

# Molecular studies in 10 cases of Rubinstein-Taybi syndrome, including a mild variant showing a missense mutation in codon 1175 of *CREBBP*

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The genetic analysis of the Rubinstein-Taybi syndrome (RTS, OMIM 180849) may shed light on mechanisms of transcription, brain function, keloid formation, and cancer.<sup>1–6</sup> RTS can be caused by heteroallelic mutations of *CREBBP* (the gene for cAMP responsive element binding (CREB) binding protein).<sup>2</sup> Human *CREBBP* resides on chromosome 16p13.3,<sup>7–9</sup> spans approximately 150 kb at the genomic level, and comprises a coding sequence of 7329 bp.<sup>10</sup> The protein (2442 amino acids) is conserved (human *v* mouse, 94% amino acid identity)<sup>10</sup> and a transcriptional coactivator.<sup>4–6</sup> Predicted domains include the nuclear receptor binding and receptor interacting domain (aa 1–170), the amino-terminal transactivation domain (N-terminal TAD, aa 228–461), the Cys/His rich region (aa 363–496), the CREB binding domain (aa 452–682), the bromodomain (aa 1108–1170), the histone acetyltransferase domain (aa 1173–1849), the trithorax consensus finger and Cys/His rich region (aa 1232–1487), the E1A oncoprotein binding domain (aa 1679–1732), the protein kinase A phosphorylation site (aa 1771), the Gln rich region (aa 1849–1999), and the C-terminal TAD (aa 1960–2162).<sup>10</sup>

Mutations of *CREBBP* reported in RTS have included chromosomal translocations, deletions at the microscopic and submicroscopic level, and molecular mutations.<sup>2 7–9 11–14</sup> Apart from a recent report,<sup>14</sup> previous studies on RTS used the protein truncation test before molecular analysis, thereby limiting the spectrum of observed molecular mutations. To date, 11 small mutations of *CREBBP* have been reported, comprising truncating mutations and one missense mutation.<sup>2 13 14</sup> Two modes of action of how *CREBBP* mutations may cause RTS have been discussed, haploinsufficiency and dominant negative effects. The haploinsufficiency mechanism is made likely by the observation that some 10% of subjects with RTS exhibit deletion of one *CREBBP* allele, and that deletions of different domains of *CREBBP* were found with the same RTS phenotype, without genotype-phenotype correlation.<sup>9 11–13</sup> The finding that microinjection of truncated *CREBBP* alleles into mammalian fibroblasts blocks transcriptional activation of a *CRE-lacZ* reporter<sup>15</sup> maintains the idea that some signs of RTS can be caused by a dominant negative inhibiting mechanism. This mechanism is also supported by studies of mice with a truncated *CREBBP* allele (aa 1–1084)<sup>16</sup> or two null alleles.<sup>17</sup>

We describe here *CREBBP* sequence variants in 10 subjects, including one (subject 1) showing a very mild phenotype of RTS (termed incomplete RTS) and a missense mutation.

## MATERIALS AND METHODS

### Subjects

The study was authorised by the Ethics Committee of the Medical Faculty of the Dresden University of Technology. We analysed a sample of 20 patients from Germany and the Czech Republic, for whom parental consent was provided, clinical signs were well documented, and material for FISH and DNA

analysis was available. All subjects had a normal chromosome analysis on Giemsa-trypsin banding at 450+ band resolution before the study. Subjects were unrelated, notably patients 3 and 4, who came from different parts of Germany. Diagnoses established elsewhere were confirmed by one of us (OB) based on clinical records and photographs. All subjects except patient 1 had a diagnosis of typical RTS. If a mutation was found and the parents were available, we also studied parental DNA. Paternity testing was not performed owing to legal restrictions. We report here 10 cases that showed allelic variants.

