A novel intragenic microdeletion in \( \textit{RUNX2} \) in a Chinese family with cleidocranial dysplasia

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Received January 19, 2016; Accepted April 22, 2016; Epub July 1, 2016; Published July 15, 2016

Abstract: Objective: Cleidocranial dysplasia (CCD) is a rare autosomal dominant skeletal dysplasia which is characterized by cranial, clavicular, and dental anomalies. Aberrations in the \( \textit{RUNX2} \) gene, which is considered to be responsible for CCD, were investigated in a Chinese family with CCD in this study. Methods: Genomic DNA was isolated from the blood samples of all 11 participants, including 3 patients in this family. Mutation analysis of \( \textit{RUNX2} \) was performed using amplified polymerase chain reaction and direct sequencing. To determine the copy number of the exon of \( \textit{RUNX2} \), real-time quantitative PCR was performed for all 3 patients and the 8 unaffected individuals. Results: A previously reported SNP (rs6921145) was found, but no causative mutation was detected in the coding regions of \( \textit{RUNX2} \) by direct sequencing analysis. Real-time quantitative PCR revealed a novel exon 4 to exon 7 intragenic deletion in \( \textit{RUNX2} \) in all 3 affected family members. Conclusions: Our findings suggest that the intragenic deletion from exon 4 to exon 7 in the \( \textit{RUNX2} \) gene is the cause of CCD in this family and most likely causes CCD by altering the protein structure of \( \textit{RUNX2} \), which then fails to regulate the transcription of \( \textit{RUNX2} \)-regulated genes. We therefore highlight the importance of considering deletions and duplications in patients without a causative mutation identified by DNA sequencing.

Keywords: Cleidocranial dysplasia, CCD, runt-related transcription factor 2, \( \textit{RUNX2} \), intragenic deletion

Introduction

Cleidocranial dysplasia (CCD; MIM 119600), also known as Scheuthauer-Marie-Sainton disease or cleidocranial dysostosis, was initially described accurately by Scheuthauer in 1871 [1]. It is a rare human autosomal dominant skeletal disease with high penetrance and variable expressivity [2, 3]. The primary clinical features of CCD include delayed closure of the cranial fontanels and sutures, rudimentary or absent clavicles, wormian bones, frontal bossing, supernumerary and late erupting teeth, wide pubic symphysis, short stature, and other skeletal anomalies [4, 5]. Although tooth anomalies and some degrees of clavicular hypoplasia seem to be the most consistent features of this disease, the phenotypic spectrum varies widely, from mildly affected individuals with only dental abnormalities to severely affected cases with generalized osteoporosis [6].

CCD is usually caused by mutations in the runt-related transcription factor 2 gene (\( \textit{RUNX2} \), MIM 600211; formerly known as \( \textit{CBFA1} \), \( \textit{PEBP2ab} \) and \( \textit{AML3} \)), which was mapped to chromosome 6p21 and belongs to a family of transcription factors with homology to the \( \textit{Drosophila} \) pair-rule gene runt [7, 8]. \( \textit{RUNX2} \) has an important regulatory role in the differentiation of osteoblasts and in the maturation of chondrocytes [9, 10]. Mice with a homozygous mutation in \( \textit{RUNX2} \) completely lack osteoblasts and bone and die of respiratory failure shortly after birth; \( \textit{RUNX2} \) haplo-insufficiency in mice produces a CCD phenotype, with a primary defect in intramembranous bone formation [11, 12]. The \( \textit{RUNX2} \) gene is over 220 kb in length and consists of 8 coding exons, with 3 known transcripts [13]. To date, more than 100 mutations in \( \textit{RUNX2} \) have been reported in association with familial and sporadic cases of CCD; these mutations include missense mutations,
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Figure 1. The pedigree of the CCD family. Squares, males; circles, females; open symbols, unaffected members; solid symbols, patients with CCD. The arrow indicates the proband.

nonsense mutations, frameshift mutations and splice mutations and large chromosomal deletions, insertions, and translocations [14]. However, stringent genotype-phenotype correlations have not been found to date [15].

To our knowledge, most of the data on RUNX2 mutations in CCD patients have been generated from Caucasian populations, and few RUNX2 mutations have been reported in Chinese CCD patients. In this study, we analyzed a Chinese family showing a typical CCD phenotype. No mutation was found in the 8 coding exons of RUNX2 by sequencing analysis. Further analysis using real-time PCR identified a novel deletion in one allele of the RUNX2 gene, which emphasizes the contribution of such deletions in RUNX2 to the etiology of this disease.

