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ARTICLE

FISH studies in 45 patients with Rubinstein-Taybi syndrome: deletions associated with polysplenia, hypoplastic left heart and death in infancy

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Rubinstein-Taybi syndrome (RTS) is a dominant Mendelian disorder characterised by mental retardation, a typical facies, broad thumbs and short stature. Previous reports indicated that 4–25% of RTS patients have a submicroscopic 16p13.3 deletion of the *CBP* gene. Using FISH and cosmid probes RT100, RT191 and RT203 we studied 45 RTS patients from Germany, the Czech Republic, Austria and Turkey and found four deletions (8.9%, pooled data including other studies: 11%). All deletions were interstitial; three spanned the *CBP* gene (RT100–RT203) and one was smaller (RT100 only). Previous studies reported no phenotype–genotype correlation between RTS patients with or without a deletion. Our findings suggest a more severe phenotype. The mean age at presentation was 0.96 years in patients with a deletion as against 11.12 years in those without. Patients A and B with a deletion died in infancy which is rare in RTS and was not observed among the other patients. Patients A and D had accessory spleens, Patient A with hypoplastic left heart, abnormal pulmonary lobulation and renal agenesis. This is the second report of hypoplastic left heart and the first report of polysplenia with RTS. The signs suggest a developmental field defect (disturbance of laterality) either as a newly recognised pattern of RTS, or alternatively a novel contiguous gene syndrome.

Keywords: Rubinstein-Taybi syndrome; chromosome 16p13.3; CBP; submicroscopic deletion; hypoplastic left heart; polysplenia

Introduction

The Rubinstein-Taybi syndrome (RTS, OMIM #180849), a well-defined disorder of characteristic facial features, broad thumbs and halluces, short stature and mental retardation, is caused in some individuals by a cytogenetically cryptic 16p13.3 deletion of *CBP* (alias *CREBBP*, the gene which encodes the CREB binding protein, a nuclear protein participating as a coactivator in cyclic AMP-regulated gene expression) and in the majority of cases by other mutations.[1,2] The frequency of chromosomal microdeletions in RTS patients was investigated using FISH in different studies in the Netherlands, Denmark and Norway,[1] Japan,[3] Great

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Britain,[4] the USA,[5,6] and France.[7] Deletions were observed in 6/24 (25%), 1/25 (4%), 2/16 (12.5%), 1/15 (6.7%), 7/64 (10.9%), and 3/30 (10%) of patients.[1,3–7] Clinical differences between patients with and without deletions were reported to be minimal or absent, but the number of patients was too small to allow firm conclusions.[7,8]

We report a prospective molecular cytogenetic study of 45 patients with RTS, four of whom were found to have a deletion. Three patients with a deletion displayed traits or distinctive malformation patterns which are unusual in RTS, including death in infancy, polysplenia and the so-called hypoplastic left heart 'syndrome' (which *sensu stricto* represents a morphogenetic sequence).

Materials and Methods

Subjects

Forty-five unrelated patients (23 female, 22 male) with the clinical diagnosis or the strong suspicion of RTS were evaluated in a prospective molecular cytogenetic study. Additionally we studied the parents of Patients A, B and C with a deletion and performed a prenatal diagnosis (of Patient C).

Six patients were known to the Institute of Clinical Genetics in Dresden and the remaining were referred from 23 different institutions in Germany (38 patients), the Czech Republic (2), Austria (2) and Turkey (3). Clinical information was obtained from referring colleagues. Age was 0–3 years in 17 patients and 4–33 years in 28 patients. In 28 patients the diagnosis at referral was 'strong suspicion of RTS' mostly in younger children, acknowledging the fact that the full form evolves with age,[9,10] and in some cases with unusual additional signs. However, all patients had RTS according to the criteria defined by Hennekam *et al*[9] and were assigned to the study group by one of the authors (OB) after rigorous examination. Several cases in whom the diagnosis was doubtful were presented to the co-authors (GKH, FM) for their opinion. If the diagnosis remained doubtful, the patient was not included in this study.

Cytogenetic Studies

All patients except newborns had a normal karyotype from blood prior to inclusion in this study. For newborns and patients with a karyotype of < 400 bands resolution a chromosome analysis of cultured peripheral blood lymphocytes after GTG banding at > 450 bands resolution was performed during the study. All results were normal.

