



# Multiplex ligation-dependent probe amplification detection of an unknown large deletion of the CREB-binding protein gene in a patient with Rubinstein-Taybi syndrome

F. Cali<sup>1</sup>, P. Failla<sup>2</sup>, V. Chiavetta<sup>1</sup>, A. Ragalmuto<sup>1</sup>, G. Ruggeri<sup>1</sup>,  
P. Schinocca<sup>1</sup>, C. Schepis<sup>3</sup>, V. Romano<sup>1,4</sup> and C. Romano<sup>2</sup>

<sup>1</sup>Laboratory of Molecular Genetics, IRCCS Associazione Oasi Maria SS, Troina, Italy

<sup>2</sup>Unit of Pediatrics and Medical Genetics, IRCCS Associazione Oasi Maria SS, Troina, Italy

<sup>3</sup>Unit of Dermatology, IRCCS Associazione Oasi Maria SS, Troina, Italy

<sup>4</sup>Department of Physics, University of Palermo, Palermo, Italy

Corresponding author: C. Romano

E-mail: [cromano@oasi.en.it](mailto:cromano@oasi.en.it)

Genet. Mol. Res. 12 (3): 2809-2815 (2013)

Received May 3, 2012

Accepted August 3, 2012

Published January 7, 2013

DOI <http://dx.doi.org/10.4238/2013.January.7.2>

**ABSTRACT.** Rubinstein-Taybi syndrome is a rare autosomal dominant congenital disorder characterized by postnatal growth retardation, psychomotor developmental delay, skeletal anomalies, peculiar facial morphology, and tumorigenesis. Mutations in the gene encoding the cAMP response element-binding protein (CREB, also known as CREBBP or CBP) on chromosome 16p13.3 have been identified. In addition, some patients with low intelligence quotients and autistic features bear large deletions. Based on these observations, we used multiplex ligation-dependent probe amplification to search for large deletions affecting the *CREBBP* gene in a Rubinstein-Taybi syndrome patient. We identified a novel heterozygote deletion removing five exons (exons 17-21), encoding the histone acetyltransferase domain.

We propose the use of multiplex ligation-dependent probe amplification as a fast, accurate and cheap test for detecting large deletions in the *CREBBP* gene in the sub-group of Rubinstein-Taybi syndrome patients with low intelligence quotients and autistic features.

**Key words:** Comparative multiplex dosage analysis; CREB-binding protein; Rubinstein-Taybi syndrome; Multiplex ligation-dependent probe amplification

## INTRODUCTION

Rubinstein-Taybi syndrome (RTS) [Mendelian Inheritance in Man (MIM, #180849)] is an autosomal dominant disease occurring in 1 of 125,000 births (Hennekam et al., 1990; Wiley et al., 2003) that is characterized by growth retardation, psychomotor development delay, broad and/or bifid distal phalanges of thumbs and halluces, atypical facial morphology, short stature, and moderate to severe intellectual disability (ID). Other features include coloboma, cardiac anomalies, keloid formation in scars, and increased risk of tumor formation (Bartsch et al., 2006). RTS results from *de novo* heterozygous point mutations or deletions of the cAMP response element-binding protein (CREB also known as CREBBP or CBP; Petrij et al., 1995) and E1A-associated protein p300 (*EP300*) (Bartsch et al., 2010) genes. Individuals reported with mutations in *EP300* have a milder skeletal phenotype, lacking typical broadening and angulation of the thumb and hallux (Bartholdi et al., 2007).

The *CREBBP* gene includes 31 exons, spans approximately 155 kb of genomic DNA and is located on chromosome 16p13.3. *EP300* also contains 31 exons, spans approximately 87 kb genomic DNA and is located on chromosome 22q13.2. CREBBP is a transcriptional co-activator that has intrinsic histone acetyltransferase activity and is involved in various signal transduction pathways that regulate the expression cell growth, differentiation, DNA repair, apoptosis, and tumor suppression (Chrivia et al., 1993; Giles et al., 1998).

