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ORIGINAL INVESTIGATION

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DNA sequencing of *CREBBP* demonstrates mutations in 56% of patients with Rubinstein–Taybi syndrome (RSTS) and in another patient with incomplete RSTS

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Abstract Rubinstein–Taybi syndrome (RSTS) is a distinct dominant disorder characterized by short stature, typical face, broad angulated thumbs and halluces, and mental retardation. The RSTS can be caused by chromosomal microdeletions and molecular mutations in the *CREBBP* gene; however, relatively few mutations have been reported to date. Here, we aimed to determine the rate of point mutations and other small molecular lesions in true RSTS and possible mild variants, by using genomic DNA sequencing. A consecutive series of patients including 17 patients from our previous study was investigated. We identified 19 causative mutations of *CREBBP* in a total of 45 patients representing three different diagnostic groups: (a) 17 mutations in 30 patients with unequivocal RSTS (detection rate 56.6%), (b) two mutations in eight patients with features suggestive of RSTS ("moderate or incomplete RSTS", detection rate 25%), and (c) no mutation in seven patients with undiagnosed syndromes and isolated features of RSTS. In general, the mutations were distributed without hot spots and most were unique; however, three recurrent mutations (R370X, R1664H, and N1978S) were identified. Furthermore, we detected 15 different intragenic polymorphisms, including two non-synonymous coding polymorphisms, L551I and Q2208H. We report not only the highest detection rate (56.6%) of *CREBBP* mutations in patients with RSTS to date, but also the second missense mutation (N1978S) in a patient with moderate or incomplete RSTS. Previous studies have identified cytogenetic deletions in the *CREBBP* gene in eight to 12% of patients and very recently, Roelfsema et al. reported *EP300* gene mutations in three of 92 (3.3%) patients with either true RSTS or different syndromes resembling RSTS. Our 56.6% detection rate of molecular mutations in *CREBBP* in patients with unequivocal RSTS supports the new concept that RSTS is a genetically heterogeneous disorder and furthermore, indicates that RSTS may be caused by gene/s other than *CREBBP* in up to 30% of cases.

Keywords CREB binding protein · Rubinstein–Taybi syndrome · Small molecular mutations

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Introduction

Short stature, typical facies, broad angulated thumbs and halluces, mild to moderate mental retardation (MR), occasional microcephaly and/or visceral malformations represent the main clinical features of the Rubinstein–Taybi syndrome (RSTS, MIM 180849). RSTS is a disorder of the chromatin-mediated activation of gene expression and belongs to a newly recognized family of disorders that are characterized by defects in chromatin structure and modification (Goodman and Smolik 2000; Hendrich and Bickmore 2001). RSTS is typically caused by mutations in *CREBBP* (gene for CREB-binding protein) (Petrij et al. 1995; Giles et al. 1997; Coupry et al. 2002). Very recently, however, Roelfsema et al. (2005) reported mutations of the *EP300* (gene for p300) in a small subset (3.3%) of patients.

Both CREBBP and p300 are transcriptional coactivators (Goodman and Smolik 2000). Conserved domains of CREBBP include the histone acetyltransferase (HAT) domain at amino acids 1232–1709 and the plant homeodomain type zinc finger (PHD-ZF) at amino acids 1237–1311 (Giles et al. 1997; Murata et al. 2001; Kalkhoven et al. 2003). A deficiency of CREBBP, caused by a deletion or a null mutation of one of the two *CREBBP* alleles in most patients, causes RSTS. However, the exact mechanism of how CREBBP deficiency leads to the clinical phenotype of RSTS is not known. In addition to studies in model animals, studies of the genetic and clinical heterogeneity of RSTS in humans may help shed light on this problem.

Previous studies have indicated low detection rates, below 47.6%, in the *CREBBP* gene (Petrij et al. 1995; Coupry et al. 2002), and have postulated the possibility of genetic and clinical (phenotypic) heterogeneity (Bartsch et al. 2002; Coupry et al. 2002; Roelfsema et al. 2005). In order to test this hypothesis, the current study used very stringent inclusion criteria to determine the frequency of mutations in the *CREBBP* gene in RSTS patients, strictly placing patients with moderate features and patients with differential diagnoses in different groups. We studied 45 patients, including 30 patients with unequivocal RSTS (characteristic appearance), eight patients with moderate features or incomplete RSTS (one or two characteristic features mild or missing), and seven patients with isolated characteristics of RSTS.

