineous Caucasian parents. She was born full term via repeat Caesarean weighing 1,980 g (<−2 SDS) with an OFC of 30 cm (<−2 SDS). Prenatal history was remarkable for growth retardation of length and skull circumference, and delayed cerebral development on fetal MRI. Prenatal chromosomal microarray was normal. She had cutis marmorata and distinctive facial features. Postnatal echocardiogram was normal and her brain MRI showed mild underdevelopment of the pons and cerebellum. Distortion product otoacoustic emissions responses were essentially absent bilaterally at 3 months of age and she was diagnosed with bilateral conductive hearing loss and small ear canals. She had feeding difficulties since birth, and was diagnosed to have gastroesophageal reflux. She had stiffening spells associated with deep breathing, which were thought to be seizures, but EEG was normal. At 8 months, during anesthesia for a frenulectomy and myringotomy tubes placement, she was found to have a small airway and anteriorly placed larynx. At 10 months (Figs. 1 and 2), her length was 68 cm (−1.5 SDS) and OFC was 41 cm (−2.1 SDS). Physical exam was remarkable for lax joints, tight heel cords, camptodactyly, and decreased patellar reflexes. She was able to sit up with assistance and was trying to roll over. She made good eye contact and had a social smile. She was not babbling yet.

Patient 11 was born at term to non-consanguineous parents weighing 2,685 g (1.8 SDS) and several dysmorphic features. A peri-membranous ventricular septal defect, atrial septal defect, and mild pulmonic stenosis together with a congenital diaphragmatic paresis were diagnosed. No surgical corrections were needed. She had a congenital dysplasia of the left hip for which she was treated with spreader pants. For a prolonged period of time, she refused to eat, for which she had a gastrostomy that was removed at 2 years of age. A squint was surgically corrected. Speech and motor delay became clear with a full-scale intellectual quotient of 64 at formal testing at the age of 5 years. A brain MRI showed mildly widened ventricles and a relatively small brain. Health issues were recurrent airway infections (including recurrent otitis), mild hearing loss, nasal speech due to velum insufficiency, and obstipation. At 7 years, her height was 127 cm (0 SDS) and OFC was 51 cm (−0.5 SDS). Several unusual facial and limb features were noted (Figs. 1 and 2) as well as a somewhat restricted mobility of the metacarpophalangeal joint of the right thumb. She had a scoliosis and two exostoses near the coccygeal bone.

Genotype

The sequencing analyses yielded 10 distinct de novo *CREBBP* missense variants in the 11 patients (Table III). The missense variants were all clustered in the end of exon 30 and the beginning of exon 31, between base pairs 5,128 and 5,614 (codons 1,710–1,872) (Fig. 3a). This region corresponds to a conserved region in the *CREBBP* protein (Fig. 3b). Five variants were located in a zinc finger domain (Table III; Supplementary data Fig. S1), the other variants were not located in a known protein domain. All variants changed a conserved amino acid (Table I), and all were predicted to be pathogenic by three different in silico prediction programs. None of the variants had been reported before, either in HGMD/LOVD databases or in large population cohorts such as ESP or ExAc.

DISCUSSION

The present study describes 11 individuals who all had missense mutations in the last part of exon 30 or the beginning of exon 31 of *CREBBP* and who had a phenotype that differed substantially from the RSTS phenotype. Some of the patients did show a few signs fitting RSTS but these were mainly general signs such as growth retardation and micrognathia, or common, less characteristic dysmorphisms such as low-set ears or micrognathia (Table II). None had the classical facial RSTS features however, including the most characteristic sign of RSTS, the grimacing smile, and none had the truly broad and/or angulated thumbs and halluces, or the broad distal phalanges of the fingers. The patients reported here shared many signs, especially a (variable) intellectual disability (which constitutes a bias however being the reason for exome sequencing), a marked speech delay, short stature, and microcephaly, although not all showed this. In several patients, autism or a behavior resembling autism was found, and sometimes self-injurious behavior was present. Other symptoms were feeding problems, epilepsy, recurrent upper airway infections, and (usually mild) impaired hearing. There were only few patients with malformations of which the most important ones were a congenital heart defect, malrotation, rectovaginal fistula, cryptorchidism, hip dysplasia, and retinal pigment dystrophy. Brain MRI findings mainly consisted of enlarged ventricles and cerebral atrophy.