Molecular Cytogenetic Studies (FISH)

Probes Probes included Chromoprobe-T16p (Cytocell Ltd.),[11] cosmid clones RT100, RT191 and RT203,[12] YAC clones 927a08 and 885f04,[13] and plasmid clone pHUR195 (succession from 16pter to q11.2). Except for Chromoprobe-T16p each probe was used with 20 normal controls. No variability of hybridisation to normal chromosomes 16 and no hybridisation signals on other chromosomes than 16 were observed. Chromoprobe-T16p is a specific probe for the 16p telomere.[11] RT100, RT191 and RT203 represent the *CBP* gene.[12] Extensive sequencing data on *CBP* have recently become available indicating a genomic length of 159 kb, a 8694 bp mRNA (accession No U47741)[14] with cen→tel transcriptional orientation and a 7329 bp cDNA encoding a protein of 2442 amino acids.[12] From the literature[2,12] and sequence data (AC004760, AC004651, AC004509, AC005564)[14] we determined that RT100 (D16S237) spans 45 kb of the 3'-region of CBP, includes cosmid RT1 used elsewhere,[1,3,4,6,7] corresponds to AC004651 and the proximal segment of AC004760 and represents codons 822–2443 (exons 14–31). RT191 (AC004509) maps 10 kb centromeric to RT100 and spans 39595 bp including codons 267–821 (exons 3–13) and the CREB binding domain.[12,14] The 36-kb clone RT203 maps centromeric to RT191 and represents a segment of the large intron between exon 2 (codons 29–266, on AC005564) and exon 3 (codons 267–325, on AC004509).[12,14] The 120-kb YAC 927a08 includes STS marker D16S423, and the 1690-kb YAC 885f04 includes markers D16S779, D16S506, D16S509 and D16S418. Because of problematic positioning of the YACs with the CEPH data[13] we used the LDB database (Table 2) which additionally enabled a rough estimate of between-probe distances, placing Chromoprobe-T16p at approximately 2 Mb telomeric of CBP and YACs 927a08 and 885f04, respectively, at 0.5 Mb and 1 Mb centromeric of CBP.[15] pHUR195 is a plasmid containing repetitive satellite II-DNA (D16Z4) specific for chromosome 16q11.2 and was co-hybridised in some experiments to facilitate the identification of chromosomes 16.

Probe Amplification and Labelling Cosmid probes were amplified using a degenerate oligonucleotide primed (DOP) PCR protocol.[16] The reaction mix without template and primer was pre-incubated with DNAse I. After DNAse inactivation (90°C, 10 min) the PCR was performed using a Taq/Pwo polymerase (Expand HF™, Boehringer Mannheim), eight unspecific amplification cycles (annealing temperature 25°C) with the degenerate primer (5'-CAGGAGGTGGTCGTCATCAGNNNNNNNAGGT-3') and 23 cycles (annealing temperature 60°C) with the truncated primer (5'-CAGGAGGTGGTCGTCATCAG-3'). YAC DNA was amplified by a different DOP-PCR protocol.[17] PCR products were labelled using tetramethylrhodamin-6-dUTP, Taq polymerase (Hybaid-AGS, Heidelberg, Germany) and 20 PCR cycles. Products were cut with DNAse I to a length of 100–500 bp and mixed with $5\times$ (w/w) Cot-1 DNA (Gibco-BRL). pHUR195 was grown in *E. coli* and labelled using nick translation. Labelled probes were purified by ethanol precipitation, dissolved in $1\times$ TE and Hybrisol VI (Oncor). Each lot of probe produced by DOP-PCR was tested on normal controls prior to use with patients.

FISH Probes were hybridised to metaphase spreads using standard FISH protocols with DAPI staining. Microscopy was performed with Axiophot™ epifluorescence microscopes (Carl Zeiss, Jena, Germany) and the ISIS™ digital imaging system (MetaSystems, Altlussheim, Germany). All individuals (45 patients, 7 relatives) were studied with probes RT100 and RT203. Twenty-four patients including Patients A–D and their relatives were additionally studied with probe RT191. The flanking probes (Chromoprobe-T16p, YAC 885f04) were

used with Patients A–D. YAC 927a08 produced poor hybridisation signals at our detection limit but enabled results for Patients C and D.

Results

Clinical Findings of Patients with a Deletion

Patient A 46,XX.ish del(16)(p13.3p13.3)(Chromoprobe-T16p+, CBP/RT100–, CBP/RT191–, CBP/RT203–, D16S776/D16S506/D16S418+) *de novo*.