Materials and methods

Patients

Diagnoses were verified by one of the authors (O.B.) based on the clinical reports at referral, photographs, X-rays, and medical records. Inclusion criteria included a detailed, clear description of the phenotype allowing the assignment to one of three different diagnostic categories; (a) (group A, 30 patients) unequivocal RSTS, here defined by the presence of the gestalt of RSTS and all of the following four "major" clinical signs and symptoms: short stature (<2 SD); typical facies (microcephaly, low anterior hairline, heavy and arched eyebrows, long eyelashes, ptosis, epicanthal folds, strabismus, nasolacrimal duct obstruction, myopia, cataracts, glaucoma, coloboma, downward slanting palpebral fissures, large nose, convex/beaked profile of nose, deviated nasal septum, columella below alae nasi, small opening of the mouth, narrow palate, micrognathia, and low set ears); skeletal abnormalities (short, broad thumbs and/or big toes with radial angulation, fifth finger clinodactyly, polydactyly, plantar crease between first and second toes, scoliosis); and MR; (b) (group B, eight patients) diagnosis of moderate or incomplete RSTS, here defined by mildness or absence of one or two of the four major phenotypic features (Cotsirilos et al. 1987; Bartsch et al. 2002); and (c) (group C, seven patients) undiagnosed conditions suggestive of mild RSTS, due to the presence of isolated clinical characteristics. Besides the major clinical features defined above, occasionally observed features of RSTS were considered for diagnosis, such as supernumerary nipples, visceral malformations, cryptorchid testes, or keloids. The inclusion criteria also included a normal karyotype at 450+ bands resolution prior to this study, and apart from a few exceptions (slides for FISH not available or FISH performed elsewhere), *CREBBP* gene deletions had been excluded by means of FISH as previously described (Bartsch et al. 1999).

The total study population comprised a consecutive series of 45 unrelated patients, aged 1 month to 58 years, from Germany, Austria, the Czech Republic, Finland, Poland, Romania, the UK, and the US. The patient population included 17 individuals, six causative mutations, identified in Table 1, and a polymorphism, identified in Table 2, from our previous report (Bartsch et al. 2002). Patient 8 from the previous report had to be excluded due to insufficient amounts of DNA, and patients 9 and 10 (Bartsch et al. 2002) were excluded because they had previously identified cytogenetic deletions. Permission for the study was granted by the Ethics Committee of the Medical Faculty of the Dresden University of Technology.

By definition, all patients (group A) showed short stature, typical facies, broad angulated thumbs and halluces, and MR. Patients (group B) had one or two characteristic features missing, enabling only a diagnosis of moderate or incomplete RSTS, and patients (group C) showed only isolated traits of RSTS. Therefore, it is possible that a subset of patients within group B may represent misdiagnoses, and many patients in group C may have different diagnoses. Clinical details of ten patients from this study have been provided previously. These included patients 1, 2, 11, 16, 17, and 22 of group A (Bartsch et al. 2002; patients 2–7), patient 5 of group B (Bartsch et al. 2002; patient 1), and patients 36–38 of group C (Pöyhönen et al. 2004). Here we describe an exceptional patient with moderate or incomplete RSTS (patient 28) who was identified in this study and seven additional patients, from whom photographs and consent were available, to highlight the clinical findings in the different diagnostic groups. Parental or patient consent was obtained for photographs. Patients 9, 25, 27, 33, and 34 (Fig. 1a–e) display the facies of RSTS at different ages from newborn to adult, and, in two cases, extreme skeletal findings (group A). Patients 28 and 30 (Fig. 1f, g) show suggestive but not characteristic facies and uncharacteristic hands (patient 28 only), illustrating "moderate or incomplete RSTS" (group B). Patient 35 (Fig. 1h) represents a patient with a differential diagnosis of mild RSTS (group C). Patient descriptions below follow the order mentioned above, in progression from group A to group C.

Patient 9, internal code A-3436-10 (the letter A represents patients from group A), was diagnosed with

Table 1 Nineteen (18 different) causative mutations detected in the *CREBBP* gene