Several molecular techniques, such as fluorescence *in situ* hybridization (FISH; McGaughan et al., 1996), denaturing high-performance liquid chromatography (Udaka et al., 2006), array comparative gene hybridization (Gervasini et al., 2010), DNA sequencing (Bentivegna et al., 2006), and real-time PCR (Coupry et al., 2004), have been used to identify mutations in the *CREBBP* gene. In this study, we reported the identification of a novel mutation in the *CREBBP* gene and demonstrated that multiplex ligation-dependent probe amplification (MLPA) is a reliable and efficient method for detecting large deletions of the *CREBBP* gene in the subgroup of RTS patients with lower intelligence quotient (IQ) and autistic features.

## MATERIAL AND METHODS

### Case report

The patient is the 3-year-old daughter of unrelated parents. Her family history shows

only a maternal uncle with ID, epilepsy, and an autistic disorder. She was born by Cesarean section for fetal distress after a pregnancy characterized by decreased fetal growth. The perinatal history was characterized by asphyxia, which was quickly resolved. Psychomotor development milestones have been reached with delay (sitting alone at 12 months), and she currently only speaks the words “mom” and “dad”, walks with support, and has not achieved sphincter control. Her personal history shows failure to thrive, and she is currently slightly below the 3rd centile for weight and height, with OFC -4SD. Facial morphology shows columella protruding below alae nasi with a thin beaked nose, short philtrum, hypertelorism with down-slanting palpebral fissures and a thin upper lip. A small pink nodular lesion, with a hard/elastic consistence and the size of a pea, was detectable over the posterior surface of the left ear. The lesion was completely removed and its histological examination allowed us to make the diagnosis of spontaneous keloid. She shows broad thumbs and the halluces are both broad and bifid on the left. She scores 12 months of mental age on the Griffiths mental development scale, corresponding to moderate mental retardation. Informed consent was obtained from the patient’s parents prior to the present study.

### **MLPA analysis**

Genomic DNA was isolated from peripheral blood lymphocytes using the salt chloroform extraction method. Quantification of DNA extracted from each sample was performed using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA in solution was quantified and checked for degradation on an agarose gel. MLPA analysis was performed using the SALSA MLPA kit P313-A1 PAH (MRC-Holland, Amsterdam, The Netherlands) as previously described by Schouten et al. (2002). The P313-A1 *CREBBP* probe mix contains probes for each of the 31 exons of the *CREBBP* gene (2 probes for exons 1, 2, and 3). In addition, it contains 3 probes for *EP300* (exons 1, 4, and 12). PCR products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer, using the Gene Mapper software from Applied Biosystems (Foster City, CA, USA). In order to efficiently process the MLPA deletion/duplication data, a spreadsheet was generated in Microsoft Excel (Microsoft, Redmond, WA, USA). First, the data corresponding to each sample (patient and control DNAs) were normalized by dividing each probe’s signal strength (i.e., the area of each peak) by the average signal strength yielded by the 10 control probes to generate a relative peak area (RPA) value for each peak. The RPA value for each probe in the patient’s sample was then compared to that of the control sample by dividing, for each peak, the patient RPA by the control RPA. The latter ratio was then used to define the following categories: i)  $\sim 1$  for the non-deleted/non-duplicated gene region, ii)  $\sim 0.5$  if deleted, and iii)  $\sim 1.5$  if duplicated. Other details regarding the normalizing method and quality test have been described previously by Cali et al. (2010).