This patient (Figures 1a,b) was born after a normal pregnancy to a 26-year-old I-para. Clinical signs (Table 1) included a birth length of 47 cm, APGAR 10/10, bushy eyebrows, a facial naevus flammaeus, simian crease on the right, hypertrichosis of the back and shoulders and a 3/6 heart murmur. Chest X-rays, cardiac sonography and catheterisation indicated severe hypoplastic left heart. At 2 weeks she decompensated and was admitted to the intensive care unit. At age 4 weeks her cardiorespiratory situation deteriorated and FISH studies were requested. Surgical intervention was not undertaken because of her poor condition. At 35 days she died of necrotising enterocolitis. Autopsy confirmed the hypoplastic left heart with cardiomegaly, left ventricular hypoplasia, endocardial fibroelastosis, atretic mitral and aortic valves, massive dilatation of right ventricle and truncus pulmonalis, persistent Botallo's duct and hypoplastic aorta ascendens. There were six right and three left pulmonary lobes and two small accessory spleens. On the right, the kidney, the uterine horn and the umbilical artery were absent; weight was 3000 g, length 47 cm, OFC 32.2 cm.

Patient B 46,XY.ish del(16)(p13.3p13.3)(Chromoprobe-T16p+, CBP/RT100–, CBP/RT191–, CBP/RT203–, D16S776/D16S506/D16S418+) *de novo*.

This first child (Figure 1c) of a healthy 24-year-old mother and non-consanguineous father was delivered by Caesarean section after foetal distress; length 45 cm, APGAR 5/6. Clinical signs (Table 1) included highly arched eyebrows, malrotated malformed ears, a long philtrum, a small mouth, bilateral simian creases and penoscrotal hypospadias (Figure 1d). Neonatal convulsions (multifocal hypsarrythmia) were treated with phenobarbital. The patient's Botallo's duct was ligated on day 7. He had frequent episodes of apnoea and gastro-oesophageal reflux, bronchopulmonary dysplasia and multiple infections including urosepsis and chronic tracheobronchitis. Fundoscopy indicated bilateral optic coloboma, excavated optic discs and myopia. Gastroscopy showed a wide open cardiac orifice. Length, weight and OFC fell below the 3rd centile. Development was severely delayed. FISH studies were requested at age 10 weeks. At 7 months he died with pneumonia, pneumothorax and multiple organ failure.

Patient C 46,XX.ish del(16)(p13.3p13.3)(Chromoprobe-T16p+, CBP/RT100–, CBP/RT191–, CBP/RT203–, D16S423+, D16S776/D16S506/D16S418+) *de novo*.

This patient (Figure 1e was the first child of a healthy unrelated couple of tall stature (mother 178 cm, father 202 cm), length at birth 54 cm (+1.7 SD), OFC 30.5 cm (–3.2 SD). At 7 weeks she was admitted to the hospital because of poor feeding. Findings (Table 1) included a large fontanelle, a frontal naevus flammaeus, posteriorly rotated ears, a small preauricular tag and a fistula on the right, small mouth, lumbar naevus flammaeus and sacral hairy patch, postaxial polydactyly of the feet (Figure 1f) and hypoplastic toenails. At 16 months development was delayed. She was able to sit, and stood and walked with support. Height was 78.8 cm (0 SD), weight 10 kg, OFC 42.5 cm (–3.5 SD). At age 6 months her mother was pregnant again; prenatal diagnosis by FISH was normal and the newborn was healthy.

Patient D 46,XY.ish del(16)(p13.3p13.3)(Chromoprobe-T16p+, CBP/RT100–, CBP/RT191+, CBP/RT203+, D16S423+, D16S776/D16S506/D16S418+).

This Turkish boy (Figure 1g) had RTS without major malformations. At 14 months he presented with failure to thrive and developmental delay, unable to sit without support. Findings (Table 1) included an open fontanelle (3 × 3 cm), a capillary haemangioma on the forehead, radial angulation (Figure 1h), umbilical hernia and cryptorchidism. Cranial CT indicated marked cerebral and cerebellar hypotrophy. Sonography showed an accessory spleen and caliectasis of the left kidney. The echocardiogram was normal. Height was 70 cm (–3 SD), weight 7000 g (–3 SD), OFC 39.5 cm (–6 SD). FISH was performed at the age of 3 years. He lived in the family and was well.

