

ARTICLE

Exon deletions of the *EP300* and *CREBBP* genes in two children with Rubinstein–Taybi syndrome detected by aCGH

Anne Chun-Hui Tsai¹, Cherilyn J Dossett¹, Carol S Walton¹, Andrea E Cramer², Patti A Eng³, Beata A Nowakowska^{3,4}, Amber N Pursley³, Pawel Stankiewicz^{3,4}, Joanna Wiszniewska³ and Sau Wai Cheung^{*,3}

We demonstrate the utility of an exon coverage microarray platform in detecting intragenic deletions: one in exons 24–27 of the *EP300* gene and another in exons 27 and 28 of the *CREBBP* gene in two patients with Rubinstein–Taybi syndrome (RSTS). RSTS is a heterogeneous disorder in which ~45–55% of cases result from deletion or mutations in the *CREBBP* gene and an unknown portion of cases result from gene changes in *EP300*. The first case is a 3-year-old female with an exonic deletion of the *EP300* gene who has classic facial features of RSTS without the thumb and great toe anomalies, consistent with the milder skeletal phenotype that has been described in other RSTS cases with *EP300* mutations. In addition, the mother of this patient also had preeclampsia during pregnancy, which has been infrequently reported. The second case is a newborn male who has the classical features of RSTS. Our results illustrate that exon-targeted array comparative genomic hybridization (aCGH) is a powerful tool for detecting clinically significant intragenic rearrangements that would be otherwise missed by aCGH platforms lacking sufficient exonic coverage or sequencing of the gene of interest.

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INTRODUCTION

Rubinstein–Taybi syndrome (RSTS) is characterized by growth retardation, dysmorphic features, skeletal abnormalities and mental retardation.¹ Broad and angulated thumbs and halluces are considered hallmarks in the clinical diagnosis. Mutations in two genes, *CREBBP* and *EP300*, have been identified in affected individuals.^{2,3} Individuals reported with mutations in *EP300* have a milder skeletal phenotype, lacking typical broadening and angulation of the thumb and hallux.⁴ *CREBBP* and *EP300* are ubiquitously expressed homologous proteins that act as transcriptional co-activators. Both genes are highly conserved and their proteins are thought to have two functions: formation of a bridge or scaffold between the DNA-binding transcription factors and the RNA polymerase II complex, and serving as histone acetyltransferases that open up the chromatin structure, a process that is essential for gene expression.⁵

Clinical implementation of array comparative genomic hybridization (aCGH) enables high-resolution human genome analyses, and the discovery of extensive copy-number variation (CNV) has led to the identification of new microdeletion and microduplication syndromes also known as genomic disorders.^{6,7} With genome-wide studies identifying an increasing number of disease genes, such as in patients with autism spectrum disorders,⁸ and as copy-number changes in the genome can be observed as small as a single exon,^{9,10} this array has evolved to a whole-genome array that can simultaneously evaluate over 1700 genes with a resolution significant enough to detect intragenic changes.

Here we report two such cases of exonic deletions detected by this array platform; one in a male with RSTS resulting from an exon deletion within the *CREBBP* gene and the other in a female with RSTS resulting from a deletion within the *EP300* gene. Although six other cases of *EP300* RSTS have been previously reported,^{3,4,11,12} this is the first patient ascertained by microarray analysis in a clinical setting.

CLINICAL REPORT

Case 1

The child was first seen in the genetics clinic at the age of 34 months because of microcephaly, short stature, developmental delay and behavioral problems. She was born to a then G 1 P 0-1 22-year-old mother via C-section at ~33 weeks. Prenatal exposures included maternal usage of Vicodin for migraines, Reglan for nausea, and multiple blood pressure medications for pregnancy-induced hypertension beginning at 14 weeks. Pregnancy was complicated by IUGR, thin placenta and premature labor at 28 weeks due to preeclampsia. Patient stayed in the NICU for 6 weeks for poor weight gain, which was partially attributed to a milk allergy. Overall, she was healthy with mild developmental delay and aggressive behaviors. She walked at 18 months and only has a few words and signs at 34 months of age. EEG and MRI were normal.

