Whole-exome sequencing of a novel initiation codon mutation in RUNX2 in a Chinese family with cleidocranial dysplasia

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Abstract
Cleidocranial dysplasia (CCD) is mainly attributable to a variant of runt-related transcription factor 2 (RUNX2) on chromosome 6p21. CCD is an autosomal dominant skeletal disorder characterized by open/delayed closure of fontanels, clavicular hypoplasia, retention of deciduous teeth, and supernumerary permanent teeth. The aim of this study was to investigate potentially pathogenic mutations in 2 Chinese families. Genomic DNA was obtained from peripheral blood lymphocytes, and whole exome sequencing and Sanger sequencing were performed to detect gene variants. Real-time quantitative PCR was performed to determine the mRNA expression level of RUNX2 in the proband of family 1. Silico algorithms and conservation analyses were used to evaluate the functional impact. We identified a novel initiation codon mutation (c.2T>C) and a previously reported mutation (c.569G>A). Familial co-segregation verified an autosomal-dominant inheritance pattern. Our findings demonstrated that the novel mutation c.2T>C causes CCD. Quantitative real-time PCR suggested that downregulated RUNX2 levels and haploinsufficiency in RUNX2 lead to CCD. These results extend the spectrum of RUNX2 mutations in CCD patients and can be used for genetic consultation and prenatal diagnosis.

Abbreviations: CCD = cleidocranial dysplasia, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, RUNX2 = runt-related transcription factor 2.

Keywords: cleidocranial dysplasia, initiation codon variant, RUNX2 gene, whole-exome sequencing

1. Introduction
Cleidocranial dysplasia (CCD, MIM 119600), with a prevalence rate of 1:1,000,000, is a rare autosomal dominant skeletal dysplasia characterized by defective skull ossification, open/delayed closure of fontanel, clavicular hypoplasia, delayed ossification of the pelvis, short stature, retention of deciduous teeth, and supernumerary permanent teeth.\cite{1,2,3,4} The phenotypes of CCD have wide variations, ranging from patients with only mild dental deformities to those with severe bone abnormalities.\cite{3,4} Environmental and epigenetic factors may contribute to the diverse phenotypes.\cite{3}

Variants in the runt-related transcription factor 2 gene (RUNX2, MIM 600211) account for 60% of the CCD cases.\cite{6,7} RUNX2 is located on chromosome 6p21 and has 8 coding exons.\cite{6} RUNX2 is a key regulator of osteoblast differentiation and bone development.\cite{7} Homozygous Runx2 knockout mice (Runx2\textsuperscript{−/−}) exhibited a lack of osteoblasts, failed in both intramembranous and endochondral ossification, and showed early lethality after birth. In contrast, heterozygous Runx2 knockout mice (Runx2\textsuperscript{+/−}) displayed a phenotype similar to that of CCD patients with respect to hypoplastic clavicles and defective skull formation.\cite{8,9}

Currently, the Human Gene Mutation Database (HGMD Professional 2020.4 http://www.hgmd.cf.ac.uk/) has 202 registered RUNX2 variants responsible for CCD with various severities, namely, missense/nonsense (80), splicing (11), small/gross deletions (69), small insertions (28), gross insertions/duplications (8), indels (2), and complex rearrangements (4).
However, no apparent genotype-phenotype correlations have been investigated, and only a few RUNX2 mutations have been reported in Chinese CCD patients.

In the present study, we have reported a novel initiation codon variant in 1 RUNX2 allele (c.2T>C) as well as a previously reported missense mutation in 2 Chinese Han families with CCD.

2. Material and methods

2.1. Ethical approval and subjects

This work was approved by the Ethics Committee of the Affiliated of Stomatology Hospital of Hebei Medical University (No: [2016]004). All participants or their guardians signed written informed consent. The probands (Fig. 1, Family 1, II-1; Fig. 2, Family 2, II-1) were initially evaluated and diagnosed with CCD by Hebei Children’s Hospital, and they came to the Hospital of Stomatology, Hebei Medical University, for dental treatment. All family members were checked by 2 experienced dentists, who performed oral and radiographic examinations.

2.2. DNA sample collection and extraction

The QIAmp DNA Blood Midi kit (Cat#51185; Qiagen, Hilden, Germany) was used to obtain genomic DNA from the peripheral blood lymphocytes of all participants, as per the manufacturer’s protocols. DNA purity was analyzed with the NanoPhotometer spectrophotometer (Implen, Westlake Village, CA) and quantified with the Qubit DNA assay kit and Qubit 2.0 fluorimeter (Life Technologies, Carlsbad, CA).