### **Comparative multiplex dosage analysis (CMDA)**

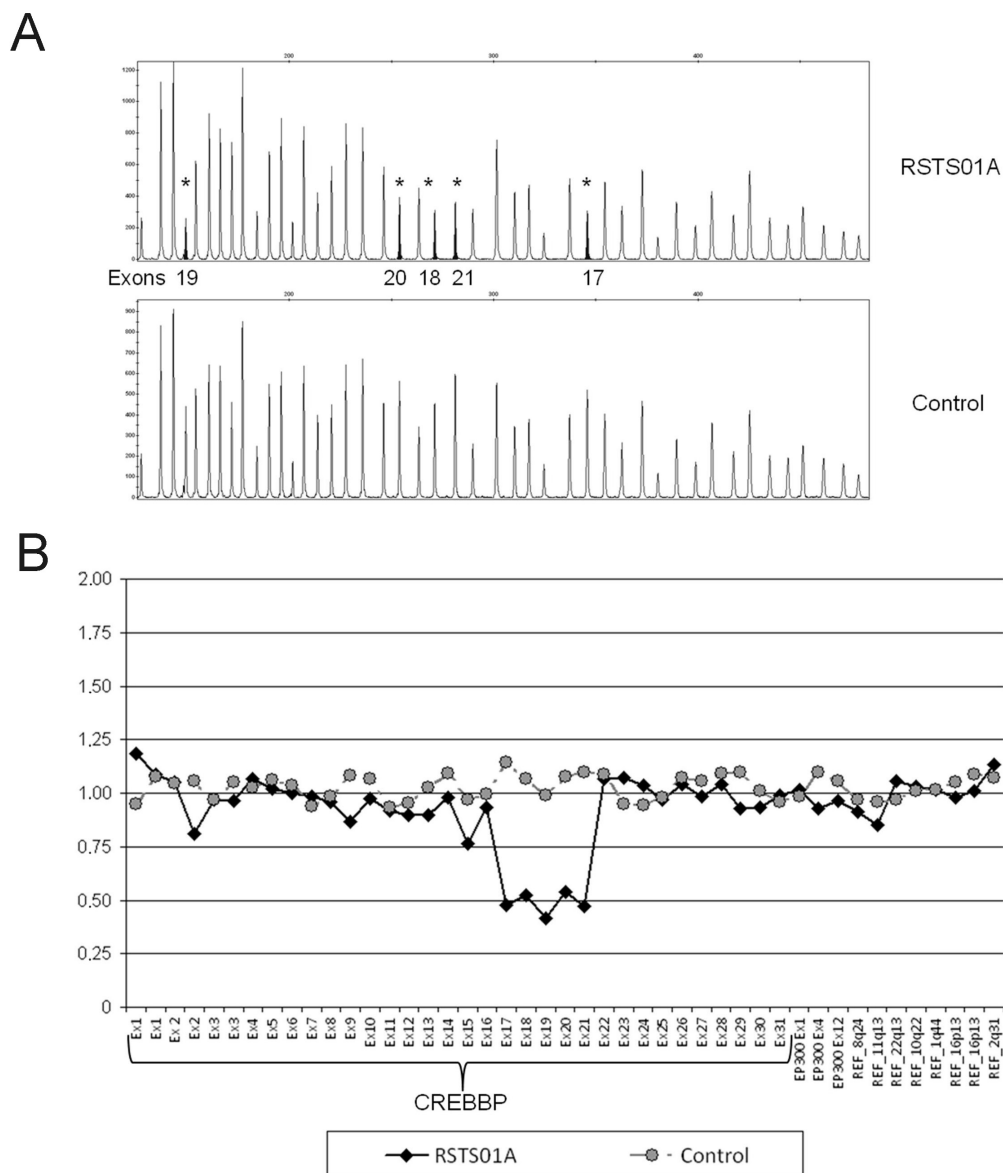
CMDA was performed as previously described by Gable et al. (2003) and Cali et al. (2010) to confirm the deletion of the exons 17-21 of the *CREBBP* gene identified by MLPA. Briefly, only exons 16 and 22 (expected normal dosage) and 17 and 21 (expected

heterozygous deletion) of the *CREBBP* gene were co-amplified in a fluorescent multiplex PCR with myelin protein zero, mutL homolog 1, and neuroligin 3 as control genes. A total of 10-15 pmol of each primer (sequencing and PCR conditions are available from the authors upon request) and 18 PCR cycles were used to ensure the reaction was kept within the exponential phase. The PCR products were resolved on an ABI3130 sequence analyzer (Applied Biosystems) according to manufacturer instructions. Results from the patient's DNA (RSTS01A) were compared separately with those from 4 (2 males and 2 females) non-deleted, non-duplicated control individuals. The RPAs for each test were calculated as described above for the MLPA. The sensitivity of this test to detect deletion/duplication was also verified by comparing the RPAs between males (RPA ~0.5) and females (RPA ~1) using the X-linked neuroligin 3 gene as control. The results of RPA were segregated into the following categories: i) ~1, for the non-deleted/non-duplicated gene region tested in the patient, ii) ~0.5 if deleted, and iii) ~1.5 if duplicated.

