Available online at www.sciencedirect.com
ScienceDirect
Genomics 90 (2007) 567–573
GENOMICS
www.elsevier.com/locate/ygeno

High frequency of mosaic *CREBBP* deletions in Rubinstein–Taybi syndrome patients and mapping of somatic and germ-line breakpoints

Cristina Gervasini [a], Paola Castronovo [a], Angela Bentivegna [a], Federica Mottadelli [a], Francesca Faravelli [b], Maria Luisa Giovannucci-Uzielli [c], Alice Pessagno [d], Emanuela Lucci-Cordisco [e], Anna Maria Pinto [e], Leonardo Salviati [f], Angelo Selicorni [g], Romano Tenconi [f], Giovanni Neri [e], Lidia Larizza [a,*]

[a] *Division of Medical Genetics, San Paolo School of Medicine, University of Milan, 20142 Milan, Italy*
[b] *Department of Human Genetics of Galliera Hospital, Genoa, Italy*
[c] *Department of Paediatrics—Genetics Unit, Children's Hospital Meyer, Florence, Italy*
[d] *Child Neuropsychiatry, Istituto G. Gaslini, Genoa, Italy*
[e] *Insitute of Medical Genetics, Catholic University, Rome, Italy*
[f] *Clinical Genetics and Epidemiology, Department of Pediatrics, University of Padua, Padua, Italy*
[g] *I Clinica Pediatrica, Fondazione Policlinico Mangiagalli Regina Elena, Milan, Italy*

Received 6 April 2007; accepted 23 July 2007
Available online 12 September 2007

Abstract

Rubinstein–Taybi syndrome (RSTS) is a rare malformation disorder caused by mutations in the closely related *CREBBP* and *EP300* genes, accounting respectively for up to 60 and 3% of cases. About 10% of *CREBBP* mutations are whole gene deletions often extending into flanking regions. Using FISH and microsatellite analyses as a first step in the *CREBBP* mutation screening of 42 Italian RSTS patients, we identified six deletions, three of which were in a mosaic condition that has not been previously reported in RSTS. The use of region-specific BAC clones and small *CREBBP* probes allowed us to assess the extent of all of the deletions by mapping their endpoints to genomic intervals of 5–10 kb. Four of our five intragenic breakpoints cluster at the 5′ end of *CREBBP*, where there is a peak of breakpoints underlying rearrangements in RSTS patients and tumors. The search for genomic motifs did not reveal any low-copy repeats (LCRs) or any greater density of repetitive sequences. In contrast, the percentage of interspersed repetitive elements (mainly *Alu* and LINEs in the *CREBBP* exon 2 region) is significantly higher than that in the entire gene or the average in the genome, thus suggesting that this characteristic may be involved in the region's vulnerability to breaking and nonhomologous pairing. The FISH analysis extended to the *EP300* genomic region did not reveal any deletions. The clinical presentation was typical in all cases, but more severe in the three patients carrying constitutional deletions, raising a question about the possible underdiagnosis of a few cases of mild RSTS.

Keywords: Rubinstein–Taybi syndrome; Microdeletion; Mosaicism; Breakpoint mapping

Rubinstein–Taybi syndrome (RSTS; MIM 180849) is an autosomal dominant disease that occurs in 1 of 125,000 births and is characterized by growth and psychomotor development delay, broad and duplicated distal phalanges of thumbs and halluces, typical facial dysmorphisms, and an increased risk of tumors [1].

The etiology of RSTS was first revealed by the presence of cytogenetic anomalies detected in a few patients, all involving chromosome band 16p13.3 [2–4]. Subsequent fluorescence in situ hybridization (FISH) studies using a panel of probes encompassing 16p13.3 showed that most patients carry microdeletions involving the CREB-binding protein gene, *CREBBP*, commonly referred to by its shorter acronym *CBP*

* Corresponding author. Fax: +39 2 50323026.
E-mail address: lidia.larizza@unimi.it (L. Larizza).

0888-7543/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2007.07.012

(MIM 600140) [5–7], which paved the way to the targeted screening of *CBP*.

Alterations in the *CREBBP* gene, including heterozygous point mutations and deletions, have been identified in 56–61% of RSTS patients [6,8–10], with deletions representing about 10% of the lesions. All of the >60 *CREBBP* microdeletions so far reported were de novo and, when parental origin could be investigated, no preferential maternal or paternal contribution of the affected chromosome 16 was observed. Various techniques have been applied, including FISH [7,11], real-time quantitative PCR [12], and multiplex ligation-dependent probe amplification, which refined mainly the point mutation repertoire by adding small intragenic deletions [13].