Physical examination noted a short, microcephalic girl who was hyperactive. Height was 83.9 cm (0.49%), weight was 11.2 kg (3%) and OFC was 44.5 cm (<2%). Dysmorphic facial features include hypotelorism, deep-set eyes with luxuriant eyelashes, downslanting

¹The Children's Hospital, Section of Clinical Genetics and Metabolism, Denver, UC Denver, Aurora, CO, USA; ²1600 Republic Pkwy, Mesquite, TX, USA; ³Medical Genetics Laboratories, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA; ⁴Department of Medical Genetics, Institute of Mother and Child, Warsaw, Poland

*Correspondence: Dr SW Cheung, Medical Genetics Laboratories, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA. Tel: +1 713 798 4991; Fax: +1 713 798 4998; E-mail: scheung@bcm.edu

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palpebral fissures, mild synophrys, hirsutism, prominent nasal tip, cupid-bow-shaped (beaked) upper lip and high-arched palate (Figure 1a). The arms, legs, fingers and toes were normal, except flat feet, hyperextensible joints and hypotonia. She demonstrated some repetitive and self-stimulating behaviors.

Case 2

This child was ascertained at birth with obvious stigmata of RSTS. He was a full-term male infant, delivered vaginally to a G3 P1 A1 34-year-old female after an unremarkable gestation. Maternal history was significant for rheumatoid arthritis for which she received methotrexate before conception. Family pedigree was unremarkable and consanguinity was denied. Birth growth parameters were normal. The NICU transfer was initiated upon development of respiratory distress and cardiac murmur, subsequently identified as due to patent ductus arteriosus and patent foramen ovale. Facial features are compatible with RSTS as shown in Figure 1b. Undescended unilateral testis, hypoplastic scrota and penis were identified, the latter responding well to testosterone injection. Microcephaly developed postnatally.

MATERIALS AND METHODS

Patients

Chromosomal microarray analysis Version 8 OLIGO (CMA V8 OLIGO) with exon coverage was performed on ~3000 samples that were referred to the Kleberg Cytogenetic Laboratory at Baylor College of Medicine (BCM) during the first 6 months of the clinical application of this array platform (June to December 2009). Informed consent approved by the Institutional Review Board for Human Subject Research at BCM was obtained from their families.

DNA samples

DNA was extracted from whole blood using the Puregene DNA extraction kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's instructions.

Clinical CMA

The CMA V8 OLIGO is a custom designed array with ~180 000 oligonucleotides of 60bp manufactured by Agilent Technologies Inc. (Santa Clara, CA, USA). This array selects the best performing oligos from the electronic library from Agilent and probes for virtually all the known microdeletion or microduplication syndromes, as well as the pericentromeric and subtelomeric regions as previously described in version 7.¹³ In addition, more than 1700 genes throughout the genome have coverage for every exon. On average, there are 4.5 oligos per exon and intron coverage if the intron is >10 kb. However, probe coverage for an individual exon may be less if the exon is small or there are few oligos that meet the selection criteria available from the electronic library of Agilent. The entire genome (between disease regions) is covered with an average resolution of 30 kb excluding repetitive sequences as determined through a combination of bioinformatics and computation. Although the majority of genes selected for exonic coverage are known disease genes, candidate genes implicated in autism, epilepsy and heart defects are also included in the array. The procedures for DNA digestion, labeling and hybridization for the oligo arrays were performed according to the manufacturers' instructions with some modifications.¹⁴ The slides were scanned into image files using an Agilent G2565 laser scanner. Microarray image files of oligo arrays were quantified using Agilent Feature extraction software (v9.0), and text file outputs from the quantitation analysis were imported into our in-house analysis package for copy-number analysis, as previously described.¹⁴

High-density research aCGH analysis of chromosome 22

The chromosome 22 array was designed to cover the entire long arm of chromosome 22 by selecting oligos from the e-library supplied by Agilent. This array contains 43 099 arrayed 60-mer oligonucleotides, representing a compiled view of the long arm of chromosome 22 at an average resolution of 1 kb while excluding the regions with segmental duplication. The procedures for DNA digestion, labeling and hybridization were performed according to the manufacturer's instructions with some modifications.¹⁴ This array was used to confirm the deletion of *EP300* in patient 1.