FISH

FISH indicated a deletion (Figure 2, Table 2) in Patients A–D and normal findings in their relatives and the other 41 patients. Patients A–C had been referred with the strong suspicion of RTS and were found to have a deletion of RT100, RT191 and RT203 (minimum

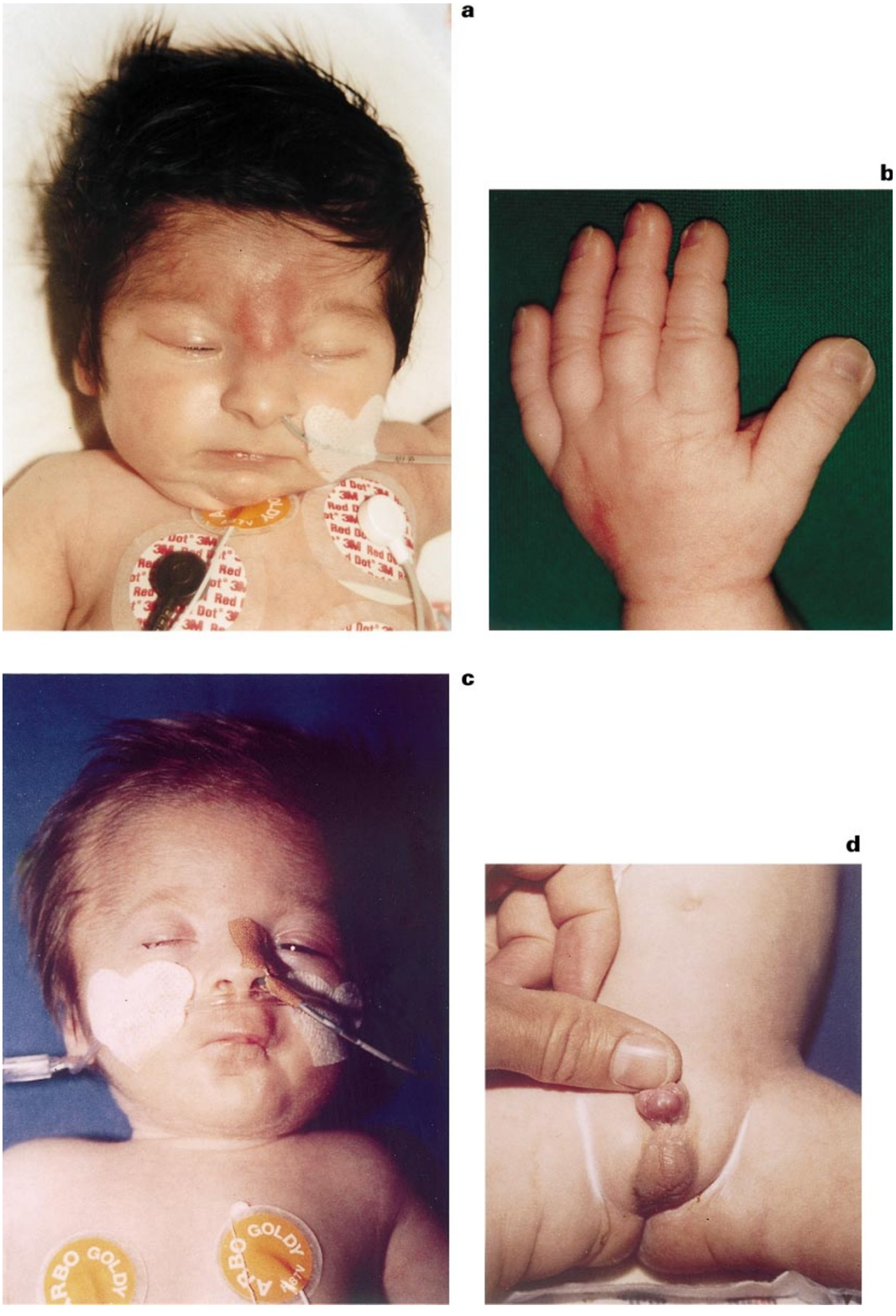

Figure 1 a,b: *Patient A at 3 weeks;* **c,d:** *Patient B at 6 weeks, note the opening of the urethra at the base of the phallus;* **e:** *Patient C at 7 weeks;* **f:** *radiogram of Patient C showing postaxial polydactyly of the right foot;* **g,h:** *Patient D at 14 months*