When the deletion breakpoints were defined by FISH mapping, a great variety of deletion ends was observed, including breakpoints inside or outside the *CREBBP* gene or one break inside and the other jutting beyond the 3′ or 5′ end [7,11,12]. No genomic motifs were revealed that might prime nonallelic homologous recombination (NAHR) recurrent deletions associated with a more severe *CREBBP*-related phenotype than that due to inactivating point mutations. With a few exceptions [11], clinical features are slightly more severe in patients with deletions [14], thus making it unlikely to define an RSTS contiguous gene syndrome characterized by a specific phenotypic spectrum, as shown by most *CREBBP* microdeleted patients.

No *CREBBP* mosaic deletions have yet been reported, although numerous somatic rearrangements (mainly translocations disrupting *CREBBP*) have been described in M4/M5 subtypes of acute myelogenous leukemia [6,15–21]. Both germ-line and somatic rearrangements affecting *CREBBP* seem to determine the loss or impairment of the multifunctional *CREBBP* protein, although the rearrangement leads to a chimeric gene as in leukemias.

During a search for other genes whose disruption might account for *CREBBP*-negative RSTS, a small subset (3%) of the tested patients was found to carry mutations in the *CREBBP*-related *EP300* gene [22]. Refined evaluation of the clinical presentation of the three identified *EP300*-mutated patients (and an additional case) revealed much milder hand and feet skeletal findings than those typically observed in *CREBBP*-mutated RSTS patients [23]. No *EP300* deletions have yet been reported in RSTS patients with a classic or mild phenotype.

We here describe six *CREBBP* microdeletions identified in Italian RSTS patients screened for *CREBBP* point mutations in parallel or previously [9]. FISH with BACs and small BAC-derived probes allowed the precise mapping of the 12 underlying breakpoints to 11 different genomic intervals, thus providing a detailed genomic view of nonrecurrent *CREBBP* deletions. Interestingly, three of the six deletions were in a mosaic condition, a novel finding in microdeleted RSTS patients. Despite the small number of deleted cells in the investigated tissues, the clinical phenotype was quite typical, thus emphasizing the dose sensitivity of *CREBBP*. We also sought *EP300* deletions in the *CREBBP*-negative patients and confirm the limited role of the second highly homologous gene in the etiology of RSTS.

Results

We report the genomic characterization of six deletions affecting the *CREBBP* gene and flanking regions, which were identified in a cohort of 42 Italian RSTS patients who underwent FISH screening as a first step in *CREBBP* mutation testing. The recruited patients then entered the *CREBBP* point mutation scan, which led to the confirmation or exclusion of *CREBBP* involvement, as previously described [9].

By genotyping the polymorphic intragenic repeats MS4, MS2, and D16S3065 (plus one SNP), we identified two deletions encompassing all or almost all of the *CREBBP* gene in two patients, respectively (patients 30 and 41) (Fig. 1), and FISH analysis using region-specific BAC clones and small *CREBBP* probes allowed us to assess the extent and approximate boundaries of both deletions. The deletion of patient 30 encompasses a 150-kb region between *CREBBP* exons 2 and 31, and that of patient 41 (about 500 kb in size) extends beyond the 5′ and 3′ ends of the gene (Fig. 1). A third deletion encompassing the whole *CREBBP* gene with very large (2.6 Mb) involvement beyond the 5′ end was identified in patient 82 by FISH analysis. All three deletions affect the maternal allele, as assessed by segregation analyses of region-specific microsatellites (data not shown).

FISH analysis also enabled the detection of three additional deletions, all in the mosaic condition (Figs. 1 and 2): two affect the 5′ end of the gene, with a span of 335 kb in patient 40 and 275 kb in patient 66; the third, which includes a significant portion of the gene, extends for 720 kb to the 3′ flanking region (patient 38) (Fig. 1). In all cases, the lymphocytes contained ≤30% deleted cells (Fig. 2). In the case of two patients (40 and 66), FISH analyses were also made of buccal smears, which allowed the scoring of almost 100 nuclei. The average percentage of deleted cells in the epithelial cell lineage was less (20%) than in the circulating lymphocytes (Fig. 2), thus confirming low-level mosaicism. The size of the identified deletions varied widely from 150 kb (patient 30) to 2.6 Mb (patient 82) (Fig. 1). FISH experiments using locus-specific probes allowed us to delimit the region breakpoints to genomic intervals of 5–10 kb. All 12 breakpoints are private, except for the telomeric breakpoints of patients 41 and 82, which map to the same interval. Three breakpoints are located in a 500-kb region telomeric to *CREBBP*, 4 are in a 2.2-Mb region centromeric to it, and 5 are embedded in the *CREBBP* gene (Fig. 1). Of the 5 intragenic breakpoints 4 (3 involved in the mosaic deletions) cluster to a small interval around exon 2 at the 5′ end of the gene.