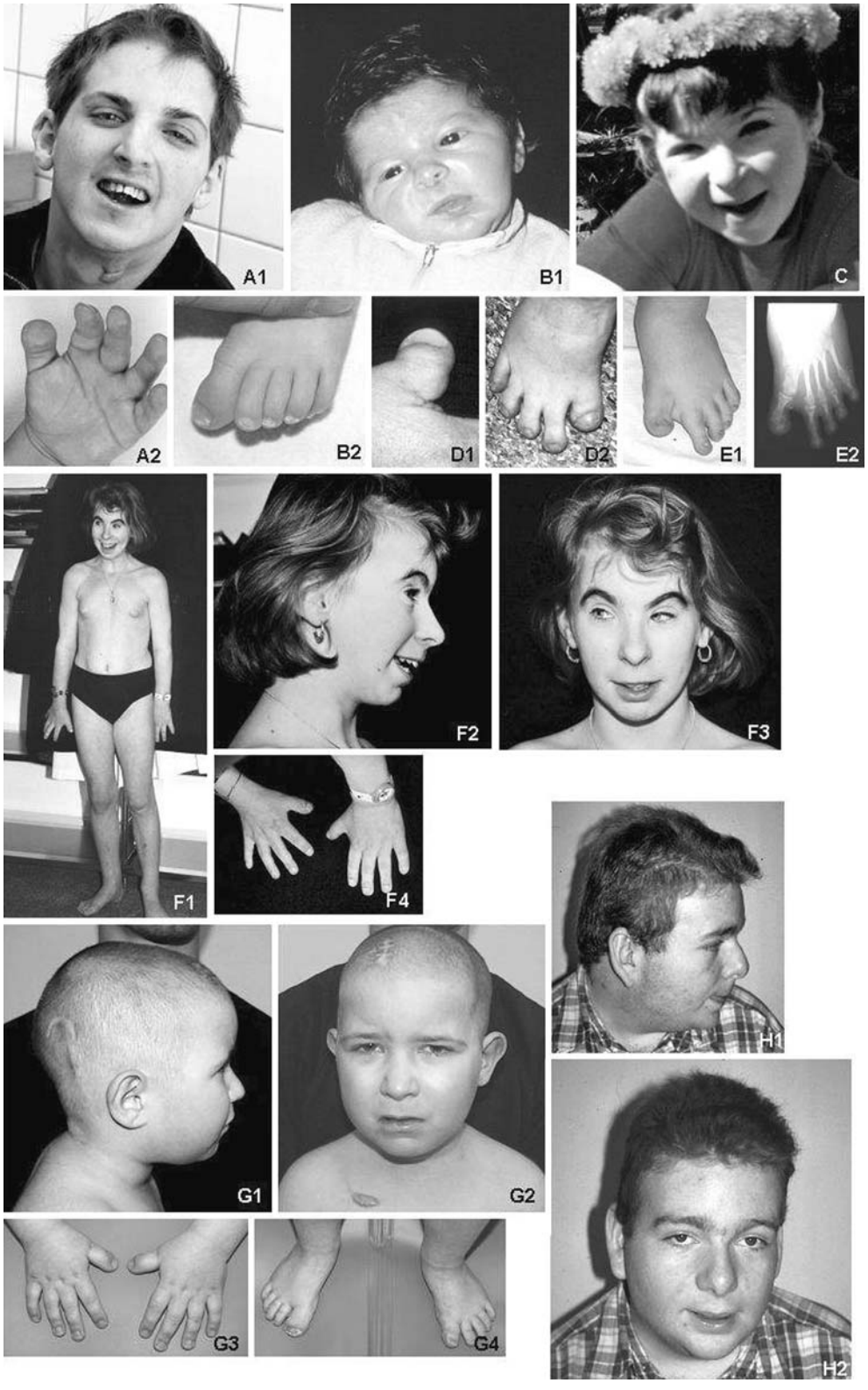
Patient	Internal code[a]	Location	Mutation			Origin
11[b]	A-3439-12	Exon 2	c.86_233del148nt	D29fs → STOP37	Molecular deletion	De novo
29	A-3513-30	Exon 2	c.474_493del20nt	Q158fs → STOP173	Molecular deletion	De novo
2[b]	A-3319-02	Exon 4	c.1108C → T[c]	R370 → STOP	Nonsense	ns[d]
22[b]	A-1925-23	Exon 4	c.1108C → T[c]	R370 → STOP	Nonsense	ns
16[b]	A-2256-17	Exon 4	c.1216 + 1G → A	Q405fs → STOP414	Splice-site mutation	De novo
25	A-3511-26	Exon 5	c.1270C → T	R424 → STOP	Nonsense	De novo
1[2]	A-3333-01	Exon 7	c.1676 + 1G → A	T558fs → STOP560	Splice-site mutation	De novo
26	A-3507-27	Exon 9	c.1891_1895delGCCTA	A631fs → STOP632	Molecular deletion	De novo
34	A-3563-36	Exon 15	c.2986G → T	E996 → STOP	Nonsense	ns
5[b]	B-3422-06	Exon 18	c.3524A → G	Y1175C	Missense	De novo
42	A-3625-45	Exon 19	c.3639 C → A	C1213 → STOP	Nonsense	De novo
40	A-3609-43	Exon 20	c.3767_3769delCAC	S1256 → STOP	Molecular deletion	De novo
45	A-3643-48	Exon 26	c.4304A → T	D1435V	Missense	De novo
9[e]	A-3436-10	Exon 26	c.4321_4322insC	R1441fs → STOP1452	Insertion	ns
23	A-3325-24	Exon 30	c.4991G → A[c]	R1664H	Missense	ns
41	A-3617-44	Exon 31	c.5635C → T	Q1879 → STOP	Nonsense	De novo
28	B-3491-29	Exon 31	c.5933A → G[c]	N1978S	Missense	De novo
19	A-3479-20	Exon 31	c.6019C → T	Q2007 → STOP	Nonsense	ns
43	A-3631-46	Exon 31	c.6050_6051insGCATGCC	P2017fs → STOP2342	Insertion	De novo

[a] *Letters A* or *B* in internal code indicate phenotype: *A* unequivocal RSTS, *B* possibly or incomplete RSTS. Criteria of classification (see Materials and methods)
[b] The mutations in these patients have been reported previously by Bartsch et al. (2002)
[c] These mutations were reported previously in other patients with RSTS (Coupry et al. 2002; Kalkhoven et al. 2003; Roelfsema et al. 2005)
[d] *ns* not studied, parental DNA not available
[e] In this patient from our previous study (Bartsch et al. 2002), the mutation was detected for the first time in this study

Table 2 Twenty-nine (15 different) polymorphisms of the *CREBBP* gene detected in this study