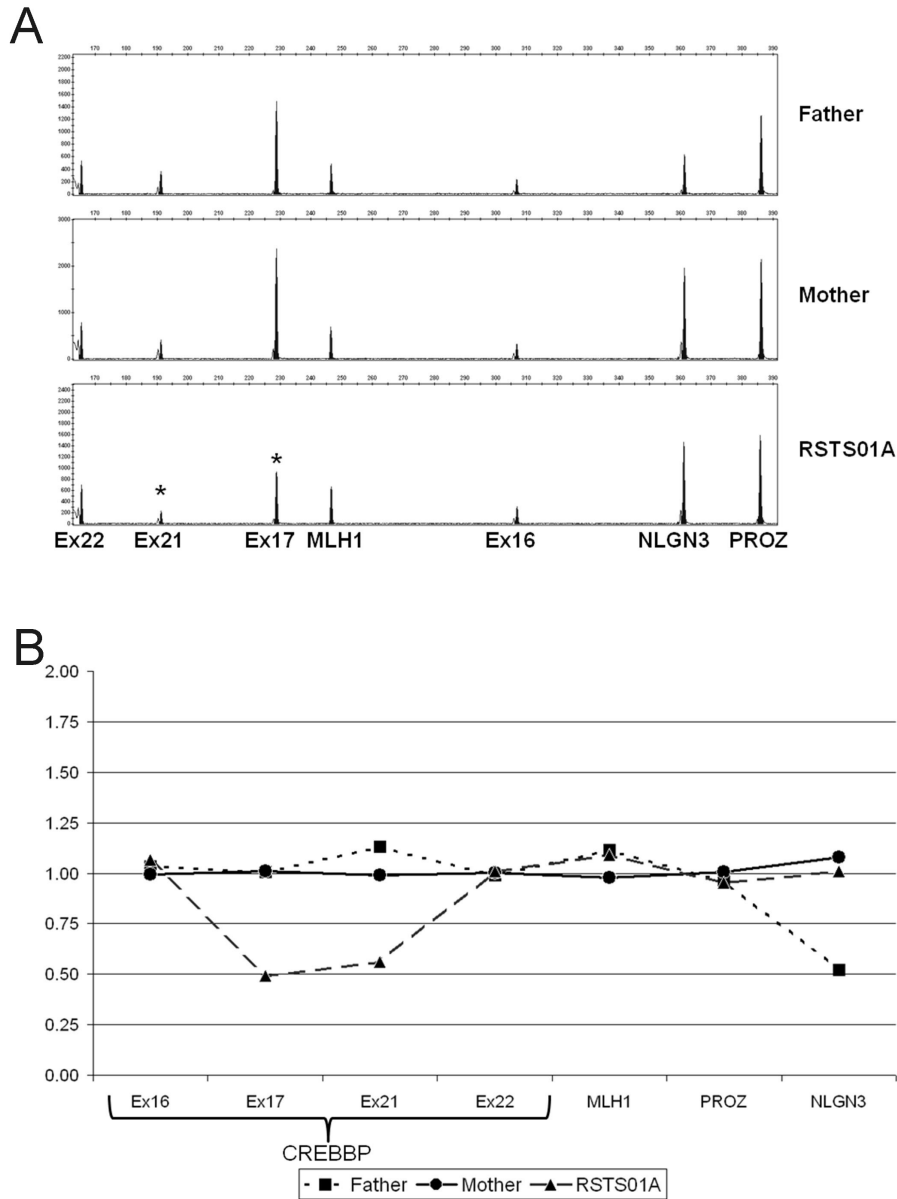
## RESULTS AND DISCUSSION

RTS underlies a wide spectrum of molecular defects affecting the expression of the *CREBBP* gene, including frameshift, nonsense, splice site, missense mutations, and (less frequently) large deletions. Facial and thumb features are associated with all mutations. Growth retardation in height and weight has been observed more frequently in patients without *CREBBP* mutations; seizures were more frequent in those with *CREBBP* mutations. The degree of ID is similar in all groups, although there is a trend toward lower IQ and autistic features in patients with large deletions (Schorry et al., 2008). These latter features led us to search for large deletions in our patient, although *CREBBP* gene deletions account only for approximately 10% of RTS patients (Petrij et al., 2000). Traditionally, FISH analysis has been employed to detect full gene deletions in the *CREBBP* gene (McGaughan et al., 1996; Bartsch et al., 1999). However, both FISH and commonly available array comparative gene hybridization analyses have limitations for the detection of small deletions. In these cases, MLPA is a suitable alternative. Moreover, MLPA can detect gene deletions in heterozygotes whereas DNA sequencing would fail due to the masking effect of the non-deleted allele (Cali et al., 2010). Based on the above considerations, we genotyped patient RSTS01A by MLPA instead of DNA sequencing, using the P313-A1 *CREBBP* kit (MRC-Holland), containing probes for all 31 exons of the *CREBBP* gene. Patient RSTS01A showed an altered amplification pattern, compatible with a heterozygous novel deletion of exons 17-21 of the *CREBBP* gene (Figure 1). This mutation was absent in parental samples, suggesting that the deletion occurred as a *de novo* event in one of the parents during gametogenesis. To confirm the preliminary MLPA finding, we developed an alternative semi-quantitative CMDA dosage, which confirmed this deletion (see Figure 2). This deletion involves the histone acetyltransferase domain, containing 44.7% of the exon variants, consistent with previous studies (Sharma et al., 2010). These findings confirmed the clinical diagnosis of RTS in the patient.