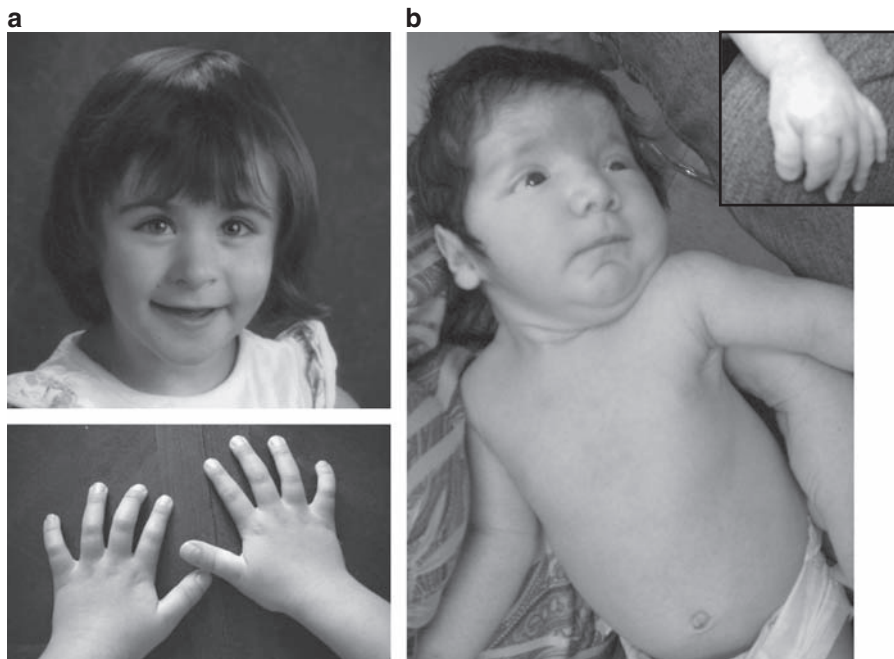


Figure 1 Clinical photos of the patients. (a) Case 1: Dysmorphic facial features include hypotelorism, deep-set eyes with luxuriant eyelashes, bushy eyebrows, synophrys, hirsutism, prominent nasal tip, cupid-bow-shaped/beaked upper lip and high-arched palate. (b) Case 2: Facial features of case 2 include hirsutism, low hairlines, glabellar nevus flammeus, ptosis, epicanthal folds, downslanting palpebral fissures, nasal septum below alae nasi and microstomia. Thumbs are broad and angulated; palmar creases are deep and simple. Halluces are broad but not angulated. Persistent fetal pads were noted on all fingers.

MLPA analysis

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed using commercially available SALSA MLPA kits, designed to detect deletions/duplications in patients with RSTS (P333-A1 EP300 and P313-CREBBP, MRC-Holland, Amsterdam, The Netherlands).¹⁵ Analysis was performed according to the manufacturer’s protocol. The amplification products were separated by capillary electrophoresis using ABI 3730xl genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). Data were visually inspected and analyzed using GeneMarker software v1.5 (SoftGenetics, LLC, State College, PA, USA).

RESULTS

Case 1

CMA V8 OLIGO revealed a loss in copy number of the long arm of chromosome 22 at sub-band 22q13.32 encompassing ~5 kb within the EP300 gene and a gain in copy number on the long arm of chromosome 21 encompassing ~160 kb (Figure 2a). Each of the oligos (red square) in the deleted region is displayed in the UCSC genome browser (build HG 18, <http://genome.ucsc.edu/>) corresponding to the four deleted exons of the EP300 gene (exons 24–27) (Figure 2b). The breakpoint for the deletion within the EP300 gene was further narrowed to be 4.43 kb using a high-resolution chromosome 22-specific array. The deletion breakpoint was mapped between 39 893 478 and 39 894 339 bp and the distal breakpoint between 39 896 990 and 39 897 907 bp (data not shown). The deletion within the EP300 gene was also confirmed by MLPA to be from exons 24 to 27 (Figure 2c). The gain in copy number on the long arm of chromosome 21 (Figure 2a) was later found to be inherited from the phenotypically normal mother and therefore thought to be likely benign. Parental CMA studies showed the deletion involving EP300 is *de novo*. The parental MLPA studies using the same probes selected

from the region encompassing the EP300 gene were also normal, consistent with the CMA findings.