### Clinical details

Patient 1 (6-21299-3422) (fig 1) is the first child of young, healthy parents from Russia. Her sister is healthy. The pregnancy was uneventful and spontaneous delivery occurred at term. The newborn was mildly hypotrophic; length was 46 cm (–1.8 SD), weight 2500 g, and occipitofrontal circumference (OFC) 32 cm (–1.7 SD). Psychomotor development was slow but within the normal range and, later, performance at school was adequate. The family emigrated to Germany when she was 15 years old. Psychological and intelligence testing before entering the German school system showed intellectual performance in the lower normal range without any indication of mental retardation. When seen by one of us (PM) at 17 years, height (161 cm, –0.9 SD) and OFC (54 cm, –0.7 SD) were normal, but obesity was present (75 kg, body mass index 28.95). Maternal height was 168 cm (+0.2 SD), OFC 58 cm (+2 SD), paternal height was 173 cm (–0.6 SD), OFC 55 cm (–0.8 SD), and midparental height was 164.5 cm. Her face was typical of RTS at the mild end of the spectrum, round and slightly dysmorphic with intermittent exotropia, subtle bilateral ptosis, a prominent beaked nose with the columella below the alae nasi, and dorsally rotated ears (fig 1A, B). Her hands showed broad thumbs with brachytelephalangism (fig 1C) and her feet were strikingly abnormal with medially deviated, broad big toes and mild clubbing of toes 2–4 bilaterally (fig 1D). Radiographs showed short and broad first phalanges of the hands and feet. Neurological examination was normal. She functioned in the normal range, spoke German well although she and her family had been native Russian speakers, graduated from secondary school at the age of 18 having passed her exams, and when recently seen at 20 years had a regular job (formal IQ data not available). The phenotype was considered very unusual. It did not represent RTS, but a mild RTS variant was suspected on the basis of the

**Abbreviations:** RTS, Rubinstein-Taybi syndrome; CREB, cAMP responsive element binding protein; TAD, transactivation domain; SNP, single nucleotide polymorphism; HAT, histone acetyltransferase



**Figure 1** Patient 1 showing a mild RTS variant (incomplete RTS). (A, B) Note typical facial dysmorphism (round face, mild bilateral ptosis, prominent beaked nose with the columella below the alae nasi, dorsally rotated ears). (C) Typical broad thumbs and brachytelephalangism. (D) Strikingly abnormal feet with medially deviated, broad big toes and mild clubbing of toes 2-4.

digital and facial signs, despite the normal stature, OFC, and development.

Patient 2 (12-3439) was born after 41 weeks of gestation; weight was 3850 g and length 53 cm. At 15 months, a dysmorphic face and lack of speech development were noted and RTS was diagnosed. At 6 years of age, he showed microbrachycephaly, a characteristic facies, broad, angulated thumbs and broad big toes, mild scoliosis, micropenis, and severe mental retardation. His general health was good; height was 110 cm ( $-1.8$  SD) and OFC 49.6 cm ( $-1.6$  SD). Radiographs showed broad ribs and clefting of vertebral body S1.

Patient 3 (2-35297-3319) was diagnosed in 1969 as the first German patient with typical RTS and was illustrated in a medical textbook.<sup>18</sup> At the age of 33 years, she was re-evaluated for this study. The hallmarks of her condition were the same as noted earlier, and included a typical face, short stature, microcephaly, broad thumbs and broad big toes, and severe mental retardation.

Patient 4 (23-04697-1925), a 14 year old girl, showed short stature (129.5 cm,  $-5.6$  SD), microcephaly (OFC 49 cm,  $-3.7$  SD), a typical face, a very narrow palate, broad, angulated thumbs, and severe scoliosis requiring 24 hour corset wearing. Cleft palate had been corrected by surgery in her first year. She had severe mental retardation with a fairly good memory and good manual motor skills. Menarche occurred at the age of 16.5 years. At 18 years, the scoliosis was stabilised by surgery and metal implants.

Patient 5 (17-43199-2256), a neonate, was diagnosed with clear cut RTS showing intrauterine growth retardation, microcephaly, typical face, broad and angulated thumbs, muscular hypotonia, and motor retardation.