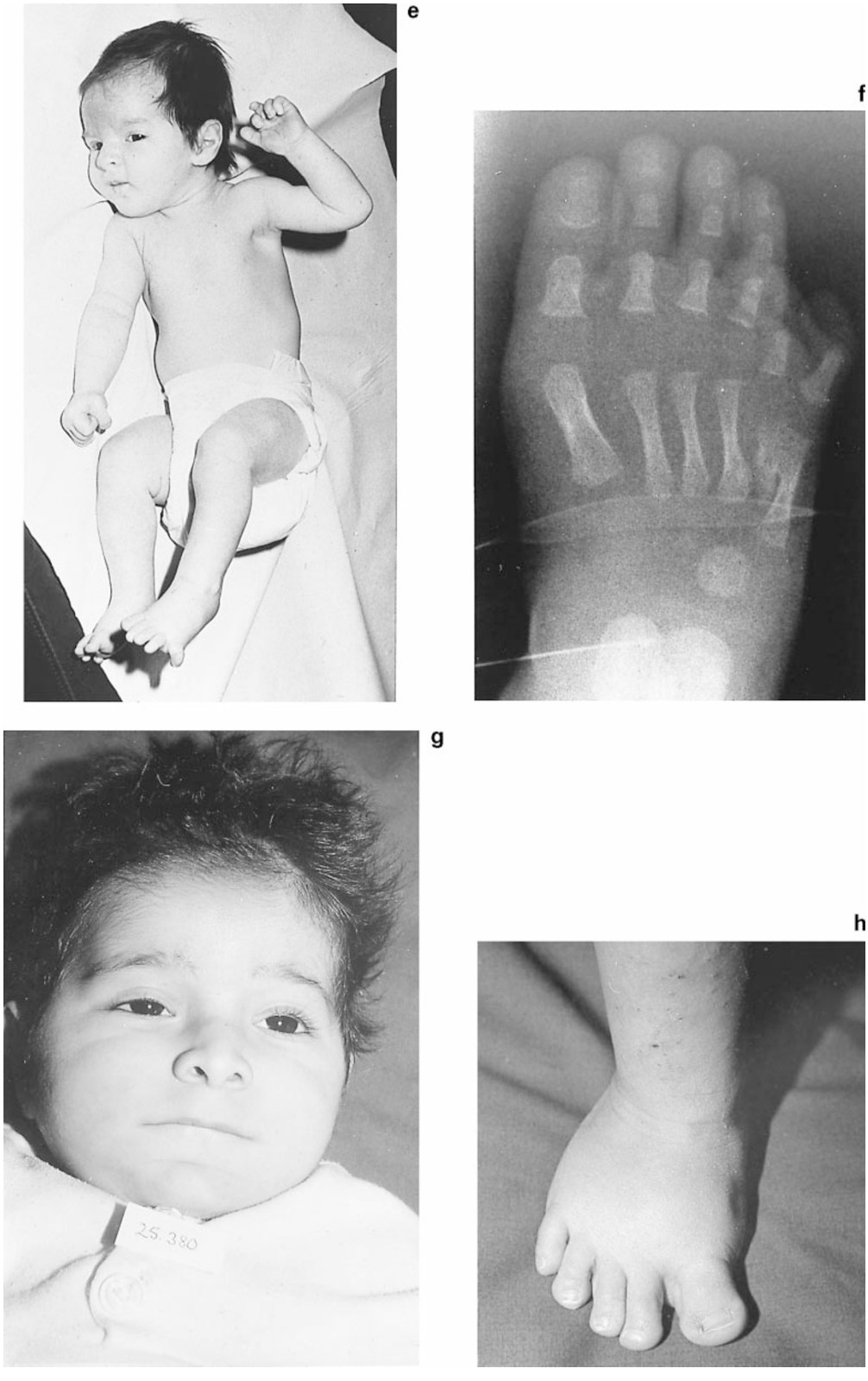

Figure 1 *continued*

deleted area, *CBP* codons 267–2443) and possibly including proximal and distal neighbouring genes of CBP. Patient D, referred with the diagnosis of RTS, had a deletion of only RT100 (codons 822–2443) indicating a breakpoint within the *CBP* gene. This deletion most likely truncated the CBP protein (expected length: 821 amino acids) and possibly included distal (but not proximal) neighbouring genes of *CBP*. No deletion of flanking probes was found in Patients A–D (Table 2), indicating that deletions were interstitial (not terminal) and most likely *de novo*. Findings with RT100, RT191 and RT203, respectively, in the parents of Patients A–C were normal providing further evidence that the deletions occurred *de novo*.