Case 2

Routine chromosome analysis demonstrated a normal male chromosome complement: 46,XY. CMA revealed a loss in copy number in the 16p13.3 region of the short arm of chromosome 16, spanning a minimum of 5 kb and a maximum of 6 kb, harboring exons 27 and 28 of the CREBBP gene (Figures 3a and b), which was verified by MLPA analysis (Figure 3c), consistent with the clinical indication of RSTS (OMIM 180849). In addition, CMA revealed a gain in copy number in the Xp22.31 region of the short arm of chromosome X, spanning a minimum of 248 kb and a maximum of 358 kb (Figure 3a), which was confirmed by FISH analysis. Parental CMA studies were performed and it was confirmed that the deletion involving exons 27 and 28 is *de novo*, whereas the CNV on chromosome X was inherited from the phenotypically normal mother. This CNV on chromosome X has also been observed in the genomic variation database (<http://projects.tcag.ca/variation/>), indicating that it is also present in the normal population and therefore thought to be likely benign. In addition, parental MLPA studies for exons 27 and 28 of the CREBBP gene were also normal, consistent with the CMA findings.

DISCUSSION

With the introduction of a microarray platform with exon coverage, we demonstrate the utility of this technology in detecting intragenic deletions: one in exons 24–27 of the EP300 gene and another in exons 27 and 28 of the CREBBP gene. These results illustrate that exon-targeted aCGH is a powerful tool for detecting clinically significant

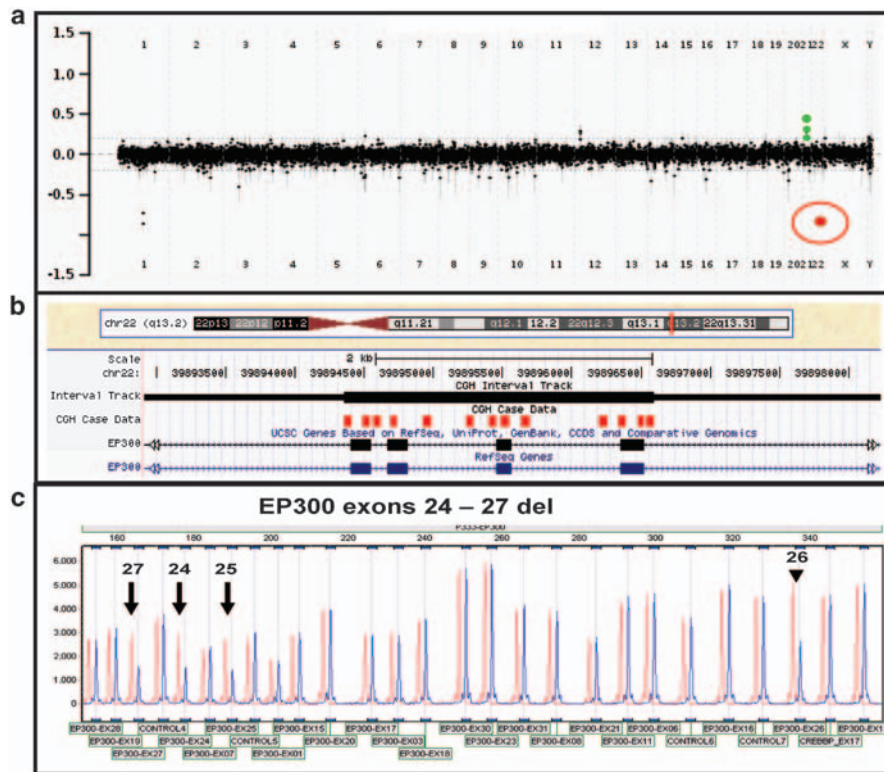


Figure 2 (a) Profile of the microarray analysis showing the deleted region as indicated in the red circle (the gain on chromosome 21 as shown in green dots is also present in the mother (data not shown)). (b) Each of the oligos (red square) in the deleted region is displayed in the UCSC genome browser (build HG 18) corresponding to the four deleted exons of the EP300 gene (exons 24–27). (c) The MLPA profile demonstrating the deletion of exons 24–27 of the EP300 gene as indicated by the arrow with the number for each exon.