We reviewed the literature concerning the *CREBBP* mapping of breakpoints underlying germ-line deletions and balanced rearrangements in RSTS patients, as well as somatic rearrangements (mainly translocations involved in tumors). Supplemental Fig. 1 shows a magnification of the genomic structure of *CREBBP* with an overview of the germ-line (top) and somatic breakpoints (middle), all of which are listed and referenced in Supplementary Table 1. The internal *CREBBP* breakpoints shown in Supplemental Fig. 1 constitute about half of the reported deletion breakpoints with defined boundaries [7,11,12]. The predominant mapping of breakpoints to the

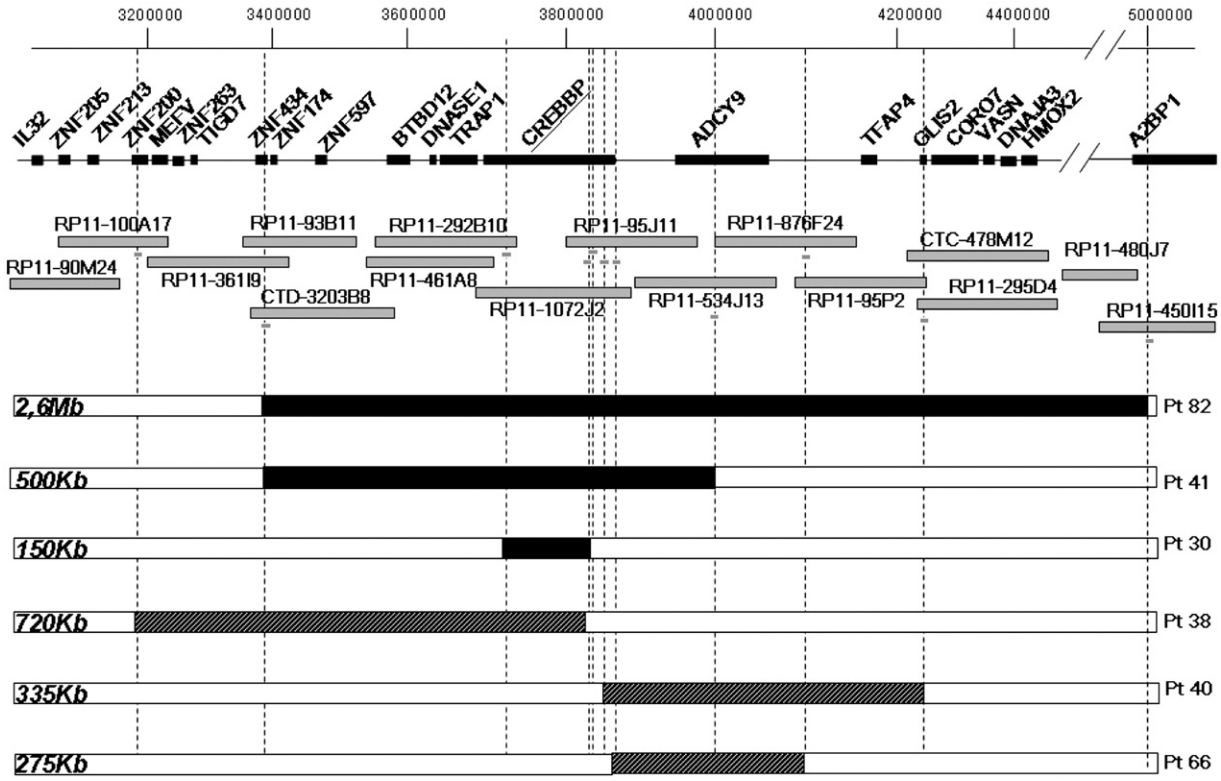

Fig. 1. Mapping of six *CREBBP* deletions to the *CREBBP* genomic region. (Top) Reference map of UCSC Web site (www.genome.ucsc.edu) with known genes (in black) and *CREBBP* underlined. The base distances from tel to cen are indicated left to right. The BAC clones used in the FISH experiments are represented by gray bars aligned under the map. The small gray boxes below the bars indicate the locus-specific probes generated by the BACs and used to narrow the breakpoint FISH mapping. (Bottom) Schematic diagram of the deletions represented by bars aligned below the physical map. The bars are black for the three constitutional deletions (top down by decreasing size) and filled with diagonal lines for the mosaic deletions (top down by decreasing size). The dashed vertical lines indicate the deletion breakpoints. The size of each deletion is shown on the left, and the patient code on the right.

Pt	Lymphocytes	Buccal cells
66	22%	21%
40	20%	17%
38	30%	/

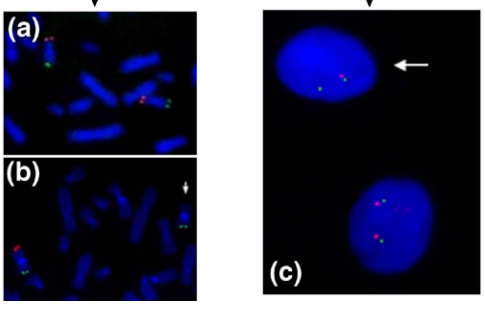

Fig. 2. Representative FISH results relating to the patients carrying *CREBBP* mosaic deletions. (Top) Table showing the percentages of deleted cells from lymphocytes and buccal cells, where available. (Bottom) FISH experiments on lymphocyte metaphases from patient 66 (a, b) and buccal cell nuclei from patient 66 (c) using BAC clone RP11-534j13 and reference subtelomeric 16q probe BAC clone RP11-566K11. The arrows point to the deleted chromosome 16 and nucleus.