Patient	Internal code[a]	Location	Mutation			Origin	dbSNP rs# cluster ID[b]
21	A-1928-22	Exon 2	c.459G → A		Synonymous	Maternal	
29	A-3513-30	Exon 3	c.939T → C[b]		Synonymous	Maternal	3025702
40	A-3609-43	Exon 3	c.939T → C[b]		Synonymous	Paternal	3025702
29	A-3513-30	Exon 7	c.1651C → A	L551I	Non-synonymous	Maternal	
40	A-3609-43	Exon 7	c.1651C → A	L551I	Non-synonymous	Paternal	
3	B-3393-04	Intron 9-10	c.1941 + 53A → T		Intron	ns[c]	
25	A-3511-26	Intron 9-10	c.1941 + 53A → T		Intron	ns	
34	A-3563-36	Intron 9-10	c.1941 + 53A → T		Intron	ns	
17[d]	A-2310-18	Exon 15	c.2973C → T		Synonymous	Paternal	
17	A-2310-18	Intron 17-18	c.3370-17_3370-16 delT		Intron	ns	
20	A-1878-21	Intron 17-18	c.3370-17_3370-16 delT		Intron	ns	
23	A-3325-24	Intron 17-18	c.3370-17_3370-16 delT		Intron	ns	
40	A-3609-43	Intron 17-18	c.3370-17_3370-16 delT		Intron	Paternal	
23	A-3325-24	Exon 22	c.3837-8C → T		Splice site	ns	
29	A-3513-30	Exon 22	c.3837-8C → T		Splice site	Maternal	
40	A-3609-43	Exon 22	c.3837-8C → T		Splice site	Paternal	
23	A-3325-24	Exon 22	c.3900C → A[b]		Synonymous	ns	129974
18	A-3478-19	Exon 27	c.4560 + 7G → A		Splice site	ns	
1	A-3436-01	Exon 31	c.5454G → A		Synonymous	ns	
36	C-3602-38	Exon 31	c.5454G → A		Synonymous	ns	
29	A-3513-30	Exon 31	c.5988C → T		Synonymous	Maternal	
40	A-3609-43	Exon 31	c.5988C → T		Synonymous	Paternal	
3	B-3392-03	Exon 31	c.6003T → C		Synonymous	ns	
42	A-3625-45	Exon 31	c.6003T → C		Synonymous	Maternal	
29	A-3513-30	Exon 31	c.6621A → G		Synonymous	Maternal	
40	A-3609-43	Exon 31	c.6621A → G		Synonymous	Paternal	
27	A-3508-28	Exon 31	c.6624A → C	Q2208H	Non-synonymous	Paternal	
38[e]	C-3582-41	Exon 31	c.6624A → C	Q2208H	Non-synonymous	Maternal	
23	A-3325-24	Exon 31	c.6711C → T[b]		Synonymous	ns	3751845

[a] *Letters A*, *B*, or *C* in internal code indicate phenotype: *A* RSTS, *B* possibly or incomplete RSTS, and *C* isolated signs of RSTS, diagnosis unknown. Criteria of classification (see Materials and methods)
[b] Polymorphism previously reported in the Entrez Single Nucleotide Polymorphism Database (dbSNP)
[c] *ns* not studied, parental DNA not available
[d] The mutation in this patient has been described previously by Bartsch et al. (2002)
[e] The mutation in this patient has been described previously by Pöyhönen et al. (2004)



RSTS at age 1 year and has been followed until the present date. At age 22 years, he had a characteristic facies (Fig. 1a1), short stature (158 cm, −2.9 SD), microcephaly (occipito-frontal circumference (OFC) 51.5 cm, −3.2 SD), keloids after thyroid surgery (Fig. 1a1), broad, angulated thumbs (Fig. 1a2), broad big toes, cryptorchidism, and mild to moderate MR.

Patient 25 (A-3511-26) was born at 41 weeks gestation. Birth weight was 4200 g (+2 SD). At age 1 month he had a typical face with low anterior and posterior

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Fig. 1 Photographs of eight patients representing the different diagnostic groups and age groups. **a**–**c** Facies, hand, and foot of patients 9, 25, and 27 (group A, classical RSTS); **a1**, **a2** patient 9 (causative mutation R1441fsX), note typical facial appearance and keloids at 22 years of age and broad angulated thumb at the age of 2 years, **b1**, **b2** patient 25 (R424X) at age 1 month, note characteristic facial dysmorphy and broad hallux, and **c** patient 27 (no mutation found) at 7 years of age, note characteristic facies. **d1**, **d2**, **e1**, **e2** A hand, two feet and an X-ray of a foot of patients 33 and 34 (group A and classical RSTS). Note extreme abnormalities of hands and feet with very short metacarpals I and metatarsals I of patient 33 (no mutation found), age 58 years, and of patient 34 (E996X nonsense mutation), age 16 years. **f1**–**f4** Full view, two facial views and hand of patient 28 with the N1978S missense mutation (group B, moderate or incomplete RSTS). Note that the facial appearance and hands, with brachydactyly of all metacarpophalangeal bones, can be observed in RSTS, but were previously believed to be uncharacteristic. **g1**–**g4** Two facial views, hands, and feet of patient 30 (group B, moderate or incomplete RSTS), age 6.5 years, no mutation detected, with short stature, broad thumbs and halluces, keloids (**g2**), MR, medulloblastoma, and a suggestive, but not characteristic facies. **h1**, **h2** Two facial views of patient 35 (age 24 years, no mutation detected) with keloids (not shown), subnormal intelligence, and facial features mildly suggestive of RSTS (group C, differential diagnosis of possibly mild RSTS)