Materials and methods

Identification and clinical assessment

The CCD family was evaluated in the Department of Prosthodontics, Stomatological Hospital of Tianjin Medical University. The study was conducted with the informed consent of all 11 participants and was approved by the Ethical Committee of Tianjin Medical University, Tianjin, China. The 11 participants belonged to 3 generations of this family, and there were 3 individuals with CCD (Figure 1). Clinical evaluations were performed on all of the participants to look for typical signs of CCD by radiographic techniques, including roentgen cephalometry to detect abnormalities in skulls and panoramic X-rays to assess the dental phenotype. In addition, somatic development was assessed using the body height and weight and was compared with normative data. Samples of peripheral blood from the 11 family members were collected into tubes containing EDTA as an anti-coagulant.

DNA extraction

Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp Blood Midi kit (Qiagen, German) according to the manufacturer's instructions. The concentration and quality of genomic DNA were determined by measuring the UV absorbance at 260 nm and 280 nm (A260/280) and by gel electrophoresis.

Sequencing and mutation screening

The nucleotide sequence of RUNX2 was obtained from GenBank (MIM: 600211). The 8 primers used to amplify the 8 coding exons of RUNX2 are listed in Supplementary Table 1. PCR reactions were performed using approximately 50 ng of template DNA, 0.4 µmol/L of each primer and 12.5 µL of Taq PCR Master-Mix (Life Feng, China) in a total reaction volume of 25 µL. The cycling conditions (Touchdown System) were 5 minutes at 95°C; 18 cycles of 30 seconds at 94°C, 30 seconds at 66°C (decreased by 0.5°C after every cycle) and 1 minute at 72°C; and 25 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 1 minute at 72°C. A final extension step of 10 minutes at 72°C was also performed. The PCR products were purified with the QIAquick PCR Purification kit (Qiagen, German), and the purified PCR products were sequenced using the forward and reverse primers. The products of the sequencing reactions were loaded onto an ABI 3730 Automatic Sequencer (Applied Biosystems, USA), which was operated by Invitrogen Corporation in Shanghai, China. The sequences were analyzed with DNAStar.

Copy number variation analysis

The copy numbers of the exons of RUNX2 were determined by real-time quantitative PCR. Three independent experiments were performed to determine the copy number variation between CCD patients and normal individuals, with duplicate samples for each experiment. The DNA was amplified in a reaction volume of 25 µL containing 12.5 µL of 2×SYBR® Premix Ex Taq™ (TaKaRa, Japan), 10 µL of primer mix
and 2.5 μL of DNA. The qPCR reactions were performed using the ABI Prism® 7900HT Sequence Detection system, and the fluorescence signal intensity was recorded on ABI Prism 7900HT Sequence Detection system and analyzed by Sequence Detector v2.3 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The formula for calculating the copy number was the following: copy number = $2^{\text{ΔCt}}$, where Ct was the threshold cycle defined as the mean cycle at which the fluorescence curve reached an arbitrary threshold, ΔCt was the Ct of RUNX2 minus the Ct of GAPDH, ΔCp was the

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Height (cm)</th>
<th>Cranial</th>
<th>Shoulders</th>
<th>Clavicle</th>
<th>Teeth</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>II5</td>
<td>40</td>
<td>165</td>
<td>frontal bossing, delayed closure of the fontanel</td>
<td>mild sloping, bilateral</td>
<td>normal</td>
<td>multiple supernumerary teeth</td>
<td>hypoplasia of the maxilla</td>
</tr>
<tr>
<td>III5</td>
<td>18</td>
<td>155</td>
<td>frontal bossing, delayed closure of the fontanel</td>
<td>sloping, bilateral</td>
<td>absence of the lateral 1/3, right</td>
<td>impacted permanent teeth, retention of the deciduous teeth</td>
<td>hypoplasia of the maxilla, hypertelorism</td>
</tr>
<tr>
<td>*III6</td>
<td>11</td>
<td>120</td>
<td>frontal bossing, wide-open fontanel</td>
<td>severely sloping, bilateral, increased mobility</td>
<td>absence of the lateral 2/3, bilateral</td>
<td>impacted permanent teeth, retention of the deciduous teeth</td>
<td>hypoplasia of the maxilla, hypertelorism, wide pubic symphysis</td>
</tr>
</tbody>
</table>

*: The proband of this CCD family.