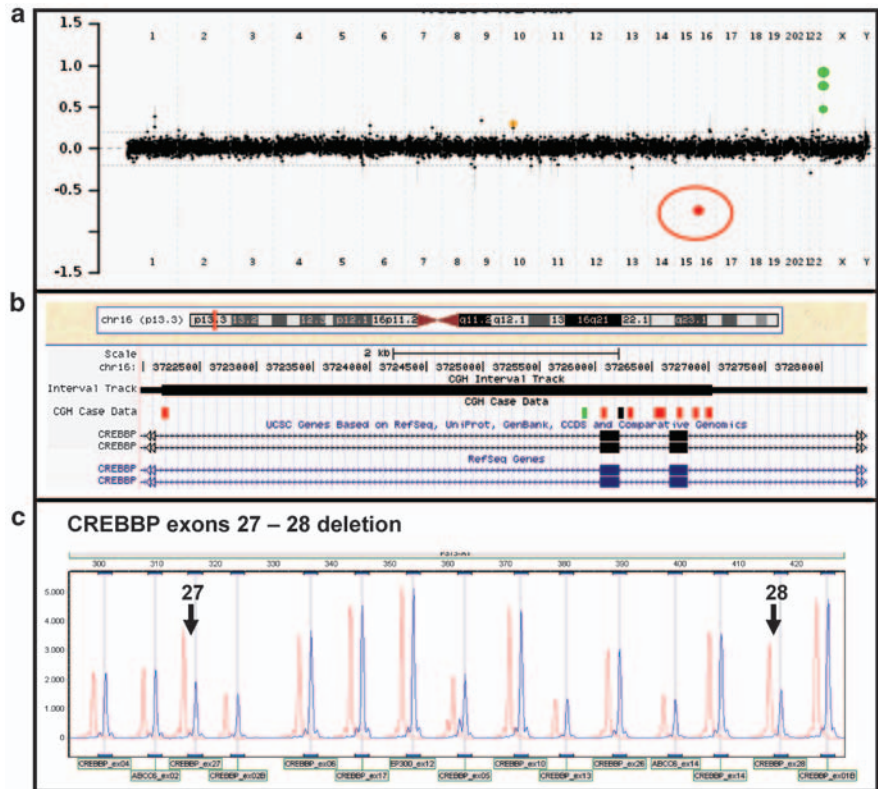


Figure 3 (a) Profile of the microarray analysis showing the deleted region as indicated in the red circle (the gain on Xp as shown in green dots is also present in the mother (data not shown), whereas the yellow dot on chromosome 10 represent a highly polymorphic region, which is also present in the Toronto database). (b) The deleted oligos displayed in the UCSC genome browser (build HG 18) corresponding to exons 27 and 28 of the *CREBBP* gene. (c) The MLPA profile demonstrating the two deleted exons of the *CREBBP* gene (exons 27–28) as indicated by the arrow with the number for each exon.

intragenic rearrangements that would be otherwise missed by aCGH platforms lacking sufficient exonic coverage or sequencing of the gene of interest. The resolution of the current clinical array used in the clinical laboratory setting is about 300–500 kb, which is not sufficient to detect intragenic changes. This version 8 array was designed with exon coverage to detect deletions and duplications with a resolution between 1000 bp and 300 kb. The continuing improvement of cost-effective high-density arrays enables detection of copy-number changes that are <1000 bp in size in the clinical setting. In fact, the design of the high-density region-specific exon array for specific disease genes such as Duchenne/Becker muscular dystrophy demonstrated the ability to detect deletions and/or duplications involving a single exon.¹⁰ An increasing number of new genomic disorders are being described as a result of detection of small intragenic copy-number changes, such as the *MEF2C*¹⁶ and *NRXN1*¹⁷ genes, from high-density clinical arrays such as the one used in this report. Although other technologies, such as MLPA, can also detect intragenic deletions/duplications, it requires that the clinical phenotype be obvious enough to indicate testing for a specific gene. Whereas, an array with this level of resolution does not have this limitation enhancing its value for diagnosing conditions with variable expressivity or that lack hallmark clinical findings.

A total of 3000 samples were referred and evaluated using this version of the exon array during a period of 6 months. The indications for the study population varied from dysmorphic features, moderate developmental delay, mental retardation, multiple congenital anomalies, congenital heart disease to no indication provided. A total of 25 cases with exon deletions were identified and confirmed by PCR or

MLPA and/or sequencing. Among them, three cases were detected with RSTS, although only one case (case 2) had the indication to rule out RSTS. The third case was not reported here due to lack of consent. However, the indications for this case were moderate developmental delay, mental retardation and dysmorphic features. CMA revealed a copy-number loss at band 16p13.3 spanning ~10 kb. This loss deletes exons 11–16 of the *CREBBP* gene. This 3.5-year-old child has normal thumbs, but his great toes appear disproportionately large bilaterally as is seen in RSTS. He also has micrognathia, inverted nipples and prominent globes.

