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A novel *RUNX2* mutation (T420I) in Chinese patients with cleidocranial dysplasia

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ABSTRACT. Cleidocranial dysplasia (CCD) is an autosomal-dominant heritable skeletal disease caused by heterozygous mutations in the *RUNX2* gene. We studied a Chinese family that included three affected individuals with CCD phenotypes; the clinical features of patients with CCD include delayed closure of fontanelles, frontal bossing, dysplasia of clavicles, late tooth eruption, and other skeletal anomalies. X-ray analysis showed aplasia of the clavicles. The *RUNX2* gene was studied by PCR and direct sequencing of the entire coding region and the exon-intron boundaries of the gene. A novel missense mutation (c.1259C→T[p.T420I]) in *RUNX2* gene exon 7 was identified; it was found in the affected individuals in this Chinese family, but was not present in an unaffected family member or in 100 unrelated normal controls. This is the first report that gives evidence that the T420I mutation of *RUNX2* is associated with CCD, expanding the spectrum of *RUNX2* mutations causing CCD.

Key words: Cleidocranial dysplasia; Mutation; *RUNX2*; Gene

INTRODUCTION

Cleidocranial dysplasia (CCD; MIM 119600) is a skeletal disorder with autosomal-dominant inheritance. The main clinical features of CCD include rudimentary or absent clavicles, delayed closure of cranial fontanels and sutures, supernumerary and late-erupting teeth, wide pubic symphysis, short middle phalanx of the fifth fingers, and often vertebral malformation (Quack et al., 1999; Lee et al., 2008; Brooks and Nikitakis, 2008). The clinical spectrum ranges from mild cases with isolated dental abnormalities to lethal neonatal cases that show pronounced skeletal deformities and osteoporosis. The locus for CCD has been mapped to chromosome 6p21, where the responsible *RUNX2* gene (named according to the approved nomenclature for the mammalian runt-related genes) has been located (Lee et al., 1997).

Over 90 mutations of *RUNX2* have been published to date in independent cases of CCD (Shen et al., 2009), including missense mutations, deletions, insertions, frameshift, and splice mutations in all exons of *RUNX2* gene except exon 0 and exon 6. However, mutational screening of the *RUNX2* gene is still far from saturation, and more novel mutations will be identified to enrich the insights into the molecular basis for the pathogenesis of CCD. Here, a novel mutation in the *RUNX2* gene was detected in a Chinese family consisting of three affected individuals with CCD.

SUBJECTS AND METHODS

This study was approved without restrictions by the Research Ethics Committee of Qilu Hospital, and authorized by all individuals and patients.

Patients

The pedigree is shown in Figure 1. The proband (III-4) is a 2.5-year-old female. She received medical attention for her large fontanelle. Upon physical examination, her height was 88.3 cm, and weight was 15 kg. She had an asymmetric face, a bossing of the forehead, a depressed nasal bridge, wide rima oculi, and delayed tooth eruption. X-ray analysis showed wormian bones (Figure 2A) and aplasia of lateral third of the clavicle (Figure 2B). The knee and hand X-rays were normal. The family history shows that there were two other affected individuals in her family, her younger brother and her father (Figure 1). The clinical features of all affected individuals are shown in Table 1.

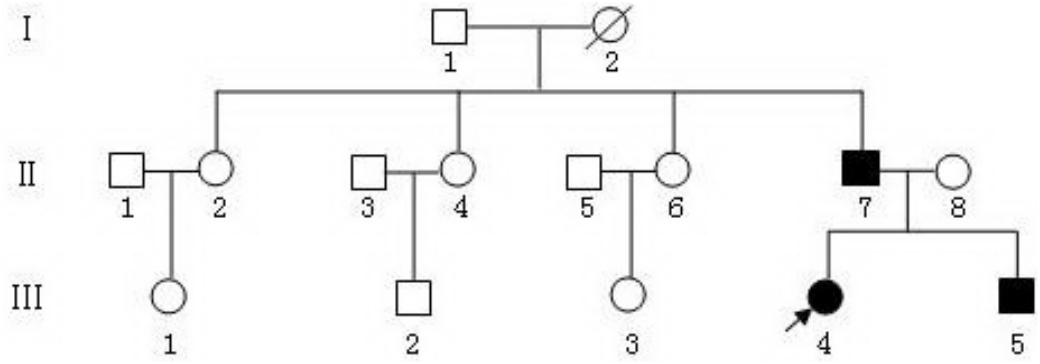

Figure 1. Pedigree of a Chinese family with cleidocranial dysplasia (proband is indicated with an arrow).

