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Rapid Communication

Disruption of the *CREBBP* gene and decreased expression of CREB, NFκB p65, c-JUN, c-FOS, BCL2 and c-MYC suggest immune dysregulation

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ABSTRACT

Genomic aberrations in the *CREBBP* (CREB-binding protein – *CREBBP or CBP*) gene such as point mutations, small insertions or exonic copy number changes are usually associated with Rubinstein-Taybi syndrome (RTs). In this study, the disruption of the *CREBBP* gene on chromosome 16p13.3, as revealed by CGH-array and FISH, suggests immune dysregulation in a patient with the Rubinstein Taybi syndrome (RTs) phenotype. Further investigation with Western blot techniques demonstrated decreased expression of CREB, NFκB, c-Jun, c-Fos, BCL2 and cMyc in peripheral blood mononuclear cells, thus indicating that the *CREBBP* gene is essential for the normal expression of these proteins and the regulation of immune responses.

1. Introduction

The *CREBBP* or *CBP* gene, which is located on chromosome 16p13.3, codes for a multidomain protein essential in embryonic development, growth control and homeostasis by coupling chromatin remodeling to transcription factor recognition [33]. The *CREBBP* gene was first described by Chrivia et al. [12] as a transcriptional coactivator. Its nomenclature is based on the interaction of the CREB protein with nuclear *CREBBP* [12]. The *CREBBP* gene has 31 exons, occupies approximately 159 kb of genomic DNA and encodes a protein of 265 kDa with 2442 amino acids [18]. *CREBBP* gene mutations lead to inactivation of one allele in patients with Rubinstein Taybi syndrome (RTs) [40,41]. The *CREBBP* gene appears to be crucial at certain stages of embryonic development, which could explain all the phenotypic changes that characterize RTs patients [19,20]. Most of the conserved domains of CREBBP function to bind a diverse array of proteins, including more than 50 vital T-cell transcriptional regulators [30]. Originally, the *CREBBP* gene was found to act as a physical link between DNA-binding transcription factors.

Furthermore, *CREBBP* facilitates transcription through intrinsic histone acetyltransferase (HAT) activity, which opens up condensed DNA and thereby allows greater access to the transcriptional machinery. CREBBP has been shown to interact with CREB, c-Jun, NF-κB and c-Fos. All of these transcription factors play a critical role in regulating the expression of many inflammatory and immune response genes [5,27,17,39,6]. Studies have shown the involvement of CREBBP in regulating the immune response and cell differentiation; failure of the gene leads to the formation of benign and malignant tumors and hematologic malignancies [4,54,11].

RTs usually occurs sporadically, although it can be inherited as an autosomal dominant disorder (OMIM 180849) [21,22]. The diagnosis is still essentially clinical and based on characteristic features [22]. The major items to look for in RTs are a beaked nose, grimacing smile, broad thumbs and big toes, and mental retardation [23]. The main clinical problems are failure to thrive, congenital heart defects, and recurrent respiratory infections [48]. *EP300*, a gene located on chromosome 22q13.3, has been identified as another gene involved in causing RTs [43,44]. Mutations in the *EP300* gene in RTs patients are rare and have only been found in eight individuals with clinical signs of incomplete or non-classical syndrome [7,58,9,16,51].

We performed an immunological screening of the major mechanisms of adaptive and innate immune response in a patient with an apparent balanced translocation involving chromosomes 2 and l6, previously described by Torres et al. [49,50], and clinical features of RTs. Further investigations using CGH-array, FISH and Western blot techniques revealed a disruption of the *CREBBP* gene that resulted in decreased expression of the CREB, NFκB p65, c-JUN, c-FOS, BCL2 and c-MYC proteins in peripheral blood mononuclear cells.

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2. Materials and methods

2.1. Clinical report

The patient is the third child of healthy unrelated parents. She was born pre-term by cesarean section; her birth weight was 1900 g (below the 5th percentile), her length was 47 cm (5th – 10th percentile), and her head circumference was 36 cm (75th percentile). Her global development was delayed: she could sit without support at 13 months, walk unassisted at 2.5 years, had toilet control at 4 years and she spoke her first meaningful words at 18 years. She had frequent feeding problems during her first five years of life. Splenomegaly was noted at 21 years of age.

At 23 years of age, the patient's weight was 61 kg (50th – 75th percentile), her height was 139.5 cm (below the 3rd percentile) and her OFC was 49.5 cm (below the 3rd percentile). She was found to have highly arched eyebrows, down-slanting palpebral fissures, a beaked nose, a *nasal septum* that extended *below* the *alae* nasi, a highly arched palate and *posteriorly* angulated *ears.* She also had broad, radially deviated thumbs and broad halluces. She presented mild leukocytosis (11,000–13,000/mm^3) despite the absence of clinical signs of infection. A high rate of B cell apoptosis and a 40–50% decrease of the total polymorphonuclear leukocyte count with dysgranulopoietic granulocytes were also observed by hemogram.