RSTS is a complex autosomal dominant disease. Point mutations and small deletions or insertions of the *CREBBP* and *EP300* genes^{3,18,19}, as well as deletions and duplications >1000 bp in length to megabases, have been shown to lead to RSTS.^{3,20} Stef *et al*²¹ also showed that a deletion as small as 3.3 kb involving a few exons of the *CREBBP* gene can be detected using a set of seven BACs and 34 cosmids region-specific array covering 2 Mb in the 16p13.3 region. It has been well established that deletions involving the *CREBBP* gene are the causative mutation in ~10% of RSTS patient. Traditionally, FISH analysis has been employed to detect these deletions. However, the resolution of FISH analysis is not sufficient for detecting smaller intragenic deletions. With the increased resolution, this exon-targeted array now offers a new opportunity to diagnose patients with RSTS due to copy-number changes in these two genes associated with RSTS in a single assay. It may also prove particularly helpful in diagnosing patients with *EP300* copy-number changes as these patients often do not present with the skeletal findings that are a hallmark feature of this syndrome. Therefore, the RSTS provides us an excellent example to

Table 1 Comparison of the features of patients with EP300 mutations

	Bartholdi patient no. 1	Bartholdi patient no. 2	Bartholdi patient no. 3	Bartholdi patient no. 4	Foley's patient	Zimmerman's patient no. 12	Our patient
Gene/mutation	p.R648X	p.Q1209fsX1226	P.S959fsX966	c.1200-?_94+?del	deletion exon 3–8	p.P2366fsX2401	Deletion exon 24–27
<i>Perinatal period</i>							
Prenancy	NR	Preeclampsia	NR	NR	Preeclampsia, HELLP	NR	Preeclampsia
Delivery	Preterm	Preterm	NR	NR	Preterm	NR	Preterm
<i>Growth</i>							
Birth measurement	BW normal BL normal Normal stature	BW decreased BL normal Short stature	BW normal BL decreased Short stature	NR	IUGR	NR	BW decreased BL decreased Short stature
Stature	Normal stature	Short stature	Short stature	Short stature	Short stature	NR	Short stature
<i>Head and face</i>							
Microcephaly	Prenatal	Postnatal	Prenatal	NR	Postnatal	Postnatal	Prenatal
<i>Ocular region</i>							
Palpebral fissures	Downslanting	Downslanting	Downslanting	Downslanting	Downslanting	NR	Downslanting
Epicantial folds	Not present	Not present	Present	Not present	NR	NR	Mild
Eyebrows	Heavy	Heavy	Heavy	Heavy	Heavy	NR	Heavy
Eyelashes	Normal	Long	Long	Long	Long	NR	Long
Strabismus	Present	Present	Discrete	Present	None	NR	None
<i>Nose</i>							
Shape	Prominent	Beaked	Prominent	Prominent	NR	Beaked	Prominent
Columella	Long	Long	Long	Long, deviated	Prominent	Long	Long
<i>Mouth</i>							
Lips	Pouting lower lip	Small lips	Pouting lower lip	Short upper lip	NR	NR	Thin upper lip
Mandible	Retrognathia	Normal	Retrognathia	Normal	NR	Retrognathia	Normal
Ears/hearing	Posteriorly rotated	Posteriorly rotated	Posteriorly rotated	NR	Performed ear drums	NR	Normal
Dental findings	Crowded	Crowded, marked caries	Crowded talon cusps	NR	NR	Protruding incisors	Small teeth
Respiratory	None	Respiratory distress	None	NR	Infections	NR	None
Gastrointestinal	NR	Reflux, chronic constipation	None	NR	Feeding difficulties, reflux	NR	None
Cardiac abnormalities	Normal	Asymmetric aortic valve	Normal	NR	NR	NR	Normal
Genitalia	Cryptorchidism	Synechia of labia majora	Normal	NR	Cryptorchidism	NR	Normal

Table 1 (Continued)