Patient 6 (1-34797-3333), a 4.5 year old boy, had short stature (97 cm,  $-3$  SD), microcephaly (OFC 47.5 cm,  $-2.7$  SD), a

typical face, bilateral congenital glaucoma, high vaulted palate, bilateral supernumerary nipples, broad angulated thumbs, right sided simian crease, undescended testes, hexadactyly of the right foot, and mental retardation. Vesicoureteral reflux had been corrected by surgery. Cranial MRI showed an enlarged posterior horn of the right ventricle, dysplasia of the right hippocampus, hypoplasia of the cerebellar vermis, and enlarged cisterna magna.

Patient 7 (18-17798-2310) was diagnosed at 2 days of age with intrauterine growth retardation, hirsutism, microcephaly, typical face, stenosis of the lacrimal ducts, typical broad and angulated thumbs, muscular hypotonia, and motor retardation. At 1 year old, she additionally presented a narrow maxilla and moderate to severe developmental delay, and the diagnosis was confirmed.

Patient 8 (5-02899-3408) showed microcephaly, a typical facial appearance, broad, angulated thumbs, complex heart defect (severe endocardial fibroelastosis, atrial septal defect, mitral valve incompetence, tricuspid aortic valve, persistent Botallo's duct), hepatomegaly, ascites, aplasia of the left kidney, and developmental retardation.

Patient 9 (18900) was diagnosed at 6 weeks of age and had microcephaly, a characteristic face, broad, angulated thumbs, bilateral hydronephrosis, undescended testes, muscular hypotonia, and developmental retardation.

Patient 10 (00801), an 11 year old girl, showed short stature, microcephaly, a typical face, broad thumbs and broad big toes, increased body hair, and severe mental retardation.

#### Fluorescence in situ hybridisation (FISH) and molecular analyses

FISH was performed as described elsewhere<sup>11</sup> using cosmid probes RT100, RT191, RT203, and RT166 (orientation on chromosome 16pter→cen) spanning the *CREBBP* gene. RT100 was

**Table 1** CREBBP sequence variations reported in this study

Nucleotide change	Exon	Patient	Mutation type	Predicted consequence
dele1-e31	1 to 31	9	Large deletion, length >120 kb	Loss of all exons
dele1-e31	1 to 31	10	Large deletion, length >120 kb	Loss of all exons
86del148nt	2	2	Out of frame deletion	aa 1-28 of CREBBP, additional 8 aa, Stop in exon 2
1108C→T	4	3	Nonsense	aa 1-369 of CREBBP, Arg 370 to Stop
1108C→T	4	4	Nonsense	aa 1-369 of CREBBP, Arg 370 to Stop
1216+1G→A	Intron 4	5	Splice site	aa 1-405 of CREBBP, additional 1 aa, Stop in intron 4
1676+1G→A	Intron 7	6	Splice site	aa 1-558 of CREBBP, additional 8 aa, Stop in intron 7
2973C→T	15	7	Polymorphism	None, Asp 991 to Asp
3524A→G	18	1	Missense	Tyr 1175 to Cys
4495C→T	27	8	Polymorphism	None, Leu 1499 to Leu

provided by F Petrij, other probes were purchased from Leiden University. RT100 covers exons e31 to e17, RT191 spans e13 to e3, RT203 includes e3, and RT166 covers e2 and e1.<sup>13</sup>