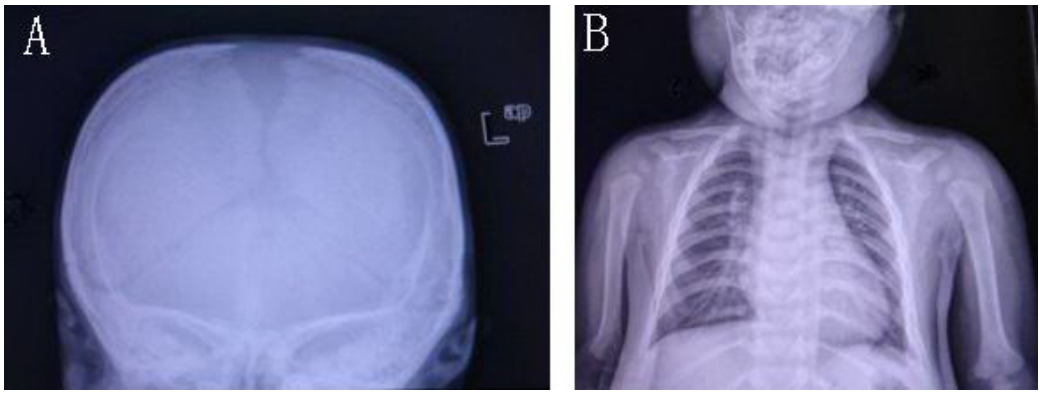

Figure 2. Radiological features of the proband. **A.** Plain X-ray film of the skull showing open fontanelles and wormian bones near the lambdoid suture. **B.** Chest film: the left clavicle is short.

Table 1. Clinical features of patients with cleidocranial dysplasia in a Chinese family.

Number	Age	Oral cavity	Craniofacial features	Skeletal system
II 7	32 years	Supernumerary tooth	Asymmetric face, large fontanelle	Height 167.5 cm, aplasia of lateral third of the clavicle, shoulder ptosis
III 4	2.5 years	Delayed tooth eruption	Asymmetric face, delayed closure of fontanelles	Height 88.3 cm, aplasia of lateral third of the clavicle
III 5	11 months	Delayed tooth eruption	Delayed closure of fontanelles, ocular hypertelorism	Height 73.5 cm, aplasia of the clavicle

Genetic analysis

Blood (3 mL) was drawn from all affected individuals, unaffected mother of the proband and one hundred unrelated normal controls. Genomic DNA was extracted from whole blood using TIANamp Blood DNA Kit (Tiangen Biotech (Beijing) Co. Ltd., China). Genomic DNA was polymerase chain reaction (PCR)-amplified with eight pairs of PCR primers for exons 0 to 7 of the *RUNX2* gene; the primers were designed based on the published sequence data (GenBank Accession #NP_ 004339.3) and spanned the entire coding region and exon-intron boundaries of the gene. The sequences of the primers used in this study are shown in Table 2.

Table 2. Primers used for polymerase chain reaction, sequencing reactions and amplification conditions.

Exon		Sense (5'-3')	Antisense (5'-3')		Fragment size (bp)	Annealing (ºC)
0	0F	CAAGGTTTGGGTATGGTTTGTATT	GACTTTTCTTCTGGTGAGGGTT	0R	484	56
1	1F	CACCTCCATCCTCTTTCCCC	GGGTTTCTGGGGTTAGAGCCG	1R	630	56
2	2F	TGGCATCACAACCCATACAC	GTCTACATTTCATCAAAGGAGC	2R	388	56
3	3F	TAAAGTGGTCATCGGAGGGT	TAGTGATTGTGTGGCAAAGGT	3R	489	56
4	4F	TGCCTCTCTATTGATGCTTGAC	GAACACATCTCCTCTGGTAGCC	4R	514	56
5	5F	CCTTTCTGAGTTTTGGGTTGC	CCAGTTGTCATTCCCTTGCC	5R	373	58
6	6F	TTGGGAGGAATAGGTAGAGG	TAAAGGTGCTGATTGAAGGT	6R	587	55
7	7F	GTGGCTTGCTGTTCCTTTATG	TATCTACCCTCTTATGGCTGC	7R	781	57