	Bartholdi patient no. 1	Bartholdi patient no. 2	Bartholdi patient no. 3	Bartholdi patient no. 4	Foley's patient	Zimmerman's patient no. 12	Our patient
Skeletal findings							
Hands	Normal	Normal	Short phalanges, broad thumbs, irregular ossification of phalanges	Normal	Slightly broad thumbs	Broad thumbs	Normal
Feet	Flat, broad halluces	Normal	Proximally implanted and broad halluces, splaying of other toes, partial duplication distal phalanx of hallux	Slightly broad halluces	Slightly broad halluces	Broad halluces	Normal
CNS	Muscular hypertonia with discrete spastic paraplegia	NR	NR	NR	NR	NR	None
Mental development							
Cognitive abilities	Nearly normal	Moderate MR	Slight MR	Slight MR	NR	NR	NR
Language	Normal	Severely impaired	Speech delay	Normal	Speech delay	Borderline normal	Speech delay
Behavior	Hyperactive	Hyperactive	Normal	NR	NR	NR	Hyperactive
Personality	Very sociable	Very sociable	NR	NR	Happy but distant	NR	Sociable
Miscellaneous	Fetal finger pads	Hirsutism	Hypoglycemia	Complex febrile seizures, growth hormone deficiency, coeliac disease, chronic anemia	Hirsutism	Pronounced overbite	Hirsutism

illustrate our capability in detecting small CNV to yet a higher level of resolution in the clinical setting, particularly if a specific gene cannot be targeted for testing based on the clinical findings.

Mutations and deletions in *EP300* are rare causes of RSTS. Our case is only the seventh case reported to date.^{3,4,11,12} The true percentage of RSTS caused by *EP300* alterations and the full phenotypic picture of *EP300* RSTS is therefore unclear. The diagnosis in our case was considered because of the typical RSTS facial dysmorphism. However, due to the absence of broad and angulated thumbs and halluces, microarray testing was initiated first. *CREBBP* testing was to be performed as second-tier testing if the microarray was normal. The facial phenotype in our patient resembles the case (patient 2) described by Bartholdi *et al*⁴ and Foley *et al*¹² with very distinct arched bushy eyebrows and hirsutism over the lumbar region. Table 1 summarizes the reported cases of *EP300* mutations, including pregnancy history and other physical and developmental features.

Interestingly, the mother of Bartholdi's second patient presented with preeclampsia, and the mother of Foley's patient had preeclampsia progressing to hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome.^{4,12} Our patient's mother also developed preeclampsia; as she went into premature labor, it is possible that there was no chance for HELLP syndrome to develop. This was the third pregnancy of the seven reported cases to have preeclampsia, which we believe is a solid association. However, a literature search revealed no genes associated with *EP300* that are known to also be associated with familial preeclampsia or HELLP syndrome. It is possible that as more genetic links are identified for preeclampsia and HELLP syndrome, an association with *EP300* may become apparent. Of note, Lachmeijer *et al*²² performed a genome scan using 293 polymorphic markers in 67 Dutch sib-pair families affected by preeclampsia, eclampsia or HELLP syndrome. Analysis in 38 preeclampsia families showed suggestive evidence for linkage on chromosome 22q at 32.4 cM (LOD score of 2.41), which is in close proximity to the *EP300* gene and on chromosome 10q at 93.9 cM (LOD score of 2.38).

In conclusion, we report on two cases of RSTS due to exonic deletions detected by a clinical microarray analysis and only the seventh case of RSTS due to an *EP300* mutation. Both our case and others in the literature with *EP300* mutations have a modified RSTS phenotype, and, in particular, we confirm the milder skeletal phenotype. In addition, we propose that preeclampsia during pregnancy may be a new feature for RSTS caused by *EP300* mutations. Together, these cases demonstrate the importance of considering *EP300* RSTS in patients who have facial features of RSTS but lack broad and angulated thumbs and halluces. As this array can simultaneously evaluate for copy-number changes in both genes, it may be considered as the first line of testing for patients with suspected RSTS. This exon-targeted array may also replace the del/dup assay by MLPA to compliment sequencing analysis for these two genes. Our case demonstrates the utility of microarray for ascertaining atypical cases and further defining the phenotypic spectrum of RSTS.

CONFLICT OF INTEREST

The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the chromosomal microarray analysis offered in the Medical Genetics Laboratories.

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