Figure 2. Radiographic analysis of the proband in this family. A: The chest X-ray indicates a cone-shaped chest, a high position of the scapular bone, and aplasia of the lateral and middle thirds of the clavicles. B: Wide pubic symphysis observed by X-ray. C: Cranial X-ray showing abnormal wormian bones and a wide-open fontanel. D: Panoramic X-ray showing dental anomalies, including the delayed loss of primary teeth and the delayed eruption of permanent teeth.
ΔCt of the patients, and ΔCn was the ΔCt of the normal individuals. Eight unaffected individuals were included in these experiments.

**Results**

**Clinical findings**

Patients with CCD were diagnosed according to the criteria reported by Mundlos in 1999 [16]. We identified a Chinese family with 18 members, spread over 3 generations, among whom there were 3 individuals affected with CCD (Figure 1). All 3 affected individuals in the CCD family exhibited delayed fontanel closure, frontal bossing, dental anomalies, and short stature. The clinical features of the patients in this CCD family are summarized in Table 1. The radiographic characteristics of the proband are shown in Figure 2.

**Molecular analysis of the RUNX2 gene**

The 8 coding exons of the RUNX2 gene, including the flanking splice recognition sequences, were screened for mutations using direct sequencing methods. The results revealed no causative mutations detected in the coding regions of the RUNX2 genes of the 3 affected individuals (II5, III5, III6). However, a previously reported SNP (rs6921145) that does not alter the normal RUNX2 polypeptide sequence was detected in the second coding exon of the RUNX2 gene (Figure 3).

Real-time quantitative PCR (RT-qPCR) was performed to determine the copy number of each of the exons of RUNX2. The qPCR results of the 3 CCD patients and 8 normal individuals revealed that there was a deletion from exon 4 to exon 7 in RUNX2. In this region, all 3 patients had a copy number of one, whereas the unaffected individuals had the normal copy number of two (Figure 4). This result demonstrated that the intragenic deletion was the cause of CCD in this family.

**Discussion**

CCD is an autosomal dominant skeletal disease caused by mutations in the RUNX2 gene [17]. Herein, we reported the results of a clinical and molecular study of 3 patients in a Chinese CCD family. In this family, CCD was transmitted as an autosomal dominant trait with high penetrance and variable expressivity. All patients exhibited typical CCD features such as short stature, delayed or open skull sutures, frontal bossing, and dental abnormalities. Some other common clinical features manifested in this family included hypoplasia of the maxilla, which was identified in all 3 patients, and hypertelorism, which was present in the 2 brothers. Vertebral alterations and wide pubic symphysis were infrequent and observed only in the proband of the family. This result confirmed the findings of previous studies showing that there is considerable phenotypic variation for CCD, even within families [18]. Interestingly, in the present family, multiple supernumerary teeth were only found in the proband’s father, and the other brothers had impacted permanent teeth and retained deciduous teeth, characteristics that are consistent with the previous reports that the spectrum of the dental abnormalities in CCD shows intra-familial variability [8, 19, 20]. Moreover, the impacted teeth and extra teeth in this family had abnormal roots with delayed development. It seems that RUNX2 mutations can induce abnormal root development, which may be one reason for the failure of the eruption of permanent teeth in CCD patients. In CCD patients, a common clinical feature and a common reason for visiting a medical clinic are dental abnormalities [21]. Therefore, it is very important for dentists to carefully examine patients to allow the diagnosis of this disease.

RUNX2, responsible for the skeletal disorder CCD, is a transcription factor of the runt family [22]. This protein family is characterized by a highly conserved region of 128 amino acids, termed the Runt domain. This domain has the unique ability of mediating DNA binding and protein heterodimerization with CBF-β [23]. The carboxyl terminus of the Runt domain contains a nuclear localization signal (NLS) that is essential for accumulation of the RUNX2 protein in the nucleus [6]. The C-terminus of RUNX2 is a region rich in proline, serine, and threonine (PST) and is thought to be the transactivation
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Figure 4. Analysis of the RUNX2 copy number using RT-qPCR. The copy number of RUNX2 was determined for each exon. II5, III5, and III6 were the patients and others were the controls.