CREBBP portion around exon 2 extended bidirectionally to IVS1 and IVS2 (enframed in all of the panels of Supplemental Fig. 1) is observed in meiotic rearrangements, whereas there is an almost exclusive clustering of mitotic breakpoints in this critical area [15,17,19,20].

We performed an in silico analysis of the sequence of the delimited region searching for specific DNA sequence motifs (palindromic elements, recombination-specific sites, LCRs) that might be associated with the rearrangements, but none was detected. Conversely, the breakpoint cluster region around exon 2 was marked by a crowding of repetitive elements, SINEs, LINEs, LTRs, and DNA elements of the Mer type. In particular, the percentage of LINEs (20%) was significantly higher than their percentage in the entire gene (8%) and contributed mainly to the overall increase in interspersed sequences in the critical region (56% vs 35% in the entire *CREBBP*) (Supplemental Fig. 1, bottom).

Table 1 summarizes the major and minor clinical signs of the six patients carrying constitutional or mosaic microdeletions, indicating the size, extent, and gene content of the deletion.

Regardless of their specific microdeletion and its constitutional or mosaic occurrence, all of the patients are affected by psychomotor delay and mental retardation and show the typical sign of broad thumbs or great toes. The clinical presentation of the three patients carrying whole gene deletions (patients 30, 41,

Table 1

Germ-line deletions	Patient No.		
	82	41	30
Size	2.6 Mb	500 kb	150 kb
Involved genes (tel–cen order)	ZNF434, ZNF174, ZNF597, BTBD12, *DNASE1*, TRAP1, *CREBBP*, ADCY9, TFAP4, GLIS2, CORO7, VASN, DNAJA3, HMOX, UBN1, PPL, NAGPA, ALG1, A2BP1	ZNF434, ZNF174, ZNF597, BTBD12, *DNASE1*, TRAP1, *CREBBP*, ADCY9	*CREBBP*
Patient gender/age at diagnosis	F/1 day	F/25 years	F/29 years
Facial dysmorphisms			
At birth	Prominent beaked nose, columella below the alae nasi, downslanting palpebral fissures, micrognathia	−	+
At diagnosis		Downslanting palpebral fissures, columella below the alae nasi	Low junction of hair, prominent beaked nose, columella below the alae nasi, micrognathia
Development			
Mental retardation	NA	+	+
Failure to thrive	+	+	+
Feeding difficulty in infancy	+	−	+
PM delay	+	+	+
Microcephaly	−	+	+
Skeletal anomalies			
Broad thumbs or big toes	+	+	+
Other	Highly arched palate	Scoliosis	Clinodactyly
Other	Hypertricosis, forehead hemangioma, laringeal synechia, hypotonia	Forehead hemangioma, myopia/strabism, obesity	Myopia/strabism, obesity, sebacee cysts
Mosaic deletions	38	40	66
Size	720 kb	335 kb	275 kb
Involved genes (tel–cen order)	ZNF205, ZNF213, ZNF200, ZNF263, MEFV, TIGD7, ZNF434, ZNF174, ZNF597, BTBD12, *DNASE1*, TRAP1, *CREBBP*	*CREBBP*, ADCY9, TFAP4, GLIS2, CORO7, VASN, DNAJA3	*CREBBP*, ADCY9
Patient gender/age at diagnosis	M/5 years	F/24 years	M/6 years
Facial dysmorphisms			
At birth	−	−	−
At diagnosis	Prominent beaked nose	Low junction of hair	Prominent beaked nose, micrognathia, columella below the alae nasi
Development			
Mental retardation	+	+	+
Failure to thrive	−	+	+
Feeding difficulty in infancy	+	−	−
PM delay	+	+	+
Microcephaly	−	+	−
Skeletal anomalies			
Broad thumbs or big toes	+	+	+
Other	Clinodactyly, scoliosis		Highly arched palate, clinodactyly
Other	Hypertricosis, disturbance of sleep	Myopia/strabism, hypotonia	Kidney abnormality

All patients have normal karyotype, except for patient 38: 46,XY,der(14)t(9;14)(p11.2;p11.2). NA, not applicable.

and 82) is generally more severe than that of the carriers of mosaic deletions (40, 66, and 38). There was no clear relationship between the size of the deletion and the manifestation of uncommon or atypical RSTS signs, thus making it difficult to attribute these signs to the haploinsufficiency of specific genes.