The exon/intron boundaries of *CREBBP* were determined using the cDNA sequence (GenBank accession No U47741) and sequence data of cosmid clones 420F6 (exons e1-e2, GenBank accession No AC005564), RT191 (e3-e13, GenBank accession No AC004509), RT102 (e14-e29, GenBank accession No AC004651), and 316H7 (e27-e31, GenBank accession No AC004760).<sup>19</sup> *CREBBP* and cosmids are represented on a human chromosome 16 sequence segment (GenBank accession No NT\_015360).<sup>19</sup> Genomic DNA was extracted from blood by standard techniques. Primers (sequences available on request) were designed using the formula  $T_m = 69.3^\circ\text{C} + 0.41 \times (\text{GC content } \%) - 650/\text{primer length}$ . PCR products were separated using gel electrophoresis and purified with the "JETQUICK gel extraction spin kit" (Genomed). SSCP analysis was performed as described elsewhere,<sup>20</sup> with 0.2  $\mu\text{l}$  <sup>32</sup>P-dCTP (2000 Ci/mmol) added to PCR reactions. PCR products of exons 3, 4, 24, and 27 were cut by restriction endonucleases (details available on request) before SSCP analysis. Fragments exhibiting anomalies in the SSCP analysis were sequenced using the thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and the LICOR 4000 sequencer (MWG Biotech), or the thermosequenase radiolabelled terminator cycle sequencing kit (Amersham Pharmacia Biotech) and the S2 sequencing system (GIBCO BRL), or the ABI PRISM Big Dye Version 2.0 dye terminator cycle sequencing kit and the ABI 310 sequencer (Applied Biosystems). Nucleic acid alterations were confirmed by sequencing in the reverse direction. In patient 2, gel electrophoresis of PCR products of exon 2 showed an abnormal shorter fragment that was cut from the gel, purified, and cloned using the TOPO TA Cloning Kit (Invitrogen). *E. coli* cells (strain DH5) were transformed using the Gene Pulser II (BioRad) apparatus. Plasmids were prepared with the JETQUICK plasmid miniprep spin kit (Genomed) and analysed with the LICOR 4000.

## RESULTS

Table 1 summarises the sequence variations of *CREBBP* found in this study. In patients 9 and 10, FISH analysis showed a chromosomal microdeletion using the RT100, RT191, RT203, and RT166 probes that, respectively, cover exons e31-e17, e13-e3, e3, and e2-e1 of *CREBBP*. These subjects have a deletion of *CREBBP* and possibly of neighbouring genes.