PCR cycling was performed on a DNA thermal cycler (Gene Amp 9700, Perkin-Elmer, USA) with 2X Hotstart Taq PCR Mastermix kit (Tiangen Biotech). In a 50-μL reaction mix, 300 ng genomic DNA was used with 2.0 μL of each primer (10 μmol/L), and 25 μL of 2X PCR Mastermix. Genomic DNA was first denatured at 94°C for 3 min, followed by 31 cycles of 94°C for 35 s, 55° to 58°C for 35 s, and 72°C for 50 s. The PCR products were extended at 72°C for 5 min. The products were gel-purified with an agarose gel DNA purification kit (Tiangen Biotech), and the purified PCR products were sequenced using the forward and reverse primers. Automated sequencing was performed on an ABI 377 automatic sequencer. Every mutation was confirmed by sequencing of the products from several independent PCRs. After a mutation within an exon was detected in the patients, this exon was PCR-amplified specifically in the proband's healthy mother and one hundred unrelated normal controls.

RESULTS

Extraction of genomic DNA and PCR of exons 0 to 7 of the *RUNX2* gene

OD_{260}/OD_{280} of genomic DNA from these blood specimens was 1.7~1.9, which meant that the genomic DNA was suitable for use as a template for PCR. PCR fragments of exons 0 to 7 of the *RUNX2* gene were checked by electrophoresis on 20 g/L agarose gels, and the gels were photographed, as shown in Figure 3.

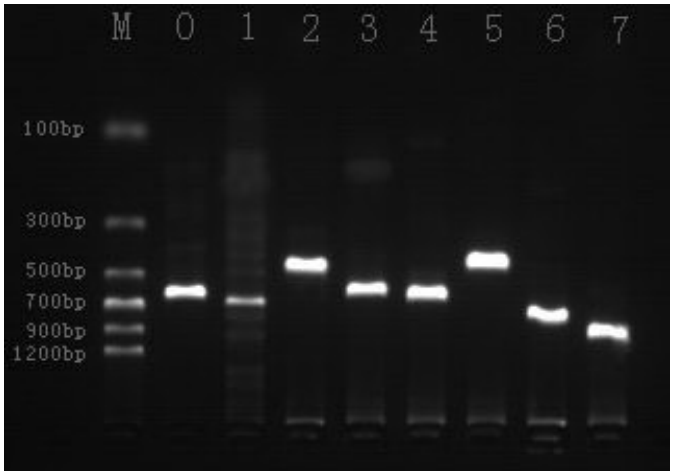

Figure 3. Electrophoresis of polymerase chain reaction (PCR) fragments of exons 0 to 7 of the *RUNX2* gene. *Lane M* = DNA marker; *lanes 0-7* = PCR fragments of exons 0 to 7, respectively.

Sequence analysis

By sequencing PCR fragments of exons 0 to 7 of the *RUNX2* gene, we detected a single base-pair variant (c.1259C→T[p.T420I]) in exon 7 in the CCD patients. This variant in the cDNA at position 1259 results in the substitution of threonine by isoleucine. It co-segregates with affected individuals of the family. There is no such sequence variant at that position in

unaffected individual and one hundred unrelated normal controls, suggesting that the sequence aberration is pathogenic (Figure 4). A threonine at that position is conserved in runt family proteins of vertebrates, suggesting that it is functionally important (Table 3).

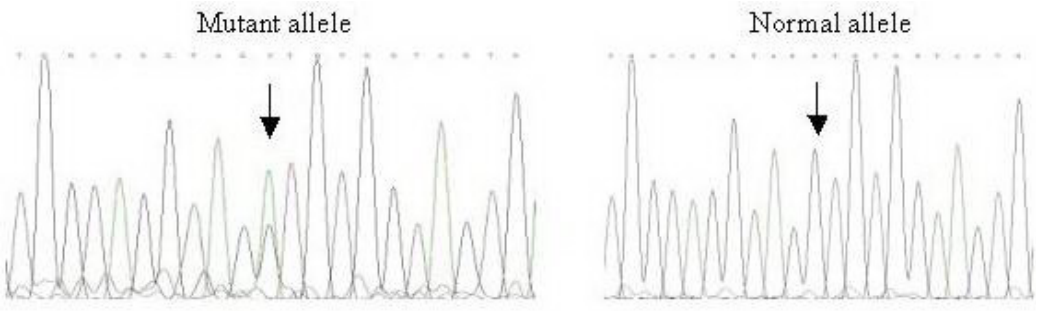

Figure 4. Sequencing of exon 7 of *RUNX2* alleles shows the heterozygous mutant allele with C > T missense mutation in c.1259C.