To establish whether deletion-type mutations might affect the *CREBBP*-related *EP300* gene, 33 patients who were negative for *CREBBP* microdeletions were tested for *EP300* microdeletions using the specific RP11-1078o11 BAC clone. None of these cases lacked the *EP300*-specific FISH signal (data not shown).

Discussion

We carried out a refined FISH characterization of the extent and boundaries of six *CREBBP* microdeletions identified in a cohort of 42 Italian subjects with a clinical diagnosis of RSTS. Possibly due to the mosaic occurrence of three deletions, our deletion rate is higher (14%) than that usually reported (10%).

The identified deletions vary widely in size and position, with only two breakpoints mapping to the same 15-kb interval, and comparison with other *CREBBP* deletions characterized in the literature confirmed the paucity of shared underlying breakpoints [7,11,12]. As breakpoint mapping has been refined on less than half of the approximately 60 described cases, comparison of the number of *CREBBP* deletions is informative but may not be fully representative. However, despite this limitation, the published background information and our genomic characterization using FISH probes, which ensures a resolution up to a few kilobases, concur in excluding a main recurrent deletion in RSTS. Consistent with this evidence in silico studies of the sequence environment of *CREBBP* and its flanking regions showed the lack of any LCR flanked by repetitive or palindromic sequences that might prime NAHR, leading to recurrent deletions. Indeed the overall deletion frequency for the *CREBBP* gene (10%) is similar to that of the neurofibromatosis type 1 gene [24,25], in which LCR-mediated deletions lead to the recurrent *NF1* microdeletion syndrome [26–31]. The described *CREBBP*-deleted patients do not associate with a RSTS microdeletion syndrome. The feature of scattered breaks clearly emerges from the analysis of the RSTS deletions described by us and those in the literature that have undergone breakpoint mapping [7,11,12]. Further characterization of the breakpoints up to the sequence of junction fragments may even differentiate apparently similar breakpoints by expanding their scattering across a critical unstable region. Only one study has so far defined the sequence junctions of two *CREBBP* partial deletions (exons 14–16 and exons 4–26) [13], and this added further evidence of the *CREBBP* genomic region's susceptibility to breakage.

Inspection of the somatic events involving *CREBBP* [17,19] has not revealed any deletions, but did reveal translocations whose *CREBBP* breakpoints confirm a kind of genomic instability characterized by scattered rather than clustered genomic motifs.

One exception to the above, however, is the breakpoint cluster observed in the 5′ region of the gene, as 4 of the 12 breakpoints (including 3 of mosaic deletions) are embedded in a 40-kb sequence between IVS1 and IVS2 (Fig. 2 and Supplemental Fig. 1). This interval includes most of the entire *CREBBP* deletion, inversion and translocation breakpoints of described RSTS patients [1,7,11–13], and most of the breakpoints of the translocations interrupting the *CREBBP* gene in the M4/M5 subtypes of acute myeloid leukemia [15,17,19,20] (Supplemental Fig. 1). There is an increased percentage of different repetitive elements (particularly SINEs and LINEs) in this sequence in comparison with the average percentage in the genome and the entire *CREBBP* gene (as has been pointed out by Coupry et al. [10] and Panagopoulos et al. [17]), but it is the general increased frequency of interspersed repetitive elements rather than that of a single type of element that may make this region a break "hot spot" in terms of both meiotic and mitotic recombination.

Nevertheless, the breakpoints clustered in this hot spot are unique and scattered, as shown by refined studies of the translocations affecting *CREBBP* in leukemia [17–21,32,33]. These studies have provided evidence showing the involvement of *Alu* elements and LINEs in the double-strand breaks, possibly repaired by a nonhomologous end-joining mechanism [17,33]. Interestingly, the involvement of *Alu* and *Alu*–LINEs has been demonstrated for two *CREBBP* partial deletion junctions [13].

Ours is the first description of a relatively high frequency of mosaic deletions (three of six) in patients with RSTS and suggests that mosaicism might be underestimated, as they were all in the group of *CREBBP*-negative cases. The limitations of the technique in detecting mosaic point mutations are known, and the low level of mosaicism scored in the tested tissues can be easily detected only by FISH analyses. Indeed mosaic patient 40, showing in two tissues an average percentage of 18.5% of deleted cells, had been previously tested for segregation from parents of intragenic *CREBBP* microsatellites, not revealing any allele loss.

Our data allow a few comments concerning the general issue of genotype/phenotype correlations. A first point regards the three patients carrying mosaic deletions (patients 38, 40, and 66), who were all referred for *CREBBP* screening because they presented with a recognizable RSTS phenotype. Given the low level of deleted cells in the tissues available for analysis, the dose sensitivity of the *CREBBP* gene is clear, as a mutation in a small portion of cells is sufficient to determine the Rubinstein–Taybi phenotype. The overall phenotype of the mosaic patients was less severe than that of those with constitutional deletions (almost borderline in the case of patient 66), which emphasizes the possibility that mild RSTS cases may be underestimated.