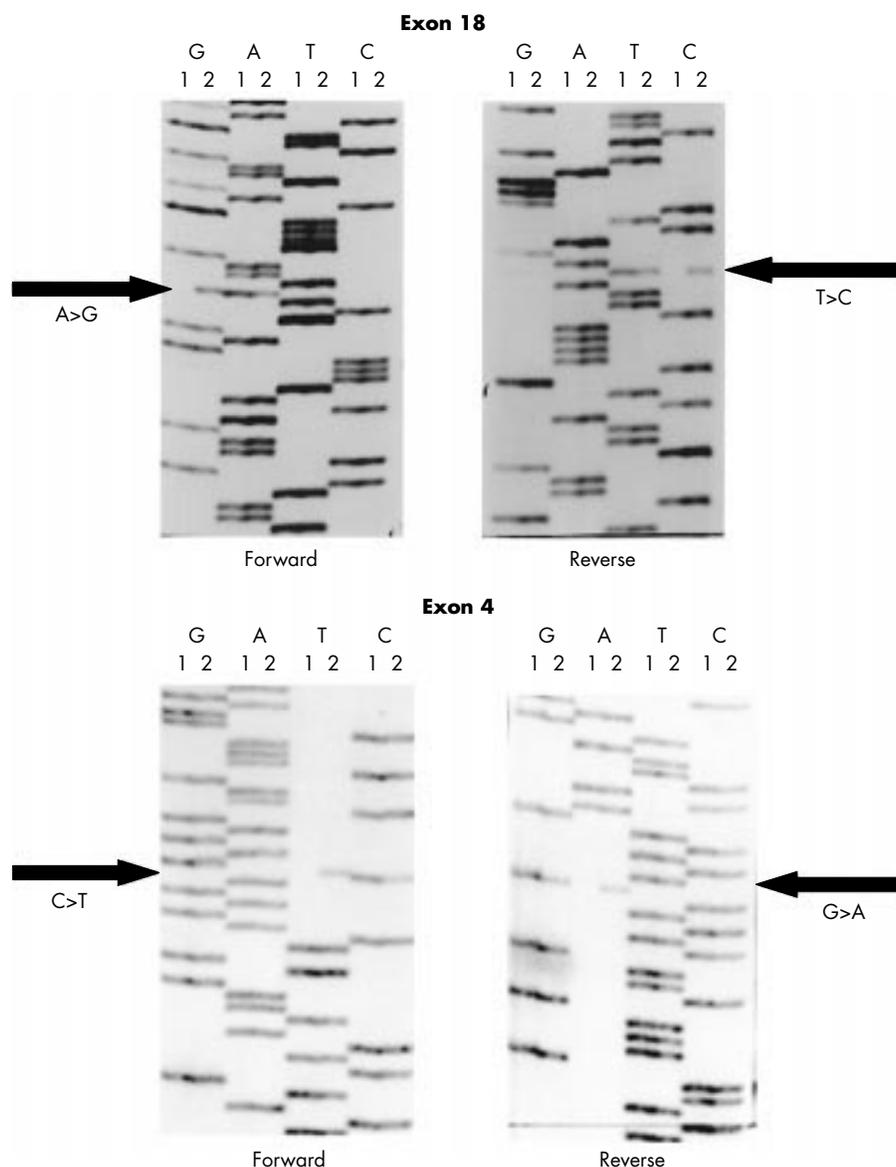
In patient 1, SSCP and DGS (fig 2, above) indicated a mutation in exon 18. The A (wild type) was replaced by a G at nucleotide 3524 (3524A→G) predicting a missense mutation from TAT (tyrosine) to TGT (cysteine) at codon 1175 (Y1175C). The site was found to be intact in both alleles of the parents, indicating that the mutation must have been de novo. SSCP and DGS analyses of other exons (e2-e8, e10-e12, e15-e16, e20, e24, and e26-e29) gave normal results.

Patients 2-6 showed heterozygous mutations predicting truncating mutations. Parental testing of patients 2, 3, 5, and 6 (the parents of patient 4 were not available) showed wild type alleles, indicating that mutations had occurred de novo. Provided that mutant mRNAs and proteins are stable, the mutations of patients 2-6 predict truncated *CREBBP* mutants without an intact CREB binding domain. These mutants support the idea of a haploinsufficiency mechanism causing "typical" RTS. In patient 2, agarose gel electrophoresis after PCR amplification of exon 2 showed two DNA fragments of about 710 bp (expected size) and 560 bp (abnormal fragment) indicating a heterozygous deletion. The shorter fragment was cloned and amplified in *E. coli*. Sequence analysis of three independent clones showed a 148 bp deletion (86del148nt) that predicted a frameshift and an extremely reduced *CREBBP* protein, retaining only the 28 N-terminal amino acids, followed by eight other aa residues owing to the altered reading frame and a stop codon. In patient 3, DNA sequencing of exon 4 showed a C→T transition at bp 1108 (fig 2, below) changing codon 370 from CGA (arginine) to TGA (stop) in one allele. The mutation predicts a truncated protein retaining only the nuclear receptor binding and receptor interacting domain (aa 1-170) and part of the N-terminal TAD (aa 228-461). Patient 4 showed the same mutation (1108C→T) as patient 3. In patients 5 and 6, sequencing of exons 4 and 7, respectively, disclosed a transition with the first nucleotide of the 5' splice consensus sequence replaced by adenine (1216+1G→A and 1676+1G→A, respectively). The alteration in patient 5 predicts a mutant *CREBBP* retaining the 405 N-terminal amino acids followed by eight other amino acids, without the CREB binding domain (aa 452-682). The 1676+1G→A allele (patient 6) predicts a mutant protein with the CREB binding domain truncated after residue 558. Patients 7 and 8 showed heterozygous transitions in exons 15 (2973C→T) and 27 (4495C→T), respectively. These sequence variants do not alter the amino acid sequence (D991D, L1499L) nor do they predict a new splice site, and therefore were interpreted as neutral or silent mutations. We could not detect pathological aberrations of *CREBBP* in patients 7 and 8 or in the remaining patients.