A domain that is necessary for RUNX2-mediated transcriptional regulation. This domain is also involved in functional interactions with various other transcription factors, co-activators, and co-repressors [24]. According to the data collected to date, mutations in RUNX2 account for up to 65% of cases of CCD, whereas micro deletions are observed in approximately 13% of patients by FISH and approximately 25% by qPCR [25, 26]. The almost two-fold higher frequency of micro deletions was found partly due to the higher resolution of the methods applied. The qPCR method is preferred to use for screening intragenic deletions or duplications in CCD patients, not only because it is comparatively cheap and easy to perform, but also because it has a much higher efficiency in identifying smaller copy number changes than array CGH and FISH do.

In the current study, we identified a novel RUNX2 microdeletion in 3 patients from a Chinese family by qPCR. This deletion created a truncated protein without most of the C-terminal domain. Without the transactivation domain, this protein was unable to interact with the other transcription factors and failed to modulate the transcription of RUNX2-regulated genes. Consequently, CCD phenotype arose as a result of haploinsufficiency of RUNX2. In addition, the report by El-Gharbawy and colleagues demonstrated patients with C-terminal deletions showed a more severe phenotype [27]. However, we did not observe this genotype-phenotype correlation in our patients.

The work described herein can also serve as a good example for other studies. Haploid insufficiency is thought to be an alternative mechanism of a disease when no mutation is found. A deletion/duplication assay, either RT-qPCR or MLPA (multiplex ligation-dependent probe amplification), would allow the efficient detection of gene deletions and duplications.

There are, however, still approximately 16% cases that lack RUNX2 mutations, a result that suggests the involvement of factors other than RUNX2 in the development of CCD [14]. In this CCD family, each affected individual has different clinical features despite carrying the same germline deletion in RUNX2. This phenomenon is consistent with the reported data that patients with variable and protean phenotypes could share a common mutation in RUNX2 [18, 19]. These data also support the hypothesis that some environmental and/or other factors might affect the final phenotypic outcome.

The analysis of the RUNX2 gene in CCD will provide important information not only for genetic counseling but also for further expanding the clinical phenotypes and mutation spectrum. Further studies are needed to define the association between the altered protein and its clinical features.

Acknowledgements

We thank these members in this family for their willingness to participate in this study. This
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study was supported by the Specialized Research Fund for the Doctoral Program of Higher Education (Projects No: 2012120212-0015).

Disclosure of conflict of interest

None.

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[22] Bufalino A, Paranaíba LM, Gouvêa AF, Gueiros LA, Martelli-Júnior H, Junior JJ, Lopes MA,


**Supplementary Table 1.** Primers used for amplification of 8 coding exons of *RUNX2* gene

<table>
<thead>
<tr>
<th>Coding Exons</th>
<th>Primers (5’-3’)</th>
<th>Primers (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: GTTTGGGTATGGTTGTATT</td>
<td>R: TTTCTCTGGTGGGTTA</td>
</tr>
<tr>
<td>2</td>
<td>F: CCCGGCCACTTC GCTACTT</td>
<td>R: GGCAGGAGGCTTGGAGGAC</td>
</tr>
<tr>
<td>3</td>
<td>F: AACACTAAGTCTGTAAAGAC</td>
<td>R: GAAGGTGCTGATTGATACAC</td>
</tr>
<tr>
<td>4</td>
<td>F: TCAATTGCTTCTTAAAGATGC</td>
<td>R: GGACATGAAAGTGACACTAC</td>
</tr>
<tr>
<td>5</td>
<td>F: TATAAAGCTTAAAGGGAAGG</td>
<td>R: GTTTGAAAGTGACACATCTCC</td>
</tr>
<tr>
<td>6</td>
<td>F: TAAGGCTGCAATGGATTGCTAT</td>
<td>R: GTCAGTGAGCATGAGGAG</td>
</tr>
<tr>
<td>7</td>
<td>F: TAGAAGCTTAGAGCTTGAAGG</td>
<td>R: CGGACAGTAACACACAGACAG</td>
</tr>
<tr>
<td>8</td>
<td>F: TGTGGCTTGTCTGATTTCATT</td>
<td>R: GATACACCTGGGCCACTGCT</td>
</tr>
</tbody>
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