Second, the constitutional deletions ranged in size from 150 kb to 2.6 Mb, with the child carrying the largest deletion not presenting an overtly severe phenotype until at least the age of 1 year. Further cases carrying a deletion covering the same genomic region should be compared to check whether the haploinsufficiency of 18 genes (in addition to *CREBBP*) is responsible for any particular additional sign and can thus be classified as "dose sensitive." The severity of the phenotype of patients with large deletions has been described by Bartsch et al. [11], but our patients did not have similar features. Critical infections manifesting as medical complications in patients with deletions including the *DNASE1* gene were not observed in our completely haploinsufficient patients 82 and 41 or in our partially haploinsufficient patient 38. Nevertheless, as suggested, specific attention should be paid to the risk of critical infections in this subgroup of deleted patients.

These considerations concerning the phenotypes of our patients (particularly that of patient 82, carrying a 2.6-Mb deletion) are not consistent with the definition of a microdeletion RSTS syndrome. The presence of genomic involvement in

a subgroup of deleted RSTS patients is unquestionable, but it is difficult to discern a different and distinguishable phenotype from that of patients carrying a point mutation.

Our patients who were negative for *CREBBP* mutations/deletions were investigated for the putative presence of an *EP300* deletion. Roelfsema et al. [22] extended the screening of 92 RSTS patients who were negative for *CREBBP* point mutations to the *EP300* gene and found three mutations causing RSTS. This very low mutation rate [34] indicates the minor role of *EP300* in this syndrome, but the recently reported mild phenotype of the four currently known *EP300*-mutated patients [23] might be reconsidered to recruit also RSTS group B patients (i.e., those with a borderline phenotype) to check the mutation rate. None of our patients tested for *EP300* deletions showed a rearrangement in this region, possibly because of the specific genomic interval and the absence of motifs promoting genomic breaks. However, as microdeletions usually represent a small subset of all mutations, and only a very small number of *EP300* point mutations have so far been detected, a larger cohort of both typical and mild/borderline RSTS patients should be tested to exclude this kind of rearrangement.

Materials and methods

Subjects and cell lines

Peripheral blood samples were obtained from all six patients after they had given their informed consent. A lymphoblastoid cell line was established in two cases (30 and 41).

Clone preparation

All of the BAC clones were supplied by Mariano Rocchi (Resources for Molecular Cytogenetics, University of Bari, Italy; http://www.biolo%20gia.uniba.it/rmc/) and the Wellcome Trust Sanger Institute Genome Campus (Hinxton, Cambridge, UK) or purchased from Invitrogen S.r.l. or the Chori BAC PAC Resource Center (Oakland, CA, USA). DNA was obtained starting from a single colony grown in 10 ml of LB medium supplemented with 12.5 μg/ml chloramphenicol (Sigma) following standard procedures.

Locus-specific probe preparation

The method, which has been previously described [35], is based on two consecutive reactions using BAC clones containing the loci of interest.

Cell culture, chromosome, and interphase nucleus preparations

Phytohemagglutinin-stimulated peripheral blood lymphocytes were set up in culture from samples using the chromosome kit “Synchro” (Celbio) and modified RPMI (Irvine Scientific) plus 5% fetal calf serum (Gibco). The cultures were stopped with colchicine after 72 h. The chromosome preparations were obtained using a standard technique.

A lymphoblastoid cell line was established from the peripheral blood of patients 30 and 41 using Epstein–Barr virus in accordance with standard procedures by means of a service provided by the Telethon Research Service “Galliera Genetic Bank.”

For the interphase nucleus preparations made from buccal smears of the mosaic deletion patients, the oral cavity was rinsed twice with drinking water and then scraped with a premoistened applicator or cytobrush. The cells were resuspended in 1×PBS supplemented with antibiotics. After a 2000-rpm spin for 5 min, 10 ml of hypotonic solution (0.5 M KCl) was added for 10 min. The nuclei were then fixed in Carnoy’s solution. The samples were uniformly smeared across a prelabeled clean slide.

FISH

BAC clones and locus-specific probes were labeled with digoxigenin–dUTP (Roche Diagnostic) using a nick-translation kit (Roche Diagnostic). The FISH experiments were performed using standard procedures [36]. The chromosomes were counterstained with DAPI in antifade (Vectashield) and then visualized using a Leitz DM-RB microscope equipped for DAPI and FITC/TRITC epifluorescence optics. The images were captured by means of a CCD camera (Hamamatsu 3CCD Camera, C5810) and visualized using Highfish software (Casti Imaging).