Molecular aberrations were compared to other reports<sup>2, 13, 14</sup> and using the nucleotide-nucleotide BLAST (blastn).<sup>21</sup> The comparison of the allelic variants of patients 1-7 identified no entries in other reports or in the human databases. The variants 86del148nt, 1108C→T, 1216+1G→A, 1676+1G→A, and 3524A→G are novel mutations, and the 2973C→T transition must be a novel coding SNP (single nucleotide polymorphism) of *CREBBP*. The 4495C→T substitution (patient 8) was identified in a human EST, AW589253, an observation in myeloid cells of 18 pooled CML cases that were positive for the *BCR/ABL* rearrangement.

## DISCUSSION

In this study, we found in eight out of 20 patients (40%) either a microdeletion or a mutation. Two patients had chromosomal



**Figure 2** Sequencing gels showing *CREBBP* mutations in genomic DNA, forward and reverse strands. (Above) Exon 18: lane 1 = normal control, lane 2 = heterozygous 3524A→G mutation of patient 1 (diagnosis, incomplete RTS). (Below) Exon 4: lane 1 = normal control, lane 2 = heterozygous 1108C→T mutation of patient 3 (diagnosis, clear cut RTS).

microdeletions of *CREBBP*, one had a 148 bp deletion, two had (identical) nonsense mutations, two had splice mutations predicting premature stop codons and gross truncations, and one had a missense mutation. Two patients had sequence variants (polymorphisms) and we could not detect their pathological aberrations. The mutations of patients 1-6 and the sequence variant of patient 7 are novel, and there has been only one previous report of a missense mutation. The 1108C→T alteration was found in two cases, patients 3 and 4, providing evidence for a mutation hotspot. The detection of the missense mutation in patient 1 was made possible by a strategy that avoided the preferential recognition of stop mutations by the protein truncation test.

As outlined in the introduction, a haploinsufficiency mechanism and dominant negative effects are currently discussed as possible modes of action of *CREBBP* mutations.<sup>2,9,11-17</sup> Patients 2, 3, 4, 5, 6, 9, and 10 had clear cut RTS phenotypes and effective null mutations or deletions, thus adding support to the haploinsufficiency model for typical RTS. In more detail, patients 2, 3, 4, and 5 had mutations predicting *CREBBP*

mutants without the CREB binding domain, retaining only the N-terminal 28, 369, or 405 amino acids. The mutation of patient 6 predicted a *CREBBP* mutant without the C-terminal portion of the CREB binding domain, with truncation at residue 558. In vitro experiments have shown that aa 586 to 679 of mouse *CREBBP* are required for CREB binding.<sup>15</sup> Therefore, and because of possible in vivo mRNA or protein instability, we assume that the *CREBBP* mutant of patient 6 represents another effective null mutation.

The major finding of this study is the very mild phenotype in patient 1 in combination with the 3524A→G missense mutation. Her digital and facial changes are typical of RTS, but she did not show several hallmarks of RTS, such as short stature, microcephaly, and mental retardation. This is the first report of incomplete RTS confirmed by a *CREBBP* mutation. At the severe end of the RTS spectrum, the report of the combination of unusually severe features, death in infancy, and deletion of *CREBBP* has suggested the possibility of a contiguous gene syndrome.<sup>11</sup> Patient 1 provides the first evidence for the presence of missense mutations at the mild end of the