Discussion

In this molecular cytogenetic study of 45 patients with Rubinstein-Taybi syndrome we used two (RT100, RT203) or three (additionally RT191) probes for the *CBP* gene and flanking probes. Four patients (8.9%) were found to have a deletion; three deletions most likely spanned CBP (Patients A–C) and one deletion truncated the gene (Patient D). Interestingly, Patients A–C were referred with a tentative diagnosis of RTS ('strong suspicion') because of young age (< 1 year) and unusual clinical signs (hypoplastic left heart). By pooling data from this series and previous studies the cumulative frequency of the 16p13.3 microdeletions is 11% (24 of 219).[1,3–7]

Most previous FISH studies of RTS used only one probe (RT1).[1,3,4,6,7] Our use of different *CBP* probes in this study did not increase the frequency of detected deletions because all deletions found included RT100 which is very similar to RT1.[12] For future FISH studies we would recommend using several cosmids for the *CBP* gene, eg RT100 (exons 14–31), RT191 (exons 3–13) and possibly 420F6 (exon 2) or 304A10 (exon 1), as well as a panel of closely flanking probes (preferably cosmids) to enable the detection of partial deletions of the gene and accurately determine the extent of the

Table 1 Clinical findings of patients with 16p13 deletions

	Patient A	*Patient B*	*Patient C*	*Patient D*	*Literature*[18]
Gender	F	M	F	M	46% F, 54% M
Gestational age at birth	40 weeks	35 weeks	40 weeks	38 weeks	
Signs of maturity	36 weeks	31 weeks			
Birthweight	2640 g (below –2 SD)	1900 g (–1.5 SD)	3030 g (–0.5 SD)	2400 g (below –2 SD)	
Age and status at last visit	died at 35 days	died at 7 months	well at 16 months	well at 3 years	
Microcephaly	+	+	+	+	95%
Facial hypertrichosis, dense hair	+	+	+	+	
Wide-spaced and down-slanting palpebral fissures	+	+	–	+	90%
Beaked or straight nose	+	+	+	+	93%
Nasal columella below alae	+	+	+	+	78%
Narrow or highly arched palate	+	+ and hypoplastic maxilla	+	not known	93%
Receding chin	+	+	–	+	75%
Broad thumbs or halluces	+	+	+	+	100%
Congenital heart defect	hypoplastic left heart, persistence of Botallo's duct	persistence of Botallo's duct, of left upper vena cavae and of foramen ovale	–	–	34%
Abnormal pulmonary lobes	+	–	–	not known	
Abnormal umbilical vessels	+	–	–	not known	
Accessory spleen	+	–	–	+	
Kidney abnormality	agenesis of right kidney	–	–	caliectasis of left kidney	52%
Hypospadias/abnormal uterus	+	+	–	–	
Feeding problems, failure to thrive	+	+	+	+	77%
Muscular hypotonia, developmental retardation	+	+	+	+	99%