Score of FISH signals in the mosaic condition

Almost 50 metaphases and 100 nuclei were scored for each sample to establish the mosaic condition. Only intact and undamaged nuclei free of cytoplasm were analyzed; nuclei with low signal intensities, diffuse signals, or no signal on either homologue chromosome were considered hybridization failures and not scored. Two small focal (or paired) signals of the same color and intensity, separated by a distance of less than the area of one signal, were considered to be a split signal from one chromosome. Interphase nuclei with one large signal of the same color and more intense fluorescence in the absence of a second hybridization signal were considered as representing the overlapping (or overposition) of two signals and were not scored. Informative mosaic samples were defined as those in which more than 5% of the nuclei had a reproducible abnormal pattern of signals compared to the expected chromosomal signals.

Haplotype analysis

Fluorescent genotyping was performed as previously described [10]. The fluorescent dye-labeled chromosome 16p microsatellite markers MS4, MS2, and D16S3065 (from centromere to telomere), and the c.5454 G → A SNP, were used for segregation analysis from parents to probands. Fluorescence was detected using an ABI 3100 sequencer. ABI Prism software (Genescan) was used for gel analysis.

In silico analysis

The in silico sequence analysis was made using the following databases and tools: Entrez Nucleotides Database, http://www.ncbi.nlm.nih.gov/Entrez/query.fcgi%3Fdb = nucleotide; MAR-Wiz, http://www.futuresoft.org/ (for searching recombination-specific sites); NCBI BLAST, http://www.ncbi.nlm.nih.gov/Blast/; UCSC Genome Bioinformatics, http://www.genome.ucsc.edu/ (for sequence homology analyses and evaluation of the G+C content, the selection of the BAC/PAC clones to be used in the FISH experiments, and the generation of locus-specific probes); and Palindrome, http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.htlm (to search for palindromic elements).

Acknowledgments

We thank the patients and their families for contributing to this study and Professor A. Federico (Department of Neurological and Behavioural Sciences, Medical School, University of Siena, Italy) for referring one of his patients to G.N. This work was supported by a grant from the Associazione Italiana Studio Malformazioni.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.07.012.

References

[1] F. Petrij, et al., Rubinstein–Taybi syndrome caused by a de novo reciprocal translocation t(2;16) (q36.3;p13.3), Am. J. Med. Genet. 92 (2000) 47–52.

[2] K. Imaizumi, Y. Kuroki, Rubinstein–Taybi syndrome with de novo reciprocal translocation t(2;16) (p13.3;p13.3), Am. J. Med. Genet. 38 (1991) 636–639.

[3] D. Lacombe, R. Saura, L. Taine, J. Battin, Confirmation of assignment of a locus for Rubinstein–Taybi syndrome gene to16p13.3, Am. J. Med. Genet. 44 (1992) 126–128.

[4] N. Tommerup, C.B. van der Hagen, A. Heiberg, Tentative assignment of a locus for Rubinstein–Taybi syndrome to 16p13.3 by a de novo reciprocal translocation, t(7;16) (q34;p13.3), Am. J. Med. 44 (1992) 237–241.

[5] M.H. Breuning, et al., Rubinstein–Taybi syndrome caused by submicroscopic deletions within 16p13.3, Am. J. Hum. Genet. 52 (1993) 249–254.

[6] F. Petrij, et al., Rubinstein–Taybi syndrome caused by mutations in the transcriptional co-activator CBP, Nature 376 (1995) 348–351.

[7] F. Petrij, et al., Diagnostic analysis of the Rubinstein–Taybi syndrome: five cosmids should be used for microdeletion detection and low number of protein truncating mutations, J. Med. Genet. 37 (2000) 168–176.

[8] O. Bartsch, et al., DNA sequencing of *CREBBP* demonstrates mutations in 56% of patients with Rubinstein–Taybi syndrome (RSTS) and in another patient with incomplete RSTS, Hum. Genet. 117 (2005) 485–493.

[9] A. Bentivegna, et al., Rubinstein–Taybi syndrome: spectrum of *CREBBP* mutations in Italian patients, BMC Med. Genet. 7 (2006) 77.

[10] I. Coupry, et al., Molecular analysis of the CBP gene in 60 patients with Rubinstein Taybi syndrome, J. Med. Genet. 39 (2002) 415–421.

[11] O. Bartsch, et al., Evidence for a new contiguous gene syndrome, the chromosome 16p13.3 deletion syndrome alias severe Rubinstein–Taybi syndrome, Hum. Genet. 120 (2006) 179–186.

[12] I. Coupry, et al., Analysis of CBP (CREBBP) gene deletions in Rubinstein–Taybi syndrome patients using real-time quantitative PCR, Hum. Mutat. 23 (2004) 278–284.

[13] T. Udaka, et al., Screening for partial deletions in the *CREBBP* gene in Rubinstein–Taybi syndrome patients using multiplex PCR/liquid chromatography, Genet. Test. 10 (2006) 265–271.