hairline, naevus flammaeus at the glabella, down-slanting palpebral fissures, bilateral coloboma, hypoplastic alae nasi, high arched palate, microretrognathia (Fig. 1b1). Supernumerary nipples, broad mildly angulated thumbs, broad big toes (Fig. 1b2), sacral dimple, and cryptorchidism were also observed.

Patient 27 (A-3508-28) was diagnosed with RSTS at age 1 year. A patent ductus arteriosus was corrected at age 2 years. At 4 years of age, her height was 92 cm (−2.6 SD), weight 13 kg (BMI 15.3 kg/cm^2, 0 SD for age), and OFC 45.5 cm (−3.3 SD). At the age of 9 years, she had a characteristic face (Fig. 1c) with low hairline, high arched eyebrows, down-slanting palpebral fissures, mild hypertelorism, prominent nasal bridge, prominent nose with hypoplastic alae, and micrognathia. She was hirsuit and had keloids, brachydactyly, broad, mildly angulated thumbs, broad big toes, and mild MR.

Patient 33 (A-3584-35) had four different neoplasms: breast cancer (age 43 years), colon cancer (age 50 years), thyroid cancer (age 54 years), and non-Hodgkin lymphoma (age 58 years). Due to her short stature and moderate MR, a DNA repair defect was suspected; however, cytogenetic studies for chromosomal breakage and molecular studies of the *NBS1* gene were normal. RSTS was diagnosed at age 58 years. Her height was estimated at 150 cm (−2.7 SD). She had a characteristic face (low hairline, frontal naevus flammaeus, hypertelorism, outward strabismus, prominent nasal bridge, prominent nose with hypoplastic alae) and very short, very broad, angulated thumbs, and very short, very broad, mildly medially deviated halluces (Fig. 1d1, d2).

Patient 34 (A-3563-36) had a history of stridor, constipation, and hydronephrosis. At age 5, she awoke very slowly from anaesthesia after renal surgery. This finding has been previously reported in patients with RSTS and easily collapsible laryngeal walls (Hennekam et al. 1990a). At the age of 13.8 years, she had short stature (136 cm, −3.4 SD) and microcephaly (OFC 49 cm, −3.4 SD). She was hirsuite and had a typical face (low hairline, high arched eyebrows, down-slanting palpebral fissures, exotropia, myopia, prominent nasal bridge, beaked nose with hypoplastic alae, short philtrum, crowded teeth, receding chin, and dorsally rotated ears). She had broad thumbs and extremely short and broad halluces (Fig. 1e1). Radiography revealed a very short metatarsal I (Fig. 1e2). She had moderate MR.

Patient 28 (B-3491-29) had a history of frequent otitis media, mild pulmonary stenosis, and mild MR. Previous differential diagnoses included Noonan, Dubowitz, and Cornelia de Lange syndromes. At 12.75 years of age, RSTS was suspected although her face and hands were not characteristic (Fig. 1f1–f3). Height was 130 cm (−3.7 SD), weight 30 kg (BMI 17.7 kg/m^2), and OFC 48.5 cm (−3.4 SD). She had a low hairline, up-slanting palpebral fissures, and thick, dark, high arched eyebrows with a significant synophrys. She had myopia (10 dpt), strabism, a columella extending below the alae nasi, high arched palate, multiple naevi, and discrete hirsutism. Her hands (Fig. 1f4) showed brachydactyly equally involving all metacarpophalangeal bones, as corroborated by the metacarpophalangeal pattern profile (MCPP; not shown). This type of brachydactyly is rare in RSTS (Hennekam et al. 1990b).

Patient 30 (B-3524-31) had a history of developmental delay. At age 4 years, he had a medulloblastoma near the 4th ventricle causing occlusive hydrocephalus. At 6.5 years of age, height was 100.5 cm (−4.7 SD), weight 18.2 kg (BMI 18 kg/m^2), and OFC 48.5 cm (−2.3 SD). He was hirsuite and had frontal bossing, thick eyebrows, myopia, and obstructed lacrimal ducts but not a typical facies (Fig. 1g1). However, he had keloids on the thorax due to shunting surgery (Fig. 1g2), broad thumbs and halluces (Fig. 1g3, g4), and cryptorchidism.

Patient 35 (C-3602-38) was a 23-year-old male with normal height (181 cm, +0.5 SD) and OFC, and normal hands and feet. He had facial features suggestive of RSTS (Fig. 1h1, h2) and keloids after surgery for undescended testes (not shown). He also had hypogonadotrophic hypogonadism and a history of poor coordination, learning difficulties (IQ formally tested at 84, particulars not available), and behavioral anomalies similar to Asperger syndrome.