2.3. Whole exome sequencing and Sanger sequencing

Whole exome sequencing was performed for the probands. Target enrichment and amplification were performed using the liquid-phase capture method with testing kits from iGeneTech.
The NovaSeq 6000 platform (Illumina Inc, Santiago, CA) was used to sequence exons from the targeted regions. With a sequencing yield of more than 14,555.7 Mb raw bases, the samples achieved a mean target depth of 140×. The reads were aligned to Genome Reference Consortium Human Build 37 (GRCh37/hg19) with Burrows-Wheeler Aligner, and single-nucleotide variants and small insertions and deletions (indels) were identified with SAMtools (http://www.htslib.org/). Single-nucleotide polymorphisms and indels were identified using the Genome Analysis Toolkit and annotated with ANNOVAR (https://annovar.openbioinformatics.org/). Candidate variants were filtered according to the following criteria: MAF < 1% and exonic. The candidate mutation of RUNX2 was verified with Sanger sequencing. Primers of exon2 of RUNX2 (reference sequence NM_022336.4) were as follows: F: 5'-GTCACTAC-CAGCCACCGAGA-3'; R: 5'-TTGGAAAAGCTAGCAGTT-TATCA-3'. Primers of exon4 were: F: 5'-AGTGGGCA-TCAAACCCCATACA-3'; R: 5'-CCTGAACCTCATCTG-GATGTATTT-3'. The reference sequence for RUNX2 was NM_001024630.4. We identified the nucleotide variant in RUNX2, and we used 100 unrelated population-matched controls.

2.4. Quantitative real-time PCR for mRNA expression of RUNX2

The cDNAs were produced; Maxima SYBR Green/ROX qPCR Mastermix (Cat#K0221; Thermo Fisher Scientific) and ABI7500 real-time PCR system (Applied Biosystems) were used to check relative mRNA expression levels, which were evaluated with the 2−ΔΔCt method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The following primers were used for qPCR: RUNX2 forward, 5'-AAGTAGCAAGGTT-CAACGATCTG-3', and reverse, 5'-TTCCCGAGGTCATCT-TACTG-3'; GAPDH forward, 5'-TGTTGTTGAAACCATA-GGAAGT-3', and reverse, 5'-TGAGTCTTTCCCAGGATAC-CAA-3'.

2.5. Bioinformatics analyses

Pathogenicity of the novel variant was predicted using Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org), polymorphism phenotyping (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), and MutationTaster (http://www.mutationtaster.org). Conservation analysis of the RUNX2 missense variants in different species was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) with reference to the following species: human (>NP_001015051.3), rhesus monkey (>XP_028703335.1), chimpanzee (>XP_016811118.1), chicken (>XP_025004234.2), rat (>NP_001265412.1), and mouse (>NP_001139510.1).

3. Results

3.1. Clinical manifestations

The clinical features of the patients and phenotypic characteristics of the probands were as follows: short stature, broad forehead, frontal bossing, orbital hypertelorism, midface hypoplasia, and protruding mandible (Fig. 1B, Fig. 2B & F). Failure of eruption of permanent teeth, retention of deciduous teeth, and supernumerary teeth were confirmed by panoramic radiograph. Tooth deformity was observed because of the failure of eruption of several supernumerary teeth in the mandible or maxilla.
3.3. RUNX2 mRNA expression

qPCR was performed to evaluate the variant effects and determine mRNA expression levels of RUNX2 of the proband in family 1. GAPDH was used as the internal reference (control group was set to 1), and the relative quantity of RUNX2 mRNA in the proband was 0.616. The results indicated that the mRNA levels of RUNX2 were downregulated in the patient.

3.4. Bioinformatics analyses

SIFT, Polyphen2, and MutationTaster predicted that the mutation (c.2T>C) was “deleterious” (0.00), “possibly damaging” (0.838), and “disease-causing” (1.00), respectively, suggesting the variant is highly pathogenic. No other candidate variants were identified in the evaluation of the exome file of family 1 to rule out the possibility of the contribution of any variation in other known causative genes for CCD. A cross-species amino acid sequence alignment of the reported mutation (c.569G>A; p.Arg190Pro) showed that Arg190 was highly conserved among humans, rhesus macaques, chickens, mice, rats, and chimpanzees (Fig. 3D).

4. Discussion

CCD is a skeletal dysplasia that ranges from classical CCD (dental abnormalities, hypoplastic/aplastic clavicles, and delayed closure of cranial sutures) to mild CCD to isolated dental anomalies without the skeletal features. Among the 202 registered RUNX2 variants (HGMD Professional 2020.4 http://www.hgmd.cf.ac.uk/index.php), 99% (201/202) cause classical CCD. In this study, the 2 probands and family 2 members all showed classic features such as short stature, midface hypoplasia, frontal bossing, retention of deciduous teeth, failure of eruption of permanent teeth, aplasia of the clavicle, and supernumerary teeth, and the proband II:1 of family 2 exhibited delayed closure of cranial sutures. These phenotypes are consistent with previous findings in which most cases exhibited classical features.