Table 3. Comparison of sequences in the vicinity of *RUNX2* T420 (bold) from various species

Species	Sequences in the vicinity of *RUNX2* T420
Homo sapiens	HYH**T**YLPPPYPGSS
Mus musculus	HYH**T**YLPPPYPGSS
Rattus norvegicus	HYH**T**YLPPPYPGSS
Gallus gallus	HYH**T**YLPPPYPGSS
Xenopus (silurana) *tropicalis*	HYH**T**YLPPPYPGSS
Danio rerio	HYH**T**YLPPPYPGST
Takifugu rubripes	HYH**T**YLPPPYPGST
Tetraodon nigroviridis	HYH**T**YLPPPYPGST

DISCUSSION

CCD is an autosomal dominant disorder and was mapped to chromosome 6p21. In 1997, the human *RUNX2* gene was identified as the CCD gene (Mundlos et al., 1997; Otto et al., 1997; Lo Muzio et al., 2007). *RUNX2* gene consists of eight coding exons and spans a genomic region of 130 kb and encodes a transcription factor that is a member of the runt family of proteins. *RUNX2* contains a DNA-binding domain (runt domain) highly homologous to the *Drosophila* pair-rule gene runt, a region of glutamine and alanine repeats in the N-terminal region (QA domain), and a region rich in proline-serine-threonine (PST), which is necessary for transcriptional activation of target genes. *RUNX2* is essential for the development of osteoblasts from their mesenchymal precursors. Inactivation of *RUNX2* in mice by gene targeting leads to a complete absence of bone. Although the cartilage anlagen develop normally, there is no differentiation of mesenchymal stem cells into osteoblasts, no ossification, and no vascular invasion of cartilage, and such mice die shortly after birth because of impaired respiratory function (Otto et al., 1997). Heterozygous *RUNX2* mutant mice display all the hallmarks of CCD, including open fontanelles and hypoplastic clavicles, but not the dental anomalies (Otto et al., 1997).

In *Homo sapiens*, Mundlos et al. (1997) found that heterozygous deletions of *RUNX2* gene were present in some CCD families, and that in other CCD families, insertion, deletion, or missense mutations of *RUNX2* led to translational stop codons in the DNA-binding domain or in the C-terminal transactivating region. Thus far, more than 90 mutations have been identified in the *RUNX2* gene. Yoshida et al. (2002) performed mutation analysis of *RUNX2* on patients with CCD, and they found that in 17 unrelated patients, 16 distinct mutations were detected in the coding region of *RUNX2*. They found that the missense mutations were clustered around the runt domain, and the runt domain was intact in 2 *RUNX2* mutants, with partial competence for transactivation remaining.

In this research, we identified a novel missense mutation c.1259C→T[p.T420I] in the C-terminal transactivating region in a Chinese family consisting of three affected individuals with classic CCD phenotypes. After searching the SNP database and the human gene mutation database, we found that c.1259C→T [p.T420I] is absent from the two databases. The proband's healthy mother and one hundred unrelated normal controls did not carry the same mutation, suggesting that the sequence aberration is pathogenic. Therefore, we demonstrated that the present patients carry a novel heterozygous mutation.

Because the T420I mutation is in the PST domain and outside the runt domain, the most likely mechanism by which the T420I mutation affects *RUNX2* function is through impaired transactivation activity, influencing transcriptional activation of target genes such as the osteocalcin gene.

Thus far, genotype-phenotype correlations have not been established for the different *RUNX2* mutations found in CCD (Cunningham et al., 2006). For example, in one Italian family with a *RUNX2* mutation, the reported clinical features ranged from minimal or absent clavicle to the presence of hypoplastic clavicles and delayed closure of the anterior fontanel, in addition to classic craniofacial features. However, Zhou et al. (1999) suggested that variable loss of function due to alterations in the runt and PST domains of *RUNX2* may give rise to clinical variability, including classic CCD, mild CCD and isolated primary dental anomalies. In our research, one criterion of CCD, short stature, was mild in this Chinese family, and this finding is not in agreement with the observation of Yoshida et al. (2002) that short stature was much milder in the patients with the intact runt domain than in those without. Further research should be performed to correlate *RUNX2* mutations in different functional domains with the CCD clinical spectrum.

In conclusion, we identified a novel mutation, T420I, in the *RUNX2* gene in a Chinese family with CCD. This mutation likely interferes with transactivation activity and would result in impaired transcriptional activation of target genes. The identification of the T420I mutation in this family further expands the clinical phenotypes and mutation spectrum, and will contribute to prenatal molecular diagnosis and preimplantation genetic diagnosis.

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