spectrum. Similar very mild clinical findings have been reported in a three generation family with nine affected members showing "a syndrome similar to Rubinstein-Taybi" and including a 22 year old female showing faciodigital signs of RTS and normal mental function; her 5-year-old son was developmentally delayed and mentally retarded.<sup>22</sup> Bonioli and Bellini<sup>23</sup> commented on this family, proposing the diagnosis of RTS. They reported a case of RTS with vertical transmission of broad thumbs in the pedigree, and pointed out that Rubinstein in 1963 had described a similar case (case 4), where the father, a paternal uncle, and three paternal aunts had broad thumbs.<sup>23</sup> The cases<sup>22-23</sup> received a MIM entry (Rubinstein-like syndrome, MIM 180850). Our molecular data (patient 1) support the concept that mild alleles or modifying factors can lead to incomplete RTS, and that the Rubinstein-like syndrome can be equated with incomplete RTS.<sup>23</sup> It makes sense to consider the partial loss of function of the mutated *CREBBP* and a dominant negative mechanism in patient 1. Provided that no other mutation is present, she must be expected to have two full length *CREBBP* proteins, one wild type allele and one with the Y1175C alteration. The mutation predicts a possible loss of HAT activity. The tyrosine at position 1175 is conserved across species and is contained within a conserved area of 79 amino acids (murine *CREBBP* aa 1160-1238 *v* human *CREBBP* aa 1159-1237, 100% conservation).<sup>10</sup> In the mouse, aa 1099-1758 of *CREBBP* have been shown to be required for full HAT activity,<sup>3,4</sup> which corresponds to the area of aa 1098-1757 in human *CREBBP*.<sup>10</sup> To date, only one missense mutation (codon 1378, 4951G→C) has been reported with RTS, and by in vitro experiments this was shown to be the first example of a defect of HAT activity in human disease.<sup>14</sup> It is tempting to speculate that the incomplete RTS (patient 1) is a disease of chromatin activation that could be explained by a partial loss of HAT activity with the Y1175C variant. However, *CREBBP* is a multifunctional protein. The HAT domain is contained within a region (aa 1099-1877) with independent transcriptional activation activity,<sup>3</sup> and *CREBBP* modifies not only histones but also other proteins, and interacts with many transcriptional factors,<sup>24-26</sup> hormone receptors,<sup>27-29</sup> and nuclear import proteins.<sup>30</sup>

Molecular studies of other cases of incomplete RTS or Rubinstein-like syndrome are warranted. Functional characterisation of *CREBBP* variants should help to determine whether phenotypes are caused by the partial loss of HAT activity, or by another disturbed function of the mutant *CREBBP* allele, and/or by modifying factor(s).

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## Key points

- Rubinstein-Taybi syndrome (RTS) is a clinically well defined autosomal dominant disorder of facial anomalies, broad thumbs, broad big toes, short stature, microcephaly, and mental retardation. RTS can result from deletions or mutations in *CREBBP* (the gene encoding the CREB binding protein) and 11 small molecular mutations have been reported.
- We report clinical and molecular findings of 10 unrelated subjects. Nine patients had RTS and one (subject 1) showed a phenotype of possibly very mild RTS. For the mutation analysis we used a direct strategy of FISH, SSCP, and genomic sequencing, thereby avoiding the loss of missense mutations that can occur with the protein truncation test.
- We identified five novel mutations (86del148nt, 1108C→T, 1216+1G→A, 1676+1G→A, and 3524A→G), two deletions of *CREBBP*, and two single nucleotide polymorphisms (2973C→T, 4495C→T). The variant 1108C→T was found in two subjects, indicating a mutation hotspot.
- Subject 1 had a 3524A→G mutation predicting a tyrosine to cysteine exchange (Y1175C) in a conserved (human *v* mouse *CREBBP*) segment of the histone acetyltransferase (HAT) domain. This is the second case of a missense mutation in *CREBBP*. The "digitofacial" phenotype of subject 1 with typical digital malformations and facial changes, but normal stature, head circumference, and development represents an unusual mild RTS variant (termed incomplete RTS) that provides insight into phenotypic variation in RTS.

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