2.2. DNA isolation

Blood samples from the patient and her parents were collected with vacuum-EDTA after informed consent (approved by the Research Ethics Committee of HC-FMUSP CAPPESQ number 1158/07). DNA extraction was carried out using the Qiagen QIAamp® DNA blood mini kit (Valencia, CA, USA).

2.3. Western blot analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from one RTs patient and controls. Total protein was extracted using a PARIS™ Kit (Applied Biosystems, Ambion, USA) and a mixture of protease inhibitors according to the manufacturer's protocol. Protein concentration was determined using a Nanovue spectrophotometer (GE Healthcare, Piscataway, NJ, USA).

Protein samples from each subject were individually loaded (60 μg per lane) onto 10% acrylamide gels, resolved by electrophoresis, and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk and subsequently incubated overnight at 4 °C with the following primary antibodies: anti-CREB (1.0 μg/mL), anti-cFOS (0.2 μg/mL), anti-cJUN (0.5 μg/mL), anti-NFκB p65 (0.5 μg/mL), anti-BCL2 (1.0 μg/mL) and anti-cMYC (0.5 μg/mL). All the antibodies used in this study were obtained from Chemicon (Millipore, USA). Primary antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were developed using Luminol Western Blotting Substrate according to the manufacturer's instructions (Millipore, USA) and detected by chemiluminescence. The obtained bands were quantitated and photographed in the LAS 4000 (Fujifilm, SA) using *Image 4000 software* (Fujifilm, SA).

3. Molecular cytogenetic analysis

3.1. Array-CGH studies

Agilent Human Genome CGH 244A microarrays (Agilent Technologies, Santa Clara, CA, USA) containing unique oligonucleotides representing 244,000 probes with an average probe spacing across the human genome of 6.4 Kb were used for the CGH-array experiments. Human genomic DNA from multiple anonymous male donors was obtained from Promega Corporation (Madison, USA) and used as a control. A microarray assay was performed using the Agilent Human Genome CGH 244A microarray according to the manufacturer's protocol version 2.0 (August 2005) for Oligonucleotide Array-Based CGH for high-throughput whole genomic DNA analysis (Agilent Technologies, Inc., Palo Alto, USA). The array was scanned and analyzed using an Agilent 2565AA DNA microarray scanner (Agilent Technologies, Inc., Palo Alto, USA) and Feature Extraction software, respectively, according to NCBI 36/hg 18 annotations.

3.2. Fluorescence in situ hybridization (FISH)

Blood samples from the patient and her parents were processed by standard cytogenetic procedures. FISH was performed on metaphase cells using different cosmid probes (RT1, RT100, RT102, RT191, RT203, and RT166) that cover the entire *CREBBP* gene except for the ~5 kb uncloned area between RT203 and RT166. A chromosome 16-specific centromeric probe was co-hybridized with probe pHUR195 in accordance with [41], with modifications. FISH with probes RP11-690I21 from 2q37.1 and RP11-90D1 from 2p16.1 was also used to investigate chromosomal breakpoints (probes were obtained from Baylor College of Medicine-Medical Genetics Laboratories, Houston, Texas) [32].

4. Results

4.1. Cytogenetic and molecular findings

The previous GTG-banding chromosome analyses of the patient demonstrated a 46,XX,t(2;16) karyotype, suggesting a balanced translocation. The parents presented normal karyotypes.

Because no apparent deletions in the *CREBBP* gene that could explain the RTs phenotype were identified, CGH-array analysis was performed to investigate the presence of microdeletions or other genomic imbalances not detected by standard techniques such as GTG-banding.

The CGH-array result showed that the patient carried benign copy number alterations, namely, losses of approximately 400 bp at 19p13.3 (794,778–795,178) also present in patient's father, and losses at 16q23 (76,938,523–76,938,923) that contain no genes or evidence in the literature associated with any pathogenic features. The patient's mother also carried a small deletion of approximately 232 bp at 16p13.3 (2,638,381–2,638,612) with no clinical significance [14,26]. Quite surprisingly, microdeletions in the 16p13.3 region were not detected in the patient as expected. Subsequent FISH analysis showed that the translocation revealed in classical karyotype analysis in fact involved the short arms of chromosomes 2 and 16. The corrected karyotype is 46,XX.ish t(2;16)(p11.2;p13.3) (RT100+,RT166−;RT100+;RT166). Therefore, we concluded that the *CREBBP* gene is disrupted by this genomic rearrangement (Fig 1).

4.2. Western blot findings

Western blot analysis showed low amounts of CREB, NFκB p65, AP-1 (c-Jun and c-Fos), Bcl2 and cMyc in the patient's peripheral blood mononuclear cells. Image quantification analysis showed that the expression of these proteins was diminished by 50% in the patient's cells when compared to two healthy controls, as depicted in Fig. 2.