[14] O. Bartsch, et al., FISH studies in 45 patients with Rubinstein–Taybi syndrome: deletions associated with polysplenia, hypoplastic left heart and death in infancy, Eur. J. Hum. Genet. 7 (1999) 748–756.

[15] J. Borrow, et al., The translocation t(8;16) (p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein, Nat. Genet. 14 (1996) 33–41.

[16] I. Panagopoulos, et al., Fusion of the MORF and CBP genes in acute myeloid leukemia with the t(10;16) (q22;p13), Hum. Mol. Genet. 10 (2001) 395–404.

[17] I. Panagopoulos, et al., Genomic characterization of MOZ/CBP and CBP/MOZ chimeras in acute myeloid leukemia suggests the involvement of a damage-repair mechanism in the origin of the t(8;16) (p11;p13), Genes Chromosomes Cancer 36 (2003) 90–98.

[18] M. Rozman, et al., Type I MOZ/CBP (MYST3/CREBBP) is the most common chimeric transcript in acute myeloid leukemia with t(8;16) (p11;p13) translocation, Genes Chromosomes Cancer 40 (2004) 140–145.

[19] H.H. Schmidt, et al., RT-PCR and FISH analysis of acute myeloid leukemia with t(8;16) (p11;p13) and chimeric MOZ and CBP transcripts: breakpoint cluster region and clinical implications, Leukemia 18 (2004) 1115–1121.

[20] O.M. Sobulo, et al., MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16) (q23;p13.3), Proc. Natl. Acad. Sci. USA 94 (1997) 8732–8737.

[21] J.L. Vizmanos, et al., t(10;16) (q22;p13) and MORF–CREBBP fusion is a recurrent event in acute myeloid leukemia, Genes Chromosomes Cancer 36 (2003) 402–405.

[22] J.H. Roelfsema, et al., Genetic heterogeneity in Rubinstein–Taybi syndrome: mutations in both the CBP and EP300 genes cause disease, Am. J. Hum. Genet. 76 (2005) 572–580.

[23] D. Bartholdi, et al., Genetic heterogeneity in Rubinstein–Taybi syndrome: delineation of the phenotype of the first patients carrying mutations in EP300, J. Med. Genet. 44 (2007) 327–333.

[24] L. Kluwe, et al., Screening 500 unselected neurofibromatosis 1 patients for deletions of the NF1 gene, Hum. Mutat. 23 (2004) 111–116.

[25] N. Kurotaki, et al., Fifty microdeletions among 112 cases of Sotos syndrome: low copy repeats possibly mediate the common deletion, Hum. Mutat. 22 (2003) 378–387.

[26] M.O. Dorschner, V.P. Sybert, M. Weaver, B.A. Pletcher, K. Stephens, NF1 microdeletion breakpoints are clustered at flanking repetitive sequences, Hum. Mol. Genet. 9 (2000) 35–46.

[27] D.E. Jenne, et al., Molecular characterization and gene content of breakpoint boundaries in patients with neurofibromatosis type 1 with 17q11.2 microdeletions, Am. J. Hum. Genet. 69 (2001) 516–527.

[28] C. Lopez Correa, H. Brems, C. Lazaro, P. Marynen, E. Legius, Unequal meiotic crossover: a frequent cause of NF1 microdeletions, Am. J. Hum. Genet. 66 (2000) 1969–1974.

[29] C. Gervasini, et al., Uncommon *Alu*-mediated NF1 microdeletion with a breakpoint inside the NF1 gene, Genomics 85 (2005) 273–279.

[30] H. Kehrer-Sawatzki, L. Kluwe, C. Funsterer, V.F. Mautner, Extensively high load of internal tumors determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2, Hum. Genet. 116 (2005) 466–475.

[31] M. Venturin, et al., Evidence for non-homologous end joining and non-allelic homologous recombination in atypical NF1 microdeletions, Hum. Genet. 115 (2004) 69–80.

[32] A. Murati, et al., New types of MYST3-CBP and CBP-MYST3 fusion transcripts in t(8;16) (p11;p13) acute myeloid leukemias, Haematologica 92 (2007) 262–263.

[33] Y. Zhang, et al., Characterization of genomic breakpoints in MLL and CBP in leukemia patients with t(11;16), Genes Chromosomes Cancer 41 (2004) 257–265.

[34] N. Zimmermann, A.M. Acosta, J. Kohlhase, O. Bartsch, Confirmation of EP300 gene mutations as a rare cause of Rubinstein–Taybi syndrome, Eur. J. Hum. Genet. 15 (2007) 837–842.

[35] P. Riva, L. Corrado, P. Colapietro, L. Larizza, A rapid and simple method of generating locus-specific probes for FISH analysis, Technical Tips Online T01618 (1999).

[36] P. Lichter, T. Cremer, A practical approach, in: D.E. Rooney, B.H. Czipolkowski (Eds.), Human Cytogenetics, IRL Press, Oxford, 1992, pp. 157–192.