Molecular analysis

The DNA sequencing of all coding exons of the *CREBBP* gene was performed as previously described (Bartsch et al. 2002), however, in order to prevent overlooking any mutations, the mutation screening using SSCP was discontinued. Furthermore, the DNA sequencing protocol was updated for use with the Big-Dye Chemistry, Versions 2.0 and 3.1, and the ABI PRISM 310 and 377 Genetic analyzer systems (Applied

Biosystems, Darmstadt, Germany). If a mutation or polymorphism was found, a second sequencing run was performed for confirmation. The origin of the mutation or polymorphism, whether de novo or familial, was established if parental DNA was available. Paternity testing was not performed due to legal restrictions. The Human gene mutation database, recent publications (Coupry et al. 2002; Kalkhoven et al. 2003; Roelfsema et al. 2005), and the Entrez Single Nucleotide Polymorphism Database were used to determine whether sequence variations were novel or previously reported. All causative mutations reported in this study were absent in 50 controls.

Results

In order to enable the investigation of a consecutive series of 45 patients, this study includes 17 individuals from our previous report (Bartsch et al. 2002). A total of 19 bona fide causative mutations were identified (Table 1). Twelve of the mutations were novel; five of the mutations (one in duplicate) have been previously reported (Bartsch et al. 2002), and two (R1664H and N1978S) represented a known mutation (Coupry et al. 2002; Kalkhoven et al. 2003; Roelfsema et al. 2005). The mutations comprised 12 different single nucleotide substitutions (one in duplicate), four deletions sized 148, 20, 5, and 3 nucleotides (patients 11, 29, 26, and 40, respectively), and two insertions sized 1 and 7 nucleotides (patients 9 and 43). Mutations were dispersed over the whole gene (Fig. 2). For 13 out of the 19 causative mutations, both parents of the affected patient were available for study, and all of these mutations were found to have occurred de novo.

Seventeen (56.6%) out of the 30 RSTS patients (group A) demonstrated a mutation. Fifteen mutations predicted a premature stop codon, leading to a significantly truncated protein and two mutations predicted an amino acid exchange in the HAT domain (D1435V and R1664H). Another two mutations (2 of 8, 25%) were detected among patients with a presumptive diagnosis of atypical or incomplete RSTS (group B). These were missense mutations predicting amino acid substitutions (Y1175C and N1978S) in conserved domains outside the HAT domain. The Y1175C mutation was present in patient 5 with a normal height and a low-normal intelligence (Bartsch et al. 2002) and the N1978S mutation was identified in patient 28 with an atypical face and atypical hands (Fig. 1f). No causative mutations were identified in the patients with isolated features of RSTS (group C).

Additionally, we have detected 29 polymorphisms of *CREBBP* in 16 patients, including 14 single nucleotide polymorphisms (SNPs) and an intronic 1-bp deletion (Table 2). With one exception, these alterations were silent, based on their presence along with a causative mutation in the patients, and because of their presence in healthy parents. The exception, the c.4560 + 7G → A alteration (patient 18), involved the non-conserved seventh dinucleotide of the intron 27 donor splice site and was predicted to be silent, because in general, the donor splice site consensus sequence shows high variability at this position. The calculation of the donor site prediction score, using Splice site prediction software showed a slight decrease from 86 to 81%, suggested that this sequence variation was most likely a polymorphism and not responsible for a leaky splice process. However, since RNA was not available from patient 18, a more detailed investigation of his splice site alteration could not be performed.

Eleven of the sequence variations have not been previously reported, including a non-synonymous coding SNP, c.1651C → A, which predicts a conservative amino acid exchange, L551I. Patient 38 who has the non-synonymous coding SNP Q2208H has been reported as part of a separate study (Pöyhönen et al.