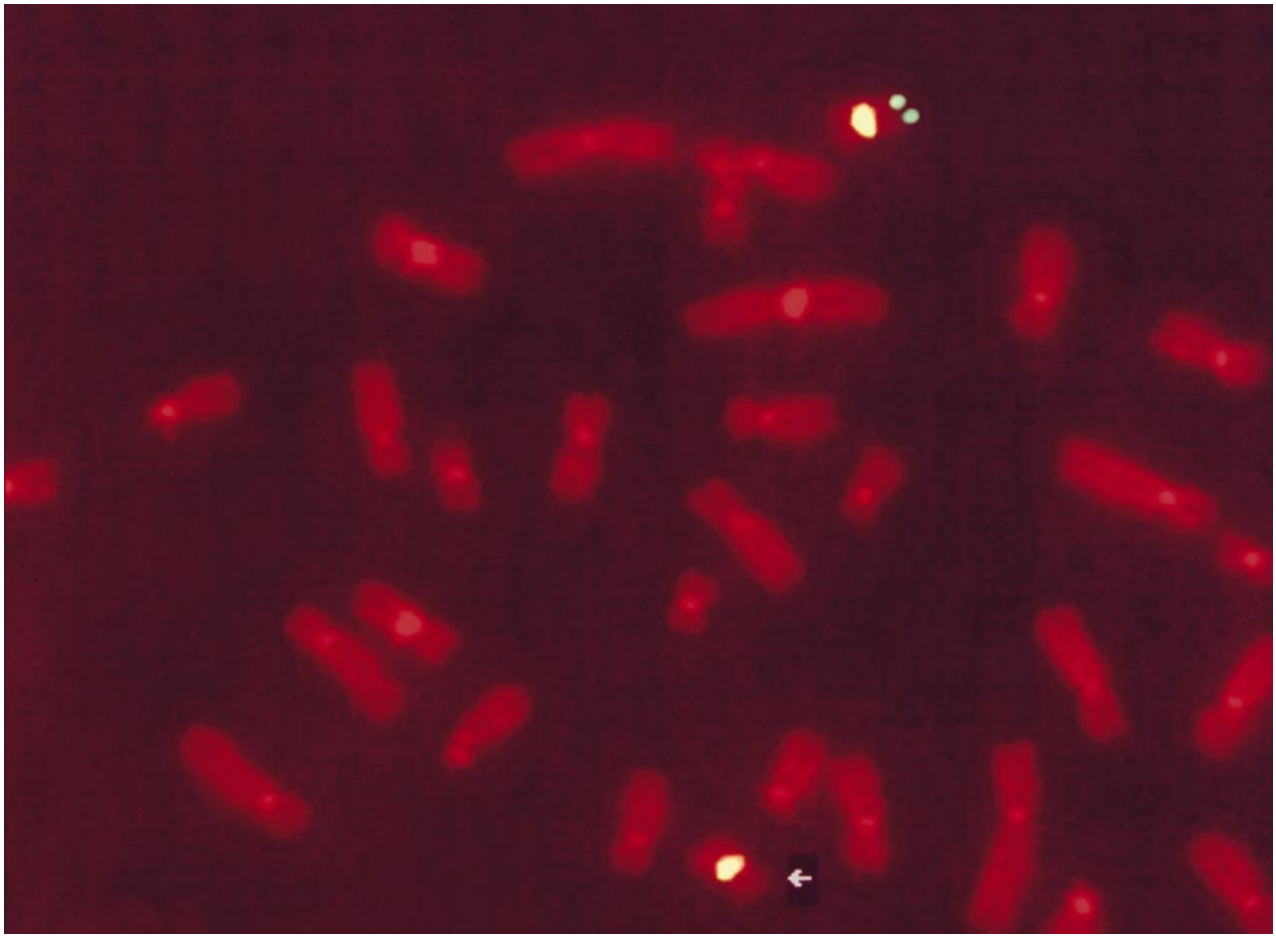

Figure 2 *Metaphase from Patient A hybridised with RT100 (3'-end of* CBP*, 16p13.3) and control probe pHUR195 (D16Z4, 16q11.2) showing the deletion at 16p13.3 (arrow) and a normal chromosome 16*

deletions. To date at least four cases of partial deletion of the *CBP* gene have been reported, 3 of the 3'-end (this study)[1,2] and 1 of the 5'-region.[5] The deletion of Patient D (RT100, codons 822–2443) most likely resulted in a truncated CBP protein (possibly amino acids 1–821). The molecular mutations documented by Petrij *et al*[2] had been C-to-T transitions changing CAG (gln) to TAG (stop) in codons 136 and 357, respectively. It is an irresistible hypothesis that the 10-kb unstable area between exons 2 and 3 (breakpoint cluster region,

Table 2 Deletions of 16p13.3 identified by FISH

Probe	*Chromosome*	*Gene/Marker*	*Distance from 16pter*[15] *comp / mcM / fcM*	*Patient A*	*Patient B*	*Patient C*	*Patient D*
Chromoprobe-T16p	16p13.3-pter	telomere 16p	0.000 / 0.00 / 0.00	+	+	+	+
RT100	16p13.3	CBP (codons 822–2443, exons 14–31) D16S237	3.439 / 16.19 / 8.33	–	–	–	–
RT191	16p13.3	CBP (codons 267–821, exons 3–13)	3.439 / 16.19 / 8.33	–	–	–	+
RT203	16p13.3	CBP (intron between exons 2 and 3)	3.439 / 16.19 / 8.33	–	–	–	+
YAC 927a08	16p13.3	D16S423	3.800 / 18.46 / 9.08	no results	no results	+	+
YAC 885f04	16p13.3	D16S776	4.158	+	+	+	+
		D16S506	4.260 / 23.68 / 11.85				
		D16S509	4.321 / 25.13 / 11.85				
		D16S418	4.418 / 28.68 / 11.85				

+: normal findings; –: deleted on one chromosome 16

BCR)[12] plays a role in the generation of the chromosomal deletions. Results from Patients A–C are compatible with a breakpoint within the BCR area but Patient D clearly has different breakpoints.