In conclusion, we propose MLPA as an inexpensive, fast, and accurate test for the initial screening for *CREBBP* gene deletions/duplications in all RTS patients displaying lower IQ and autistic features.



**Figure 1.** Multiplex ligation-dependent probe amplification (MLPA) analysis of the *CREBBP* gene performed with the SALSA MLPA kit P313-A1 in patient (RSTS01A) with Rubinstein-Taybi syndrome. In this patient, the MLPA revealed the deletion of exons 17, 18, 19, 20, and 21. **A.** The electropherograms show results referring to probes obtained for i) patient (RSTS01A) and ii) a negative control (“Control”). The asterisks above peaks indicate the deleted exons. **B.** Graph displaying the ratios between the patient’s and control’s relative peak areas (RPA) determined in the 16p13.3 region. Reference values are as follows: RPA ratio  $\sim 1$  = normal dosage; RPA ratio  $\sim 0.5$  = heterozygous deletion. See text for more details.



**Figure 2.** Comparative multiplex dosage analysis (CMDA) performed on patient RSTS01A bearing a deletion of the *CREBBP* gene removing exons 17-21. **A.** In the electropherogram, the asterisks above the peaks indicate deleted exons 17 and 21 borne only by the patient but not by her parents (*de novo* mutation). Ex16, 17, 21, and 22 refers to exons of the *CREBBP* gene. **B.** Graph displaying the ratios between the RSTS01A patient's and controls' relative peak areas (RPA) determined for the exons 16, 17, 21, and 22 of the *CREBBP* gene (see text for explanation). Reference values are as follows: RPA ratio  $\sim 1$  = normal dosage; RPA ratio  $\sim 0.5$  = heterozygous deletion. mutL homolog 1 (MLH1), myelin protein zero (PROZ), and neuroligin 3 (NLGN3) are control probes used in the CMDA.

## ACKNOWLEDGMENTS

Research supported by the Italian Ministry of Health: Current Research 2012 entitled “Genetic diseases with intellectual disability” and “5 per thousand” Funding.