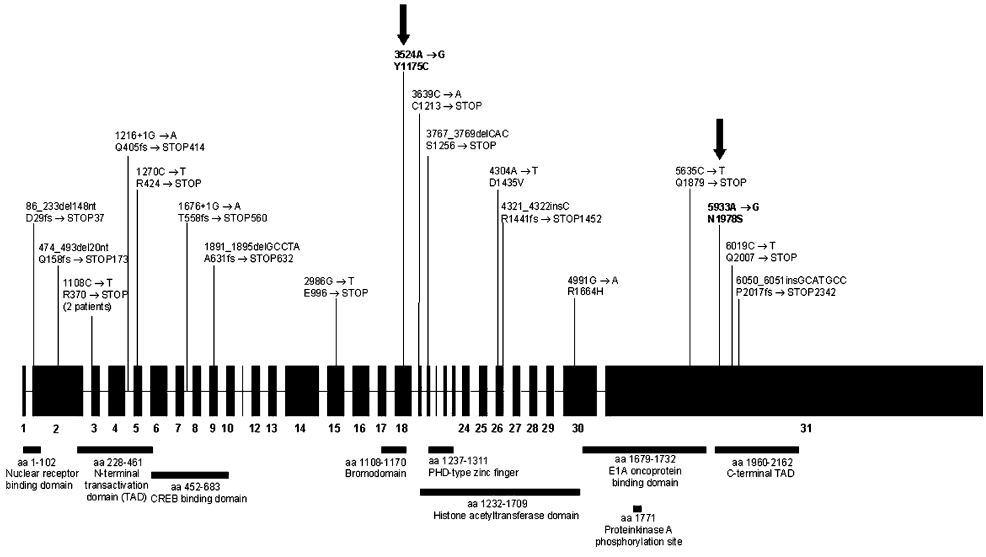

Fig. 2 Locations of the 19 causative *CREBBP* gene mutations. The 1108C → T mutation was present in two subjects. Exons are drawn to scale and selected exons have been numbered (introns and UTRs not drawn to scale). The histone acetyl transferase domain and the plant homeodomain (PHD)-type zinc finger are indicated according to Kalkhoven et al. (2003), other domains according to Giles et al. (1997). Mutations are distributed without particular hot spots. *Arrows* highlight missense mutations of patients with incomplete or atypical RSTS

2004), and the SNPs c.939T → C, c.3900C → A, and c.6711C → T were already listed in electronic databases (dbSNP IDs rs3751845, rs129974, and rs3025702). Interestingly, patients 29 and 40 shared five different polymorphisms that were inherited from their parents, including three SNPs (c.1651C → A, c.3837−8C → T, and c.6621A → G) that were not seen in any other patients or families. The two families lived in Düsseldorf, Germany, and Warsaw, Poland (distance 1000 km) and appeared to be unrelated, but extended pedigrees were not available.

Discussion

We have investigated a series of 45 patients including 17 individuals from our previous study (Bartsch et al. 2002). By sequencing all exons and splice sites of the *CREBBP* gene, 18 different mutations were identified in a total of 19 patients, 17 with RSTS and two with incomplete RSTS. Twelve of these mutations have not been described previously. The patients represented three different diagnostic groups: 30 patients had classical RSTS (group A), eight had a probable diagnosis of atypical or incomplete RSTS (group B), and seven had no known diagnosis but showed isolated signs of RSTS (group C). In 13 cases with a mutation, parental materials were available. In all of these cases the de novo status of the mutations could be inferred, because the mutations were absent in the parents.

In agreement with previously reported mutations in *CREBBP* (Petrij et al. 1995, 2000; Murata et al. 2001; Bartsch et al. 2002; Coupry et al. 2002; Kalkhoven et al. 2003) most mutations in this study (15 of 19, 79%) predicted premature stop codons. Four missense mutations (21%) were identified, and are most likely pathogenic. Patients with RSTS usually have de novo mutations and we could confirm de novo origin for three of the missense mutations. The parental DNA of the fourth individual, patient 23, was unavailable so that we were unable to establish the origin of the mutation. The missense mutations changed conserved domains of CREBBP and altered amino acid residues that are identical in the mouse. One mutation marginally preceded the HAT domain, two were located within the HAT domain, and one was located within the C-terminal transactivation domain. In two other patients, splice signals were weakened by single base substitutions that changed nucleotides at invariable positions (c.1216 + 1G → A, c.1676 + 1G → A). The effects of some splice mutations can be difficult to predict, but in these two cases the mutations altered the conserved first position of the donor splice site, which is always guanine, to adenine, effectively reducing the splice site prediction score from ≥99% to zero. Moreover, the de novo origin was confirmed by studies of parental DNA.

The mutations were distributed throughout the *CREBBP* gene without particular hot spots. Most mutations were novel, but three recurrent mutations were observed, R370X, R1664H, and N1978S (Coupry et al. 2002; Kalkhoven et al. 2003; Roelfsema et al 2005). We could not investigate the de novo status of the R1664H missense mutation (patient 23), because parental DNA was not available. However, Kalkhoven et al. (2003) reported a previous patient with RSTS and this mutation, which is in the HAT domain and alters a conserved arginine residue (man, mouse) (Giles et al. 1997) to histidine. The parents of the previous patient, 100 healthy controls analyzed by DGGE (Kalkhoven et al. 2003), and all of our controls did not display the R1664H mutation. Thus, this is most likely a pathogenic missense mutation in *CREBBP* that changes the HAT domain (amino acids 1232–1709), but does not alter the plant homeodomain (PHD) zinc finger (amino acids 1237–1311) or the postulated coenzyme A binding site (amino acids 1459–1541). Interestingly, we observed the recurrent N1978S missense mutation in patient 28 with moderate or incomplete RSTS (group B). The predicted amino acid substitution is located outside the HAT domain, so that a slightly different phenotype is conceivable. However, Coupry et al. (2002) did not report clinical details of their patient and therefore, the phenotypic spectrum with the N1978S mutation cannot be further clarified at present.