The facial characteristics in the present cohort resembled one another in some patients, and differed in others (Figs. 1 and 2). Patients 9–11, and to some extent patients 7 and 2, resembled one another more than did the other patients. Especially the telecanthi, ptosis, short palpebral fissures, depressed nasal ridge, short nose, anteverted nares, short columella, and long philtrum characterized their faces. The strong resemblance of patients 9 and 10 who shared the same mutation underlines this correlation. The mutations of patients 9–11 were located in the same small part of exon 31, whereas those of patient 7 and especially 2 were located outside this site. We, however, cannot exclude that the amino acids to be spatially close to one another and may interact with one another accordingly. Patients 6–9 had an everted vermilion of the upper lip

that caused their mouth to resemble a cupid bow, whereas this configuration was absent in the other patients. Other characteristics could be found in several other patients as well such as the telecanthi or epicanthi, full cheeks, narrow jaw, and abnormally positioned and formed ears, of which especially the prominence of the upper part was remarkable. The same held for the distal limbs: only the presence of sandal gaps was common (Fig. 2). Several patients had an unusual deviation of the distal phalanx of the big toes, and the big toes could be markedly narrow. Within the present patient cohort, patient 3 was the patient who must have resembled RSTS the most. It is unfortunate that no clear photograph can be presented for this patient as no permission could be obtained from the parents. A single picture (of very poor quality) that was available to us indicated a phenotype that did resemble RSTS only remotely. The patient was evaluated in person by one of us (DL) with strong clinical experience in RSTS, and it was concluded that the patient did not have RSTS.

The variation of the phenotype may seem surprising, but in fact can be expected. Before the use of exome sequencing, syndromes were delineated by grouping patients together because they resembled one other to a large extent. Thereafter, the variation of the syndrome became gradually apparent. Especially after the detection of the genetic cause underlying a syndrome, the variability of this syndrome was expanded, sometimes even strikingly. The present series of patients was grouped together in an opposite manner, by their common genotype. By doing so, the full variability of the phenotype is evident right from the start. Hallmarks of syndromes delineated this way will, therefore, need larger series of patients to become clear. This course of events may be compared to what has been experienced in comparative hybridization array analyses. Some patients with recurrent copy number variants (CNVs) were found to display a wide variety of clinical features ranging from affected to normal. It took years before a common phenotype could be defined in some of these [Hashemi et al., 2015; Torres et al., 2016]. Similarly, it may take a longer period of time before the phenotype associated with *CREBBP* missense variants in this region will become reliably evident. One may even argue that the phenotype is variable by definition since clinicians would otherwise have recognized the combination of findings already a long time ago. We are, therefore, careful not to conclude already what the hallmarks are of the phenotype caused by the present mutations in *CREBBP*.

CREBBP mutations, resulting in haplo-insufficiency, have long been known to cause RSTS [Petrij et al., 1995]. These mutations include nonsense, frameshift, and splice site mutations located throughout the gene, as well as exonic deletions ranging from one to all exons, and tandem duplications of one or more exons. Recently a single individual by an unusual phenotype was described with a splice site mutation in intron 20 [Dauwerse et al., 2016]. Also many different missense mutations affecting the histone acetyltransferase (HAT) activity have been described ([Roelfsema and Peters, 2007], LOVD, and HGMD). Other clearly pathogenic missense mutations causing RSTS are rare, with a few examples known in exon 1 affecting the nuclear localization signal (NLS) and in exons 16–18 affecting the Bromodomain [Bentivegna et al., 2006] and LOVD). In local registries, the groups in the Netherlands and France have found in total 81 missense mutations in *CREBBP* and all were located in one of these three parts of the gene. In the literature, a few other missense mutations have been reported, but based on current knowledge these would now be classified as neutral variants, such as c.1651C>A p. (Leu551Ile); c.2678C>T p. (Ser893Leu) [Schorry et al., 2008], and c.2941G>A p. (Ala981Thr) [Coupry et al., 2002]. In addition, there has been a report containing three patients with minimal overlap with the RSTS phenotype (not supported by photographic evidence) with a missense variant in *CREBBP* in exon 31 [Sharma et al., 2010]. However, the nomenclature used in the latter publication was unclear, the assignment of mutations on a DNA level and protein level differed, and also the location in the various functional sites of *CREBBP* was not performed according to current standards. We, therefore, remained in doubt whether these variants were truly present in these patients. In our own experience, we have never identified a de novo missense variant in exon 30/31 of *CREBBP* in the more than 300 patients suspected of RSTS, which supports the idea that missense variants in this region do not cause a classical RSTS phenotype. Truncating mutations in the last part of exon 31, however, did occur in patients suspected to have RSTS. All variants identified in the 11 patients presented here were located in a conserved region, and all were conserved amino acids themselves. In addition, all variants were absent in the parents, and were also absent in large cohorts like the ESP and ExAc.