## REFERENCES

- Bartholdi D, Roelfsema JH, Papadia F, Breuning MH, et al. (2007). Genetic heterogeneity in Rubinstein-Taybi syndrome: delineation of the phenotype of the first patients carrying mutations in EP300. *J. Med. Genet.* 44: 327-333.
- Bartsch O, Wagner A, Hinkel GK, Krebs P, et al. (1999). 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: 748-756.
- Bartsch O, Rasi S, Delicado A, Dyack S, et al. (2006). Evidence for a new contiguous gene syndrome, the chromosome 16p13.3 deletion syndrome alias severe Rubinstein-Taybi syndrome. *Hum. Genet.* 120: 179-186.
- Bartsch O, Labonté J, Albrecht B, Wiczorek D, et al. (2010). Two patients with EP300 mutations and facial dysmorphism different from the classic Rubinstein-Taybi syndrome. *Am. J. Med. Genet. A* 152A: 181-184.
- Bentivegna A, Milani D, Gervasini C, Castronovo P, et al. (2006). Rubinstein-Taybi Syndrome: spectrum of CREBBP mutations in Italian patients. *BMC Med. Genet.* 7: 77.
- Cali F, Ruggeri G, Vinci M, Meli C, et al. (2010). Exon deletions of the phenylalanine hydroxylase gene in Italian hyperphenylalaninemics. *Exp. Mol. Med.* 42: 81-86.
- Chrivia JC, Kwok RP, Lamb N, Hagiwara M, et al. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 365: 855-859.
- Coupry I, Monnet L, Attia AA, Taine L, et al. (2004). Analysis of CBP (CREBBP) gene deletions in Rubinstein-Taybi syndrome patients using real-time quantitative PCR. *Hum. Mutat.* 23: 278-284.
- Gable M, Williams M, Stephenson A, Okano Y, et al. (2003). Comparative multiplex dosage analysis detects whole exon deletions at the phenylalanine hydroxylase locus. *Hum. Mutat.* 21: 379-386.
- Gervasini C, Mottadelli F, Ciccone R, Castronovo P, et al. (2010). High frequency of copy number imbalances in Rubinstein-Taybi patients negative to CREBBP mutational analysis. *Eur. J. Hum. Genet.* 18: 768-775.
- Giles RH, Peters DJ and Breuning MH (1998). Conjunction dysfunction: CBP/p300 in human disease. *Trends Genet.* 14: 178-183.
- Hennekam RC, Stevens CA and Van de Kamp JJ (1990). Etiology and recurrence risk in Rubinstein-Taybi syndrome. *Am. J. Med. Genet. Suppl.* 6: 56-64.
- McGaughran JM, Gaunt L, Dore J, Petrij F, et al. (1996). Rubinstein-Taybi syndrome with deletions of FISH probe RT1 at 16p13.3: two UK patients. *J. Med. Genet.* 33: 82-83.
- Petrij F, Giles RH, Dauwerse HG, Saris JJ, et al. (1995). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature* 376: 348-351.
- Petrij F, Dauwerse HG, Blough RI, Giles RH, et al. (2000). 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: 168-176.
- Schorry EK, Keddache M, Lanphear N, Rubinstein JH, et al. (2008). Genotype-phenotype correlations in Rubinstein-Taybi syndrome. *Am. J. Med. Genet. A* 146A: 2512-2519.
- Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30: e57.
- Sharma N, Mali AM and Bapat SA (2010). Spectrum of CREBBP mutations in Indian patients with Rubinstein-Taybi syndrome. *J. Biosci.* 35: 187-202.
- Udaka T, Kurosawa K, Izumi K, Yoshida S, et al. (2006). Screening for partial deletions in the CREBBP gene in Rubinstein-Taybi syndrome patients using multiplex PCR/liquid chromatography. *Genet. Test.* 10: 265-271.
- Wiley S, Swaney S, Rubinstein JH, Lanphear NE, et al. (2003). Rubinstein-Taybi syndrome medical guidelines. *Am. J. Med. Genet. A* 119A: 101-110.