In this study, we found a previously reported missense variant (p.Arg190Pro) in RUNX2 that has been previously found to be responsible for CCD, but the mutation detected in the initiation codon (c.2T>C) has not yet been reported. The computational programs all predicted that the c.2T>C missense change is damaging to the resultant protein function and structure. Further, according to the criteria for classifying pathogenic variants proposed by ACMG, the initiation codon variant should be considered as very strong evidence of pathogenicity (PV51).

On the basis of Kozak principles, we hypothesized that the translation is mostly initiated 304 nucleotides downstream for type 2 RUNX2 (NM_001015051.3; Fig. 4). Consequently, this initiation codon mutation results in a frameshift mutation and truncated proteins (lack of the Q/A domain and part of the runt domain), which is consistent with the previous consensus that most mutations that cause premature termination in the runt domain produce a classic CCD phenotype. It is also possible that the mRNAs are degraded via the nonsense-mediated mRNA decay quality-control mechanism.

The etiology of CCD is heterozygous variants in RUNX2, which encodes a transcription factor essential for osteoblast differentiation. Previous studies have shown that bones are malformed in case of a homozygous deletion in this gene in animals. Thus, the knockout mice with Runx2 lack osteoblasts and bones, whereas the heterozygous mice

### Table 1

| Clinical and genetic features of the patients with cleidocranial dysplasia. |
|-----------------------------|-------------|-------------|-------------|
| Clinical features           | Family 1    | Family 2    |
| Age                         | 15          | 10          | 20          |
| Sex                         | F           | M           | F           |
| Height (cm)                 | 162         | 140         | 166         |
| Family history              | +           | +           | +           |
| Short stature               | –           | +           | +           |
| Delayed or open skull suture| –           | +           | +           |
| Wormian bone                | +           | +           | +           |
| Frontal bossing             | –           | –           | –           |
| Hypertelorism               | +           | +           | +           |
| Hypoplasia of the maxilla   | +           | +           | +           |
| Clavicle hypoplasia         | +           | +           | +           |

### Wide pubic symphysis

| Clinical features           | Family 1    | Family 2    |
|-----------------------------|-------------|-------------|
| Short middle phalanx of the 5th finger | –           | +           |
| Vertebral alteration (scoliosis) | –           | –           |
| Delayed eruption of permanent teeth | +           | +           |
| Impacted tooth              | +           | +           |
| Malocclusion                | +           | +           |

### Gene features

| Mutation type               | Family 1    | Family 2    |
|-----------------------------|-------------|-------------|
| Codon                       | M2T         | R1900       |
| Nucleotide change           | c.2T>C      | c.569G>A    |
| Location                    | A01         | Runt        |

+: presence; -: absence.
Quantitative real-time PCR results showed that the RUNX2 level was downregulated to 60% in patient II:1 family 1. Previous studies have shown a critical gene dosage requirement of functional RUNX2 for the formation of intramembranous bone tissues during embryogenesis. A decrease to 70% RUNX2 levels

Figure 3. Sequencing results show RUNX2 mutations in the 2 families. (A) A novel transition from thymine to cytosine at the second base of the initiation codon c.2T>C in RUNX2 in family 1. (B) A previously reported mutation c.569G>A, p.R190Q in the proband, his elder sister, and their mother. (C) Schematic representation of RUNX2 structure and annotated mutations. (D) The sequence alignment results show that the residues c.569G>A in RUNX2 is conserved across 6 species: the mutant allele is boxed.
indicates CCD; in contrast, >79% levels produce a normal skeleton. Our result is consistent with those of previous studies and confirms the mechanism underlying this case is haploinsufficiency.

Of the previously reported 202 RUNX2 variants, 63% (51/80) of the missense/nonsense mutations occurred in the runt domain, which is the most important variation hotspot of RUNX2. In this study, we located the variant (p.Arg190Pro) in the runt domain, confirming the above-mentioned point. Sequence alignment results showed that Arg190 is highly conserved. A functional study showed that the R190Q variant exhibited no DNA binding and markedly reduced transactivation activities. This genotype is correlated with the classic CCD phenotype and a review of seven previous cases. Mol Syndromol 2015; 6:83–6.

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5. Conclusion

This study demonstrated that a novel heterozygous initiation codon variant (c.2T>C) in RUNX2 causes CCD. This study expands the pathogenic variant spectrum of RUNX2 and could help in genetic counseling and prenatal screening and contribute to disease status prediction for CCD families.

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Figure 4. Prediction of the truncated protein of RUNX2 (c.2T>C). We hypothesized that the translation is mostly initiated 304 nucleotides downstream.
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