It may be suggested that the present patients belong to the subset of patients that is categorized as "atypical RSTS" (OMIM #180849). This category includes patients with a variable presentation. Some were patients in whom no molecular results could be defined, as the molecular cause of RSTS was not known at the time of their description. The cause of the phenotype in these patients remains uncertain, and could well have been mutations in *EP300*, which can cause this phenotype [Fergelot et al., submitted]. The second category consists of a single patient described as having a "mild variant of RSTS" [Bartsch et al., 2002]. These authors described the patients as having classical facial and distal limbs manifestations, and we concur. The patient was considered to be affected in a mild way only because of growth parameters and apparently normal cognition (she was not formally tested). Such normal growth is not uncommon in patients with RSTS [Beets et al., 2014], and we may expect that also in genuine RSTS, in which cognition can be very variable, normal cognition should occur occasionally. The last category consists of a family with a missense mutation in exon 14 of *CREBBP* [Bartsch et al., 2010]. This proband and the mother have seen by one of us (RCMH) in the past as well, and it was concluded at that time that the phenotype in these patients was not classical RSTS but still showed sufficient overlap that the patients should be categorized as having RSTS. This contrasts with the patients in current report, in whom RSTS could not be diagnosed. We conclude the present series of patients cannot be classified as "atypical RSTS."

The site of the missense variants in exon 30/31 overlaps with the ZNF2 (Zinc finger, ZZ-type) and ZNF3 (Zinc finger, TAZ-type) domains, which contain important cysteine residues that mediate Zn^{2+} binding. They represent a unique use of zinc to stabilize a helical fold that mediates binding interactions with numerous transcriptional regulatory proteins [Ponting et al., 1996; De Guzman et al., 2000]. The ZNF2 domain of *CREBBP* (residues

1,701–1,742; as per SMART) binds two zinc ions [Legge et al., 2004] (Supplemental material Fig. S1). The Cys^{1710} residue is indispensable for coordination of one of the two zinc ions via two Cys-X-X-Cys motifs, and its substitution (as was the case in patient 1, Supplemental data Fig. S1) was predicted to dramatically perturb the proper folding of the domain. Four other mutations (i.e., p.Arg1786Pro, p.Cys1819Phe, p.Cys1826Trp, p.Cys1838Tyr in patients 3, 4, 5, and 6, respectively) were found to affect the ZNF3 domain of *CREBBP* (residues 1,766–1,844). These mutations were also predicted to strongly affect the stability of the structural organization of the domain [De Guzman et al., 2000; Legge et al., 2004]. In particular, one mutation involved one of the invariant cysteine residues of the domain (Cys^{1838}), and a second change involved Arg^{1786}, which is adjacent to the second conserved, invariable cysteine residue in the ZNF3 domain (Cys^{1785}). The remaining two mutations resulted in the substitution of other cysteine residues, and were also predicted to cause structural perturbations in the domain (Supplemental data Fig. S1). Overall, these data strongly suggest that this mutation cluster specifically affect the binding properties of the two zinc finger domains to *CREBBP* partners by affecting their proper folding. Additional functional studies are needed to prove this hypothesis.

Each of the current patients has been examined by a clinical geneticist, who performed exome sequencing (and Sanger sequencing in one patient), detected the variant in *CREBBP* in a patient who did not resemble the RSTS phenotype, and subsequently remained in doubt whether the variant was causative for the phenotype in their patient or not. Only by grouping the experience of the individual clinicians and molecular geneticists did it became clear that the chance became small that the variant would not have a meaning. Subsequent resemblance among at least some of the patients, including the two with the same mutation, added further evidence that the variants were causative. This experience emphasizes the importance of databases containing detailed information of both phenotype and genotype, and the need for international collaborations to recognize new entities.

A major reason for syndrome diagnostics is the importance of a diagnosis for providing adequate information to the patient and family on the characteristics, prognosis, and suggested surveillance of the disorder. The family of a patient with a *CREBBP* mutation is likely to be informed in this respect using all information that is available on RSTS. However, the present patients may differ in any aspect of RSTS, and knowledge known in classical RSTS may not be applicable to the present patients. Patients with missense mutations in this specific *CREBBP* region have a distinct clinical phenotype, with other somatic, cognitive, and behavioral signs and symptoms, and families should be informed accordingly.

We conclude that patients with missense mutations in the last part of exon 30 and the beginning of exon 31 of *CREBBP* show a phenotype that differs substantially from that in RSTS patients with mutations elsewhere in *CREBBP*. The difference is substantial and in our opinion the patients should not be classified as "atypical RSTS" or "RSTS-like." We expect that in a similar way, variants with an uncertain meaning due to the discrepancy between the phenotype of the patient under study and the phenotype known to be associated with mutations in the gene, will be seen in many others genes as well. We stress the importance of international collaboration in gathering phenotypes and genotypes, to identify the meaning of such findings. Only by this means, patients and families can be adequately informed about the diagnosis and receive optimal clinical care.

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INTERNET RESOURCES

SMART, http://smart.embl-heidelberg.de/

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