A previous study demonstrated by flanking markers that all six deletions were located between cosmid 26 (PKD1/D16S125, telomeric) and cosmid N2 (D16S138, centromeric).[1] In our study all deletions fell into the area between Chromoprobe-T16p (16p telomere) and YAC 885f04 (D16S776, centromeric). Thus to date all 16p deletions in RTS patients studied with distal flanking probes were found to be interstitial (10 out of 10 cases). The absence of familial subtelomeric translocations is in striking contrast with the findings in other microdeletion syndromes (Wolf-Hirschhorn, Cri du chat or Miller-Dieker syndrome), supporting the very low recurrence risk of RTS in sibs and the hypothesis that monosomy of large segments of chromosome 16p13.3 may be lethal because of critical gene(s) between *CBP* and the 16p telomere.[1,9]

Interestingly, RTS patients with a deletion were younger at presentation ($n = 4$, mean age 0.96 years) than patients without a deletion ($n = 41$, mean age 11.12 years). Wallerstein *et al*[6] found a similar trend of 4.6 years mean age in patients with a deletion ($n = 7$) and 11.7 years in other patients ($n = 57$). These age differences may correspond to differences of phenotypes. In our series, two out of four patients with a deletion died during the observation period, whereas no patient without a deletion died. Early death is uncommon in RTS.[18] Moreover, some 16p13.3 deletions in RTS patients may exceed 650 kb in length.[2] Given the genomic length of CBP of 159 kb[12] and the estimated average density of 1 gene per 50 kb in the human genome (60 000 genes/3000 Mb), it appears likely that some RTS patients with a 16p13.3 deletion have a contiguous gene syndrome. This additional genetic imbalance may possibly result in additional malformations, reduced fitness, shorter life expectancy or a younger age at diagnosis than in RTS patients with a molecular mutation of *CBP*.

To the best of our knowledge, Patient A represents the first case of hypoplastic left heart (HLH) and 16p13.3 deletion. Congenital heart defects, usually patent Botallo's duct or pulmonary valve stenosis and less frequently ventricular or atrial septal defects or coarctation of aorta, are well known in RTS and occur in 17–38% of patients.[8,18,19] But the combinations of RTS and HLH (Patient A) is unusual and was previously described in only one patient not studied by FISH.[20] HLH is a rare heart defect ranging in severity from aortic arch hypoplasia with left ventricular underdevelopment to the severe form with rudimentary left ventricle and atretic valves (Patient A).[21] The genetic basis is largely unknown; isolated HLH occurs with recessive inheritance, and syndromic HLH with dominant (Apert and Holt-Oram syndromes), recessive (short rib–polydactyly syndrome type 3, Ellis-van Creveld syndrome) and chromosomal disorders (monosomy 2q, 4p, 4q, 11q, 22q11, X; trisomy 12p, 13, 16q, 18, 21).[22]

The polysplenia of Patients A and D is even more interesting. Polysplenia has not been described with RTS previously, but RTS is a multisystem disorder characterised by abnormal patterns in embryogenesis and thus may include polysplenia. The polysplenia and distinctive malformations of Patient A is a reminder of Opitz's polyasplenia concept ('midline developmental field defect')[23,24] and may represent a newly recognised pattern of defective laterality in RTS (possibly including accessory spleens, HLH, abnormal pulmonary lobulation, renal agenesis and absence of uterine horn). Alternatively the disturbance of laterality and/or the severe clinical course of some patients with a deletion may represent a new contiguous gene syndrome.

Drosophila CBP is a co-activator of *cubitus interruptus*, a component of the hedgehog signalling pathway, and a prerequisite for *dorsal*-dependent *twist* gene expression.[25,26] These genetic pathways of embryogenesis have been implicated in the formation of some of the patterns of the RTS phenotype.[7,26] Recently a mouse model of RTS with a truncated CBP protein (amino acids 1–1084) and classic signs of RTS in the $CBP^{+/-}$ mice was established which may become a powerful tool for studying the role of *CBP* in embryonic development.[27]

Our results emphasise the need for refined FISH studies in a larger number of RTS patients with a deletion, and of complementary molecular investigations in RTS patients without a deletion by FISH.

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