The low mutation detection rate in patients with RSTS remains an enigma. The current study indicates that *CREBBP* sequencing can reveal pathogenic mutations in 56.6% (17 out of 30) patients with RSTS, a high rate not previously reported (Petrij et al. 1995; Coupry et al. 2002; Roelfsema et al. 2005). On the other hand, the frequency of patients (43.3%) not showing a small molecular mutation in the *CREBBP* coding area provides evidence that RSTS could be caused by gene/s other than *CREBBP*. The CREBBP and p300 share similar cellular functions (Goodman and Smolik 2000) and recently, three (3.3%) mutations of *EP300* (gene for p300) were identified in a series of 91 patients (Roelfsema et al. 2005). Hence, small molecular mutations of *CREBBP* (this study) and *EP300* mutations (Roelfsema et al. 2005) have been identified in 56.6 and 3.3% of patients, respectively. Gross deletions of *CREBBP* have been detected by use of FISH in 8.3% (4 of 45 and 14 of 171 subjects, respectively) (Bartsch et al. 1999; Petrij et al. 2000), and additional, smaller *CREBBP* deletions and a duplication have been identified using techniques including Northern blot, Southern blot, and microsatellite analysis, reverse transcriptase-PCR (RT-PCR), real-time quantitative PCR, and multiple ligation-dependent probe amplification (MLPA) (Coupry et al. 2002, 2004; Roelfsema et al. 2005). However, all these approaches do not satisfactorily explain the lack of mutations in 25–30% of patients with RSTS. Mainly two possibilities, both equally atttractive, could explain the low mutation detection rate, (1) the presence of unusual mutations, such as alterations affecting the long-range control of gene expression (Kleinjan and van Heyningen 2005), or deep intronic mutations causing exon skipping (Pagani et al. 2002),

the activation of cryptic exons (Suminaga et al. 2002), or the generation of additional exons (King et al. 2002); and (2) the genetic heterogeneity of RSTS.

In patients with RSTS caused by other genes such as *EP300*, one might expect subtle phenotypic differences as compared with individuals with RSTS caused by *CREBBP* haploinsufficiency. Possible phenotypic differences could include differences in the skeletal dysplasia and an increased prediposition to cancer as has been already suggested (Coupry et al. 2002). Therefore, we revisited the phenotypes of the patients of group A and identified two individuals with extreme skeletal changes (Fig. 1d, e): patient 34 had a *CREBBP* nonsense mutation, but no causative alteration was identified in patient 33. Patient 33 also had a history of four different malignant tumors (non-Hodgkin lymphoma; breast, colon, and thyroid cancer). The RSTS has been associated with an increased risk for neural and developmental tumors, especially of the head, and for leukemia and lymphoma (Siraganian et al. 1989; Miller and Rubinstein 1995; Wiley et al. 2003). Hence, the lymphoma of patient 33 is a characteristic neoplasm in RSTS, but the breast, colon, and thyroid cancers are not. In patients such as this, studies of *EP300* and possibly other gene/s may be warranted.

Evidently, moderate or incomplete RSTS (Cotsirilos et al. 1987; Bartsch et al. 2002) is a genuine variant of RSTS. Including this study, two mutations in *CREBBP* have been identified among these individuals. Patient 5 was previously reported as the first case of incomplete or atypical RSTS confirmed by a *CREBBP* gene mutation (Bartsch et al. 2002) and patient 28 represents the second case. Both individuals had de novo missense mutations predicting exchanges of single amino acids. This is not surprising, given the numerous examples of missense mutations leading to milder phenotypes as compared to truncating mutations. We therefore suggest that patients with incomplete RSTS should be studied for molecular *CREBBP* mutations.

None of the seven individuals with isolated features of RSTS displayed a causative *CREBBP* mutation. However, apart from patient 35 (Fig. 1h), whose phenotypic similarities to the RSTS included a facial appearance mildly suggestive of RSTS and keloids, but excluded any skeletal changes, these individuals had facial appearances that differed from RSTS. In fact, three of these subjects have been previously described in a separate study as cases of a possible new syndrome that shows phenotypic overlap with RSTS, and represents another differential diagnosis (Pöyhönen et al. 2004).

Finally, we identified 15 sequence variations (Table 2), which most likely represent polymorphisms. These alterations included two non-synonymous coding SNPs that were also present in the non-affected parents and predicted only marginal amino acid changes (L551I and Q2208H). The c.4560 + 7G → A sequence variation was difficult to interpret, because it was novel and no additional or causative mutation was identified (patient 18). Parental DNA and RNA samples were not available to investigate whether this variation is associated with defective splicing. The c.4560 + 7G → A variation changes the seventh position of the donor splice site after exon 27 from guanine to adenine. As mammalian donor splice sites are not conserved at this position, it is most likely a silent alteration.

In conclusion, the current study demonstrated 17 (56.6%) mutations of the *CREBBP* gene in a series of 30 consecutive patients with typical RSTS. Two additional mutations, both predicting single amino acid exchanges instead of protein truncations, were identified in individuals with incomplete RSTS, leading to the identification of the second patient with incomplete RSTS confirmed by a de novo missense mutation in the *CREBBP* gene. The mutations were distributed over the *CREBBP* gene without particular hot spots and most were unique, but three recurrent mutations (R370X, R1664H, and N1978S) were identified. In conjunction with the current literature, our data indicate that a considerable subset of cases of RSTS, up to 30%, may be caused by gene/s other than *CREBBP*.

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